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Lowering polyhydroxyalkanoate bioproduction costs in mixed cultures through integrated optimization of organic loading rate, sludge retention time and biomass withdrawal

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ABSTRACT

Polyhydroxyalkanoates (PHA) are a sustainable alternative to conventional plastics, where mixed microbial cultures can be advantageous for PHA production from industrial waste streams, but lower costs are necessary to facilitate industrial application. This study assesses a novel optimisation strategy for the biomass withdrawal, organic loading rate and solids retention time, with the purpose of lowering bioproduction costs. Conventional and feast-phase biomass withdrawal strategies were compared to determine the most cost-effective operational option. Results demonstrated that increasing the organic loading rate led to an improvement of PHA productivity in each selection reactor, due to increased growth. It was observed that at low solids retention time, the incorporation of an accumulation stage did not improve the productivity of the polyhydroxyalkanoate production process using the feast-phase biomass withdrawal strategy, reducing capital costs.

Economic assessment of the two different operational modes was performed, where the results demonstrated that the feast-phase biomass withdrawal strategy at an SRT of 0.8 d was the configuration resulting in the lowest total annual costs and lowest break-even PHA price (1.88 €/kg PHA-crude), due to elimination of the accumulation stage. Overall, this work contributes towards sustainable and cost-effective polyhydroxyalkanoate production from wastes in mixed cultures.

Introduction

Growing awareness about environmental issues such as waste and resource management and the accumulation of petrochemical plastic wastes in the environment has prompted much interest in biologically derived and biodegradable polymers [1,2,3]. Polyhydroxyalkanoates (PHAs) are fully bio-based (produced by bacteria, using renewable resources as feedstocks), biodegradable and biocompatible polymers that have the potential to replace conventional petrochemical plastics since they exhibit similar thermoplastic and elastomeric properties [4,5,6].

The utilization of mixed microbial cultures (MMCs) for PHA production as compared to pure cultures allows eliminating the costs of refined substrates and the need for a sterile production environment [7,8]. An MMC PHA production process from waste streams usually consists of three stages: 1) acidogenic fermentation of the waste stream to produce volatile fatty acids (VFAs), which then can be used as PHA precursors in the subsequent steps; 2) culture selection to obtain a population with a high capacity to store PHA; and 3) the PHA production stage, where VFAs from acidogenic fermentation are used to feed the selected culture to reach its maximum PHA content [9,10].

Usually, the limiting stage regarding the PHA production process productivity is the culture selection stage, since a robust culture and strong selective pressure for PHA-producing bacteria, paired with high biomass productivity, are often pointed as determining factors for obtaining high PHA storage rates, yields and global PHA productivity [11,9]. Sequencing batch reactors (SBR) operated under feast and famine conditions are usually used to provide a competitive advantage for microorganisms to store the substrate as PHA and reuse it for growth

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during the famine phase, thus selecting a culture that is high in PHA-storing bacteria [12,8].

In the conventional MMC process, the biomass is withdrawn at the end of the famine phase (conventional BW), where the biomass is depleted in intracellular PHA, and then used as inoculum for the accumulation reactor. However, if the biomass withdrawal (BW) in the selection reactor is shifted to the end of the feast phase (FeBW), where the biomass is high in PHA, the accumulation reactor could potentially be eliminated. Several authors have implemented the feast phase BW (FeBW) in selection reactors to start the PHA accumulation stage with a higher initial PHA content and improve productivity [13,14,15]. However, the combination of operational parameters that maximizes PHA productivity in FeBW has yet to be established and should be compared to conventional BW to examine process cost-effectiveness through an integrated comparison.

Previous work [16] established the effect of operating a selection reactor under the feast and famine strategy with the alternative FeBW vs the conventional BW under constant operational conditions, finding a higher biomass production rate and PHA productivity with conventional BW with the particular organic loading rate (OLR) and solids retention time (SRT) tested in that study. The OLR and SRT are two important parameters impacting PHA by MMCs [8–10,17–19]. However, previous evaluations of OLR and SRT have been carried out using conventional BW only. Increasing the OLR and reducing the SRT could potentially lead to improving the overall productivity of the process operated with FeBW and merits further study due to its potential in reducing bioreactor capital costs. The objective in this work is to evaluate which OLR and SRT conditions can promote by-passing of the accumulator reactor, directing the biomass with high PHA from the end of the feast phase directly to downstream processing, and evaluating what would be the process and economic benefits of this strategy vs conventional BW strategies.

Since different process designs result in different demands of energy and materials, economic comparisons can assist the decision-making process regarding which design option can be preferable under a given set of operational conditions [20]. Several economic comparisons regarding PHA production by MMCs exist in the literature [21,22,23,24], however none compare the impact of the biomass withdrawal strategy and the process configuration on the cost effectiveness of the process.

This work aims to critically assess the impact of key operational conditions, such as the OLR and SRT, on both the conventional BW and FeBW operational strategies, from both a kinetic and economic viewpoint, to establish favourable combinations of conditions that can increase PHA productivity at low costs. PHA accumulation assays were performed to evaluate the potential of the cultures to store PHA and the global PHA productivity of both systems was used as a comparable parameter for measuring the efficiency of the PHA production process in MMCs. Additionally, a comparison of bioproduction costs between the different process configurations resulting from different BW strategies was performed in order to gain greater insight into the comparative viability of each operational strategy, based on the data originating from this work.

Methods and materials

Culture selection reactors

The setup design consisted of two 2L-SBR inoculated with surplus activated sludge collected from the municipal wastewater treatment plant of Mutela, Almada, Portugal, as previously described by Cruz et al. [16]. The reactors were operated with a hydraulic retention time (HRT) of 1 d at SRT of 2.6, 2.3, and 1.7 d, or an HRT and SRT of 0.75 d, where the OLR was increased from 100 to 200 $Cmmol.L^{-1}.d^{-1}$ between the first and the second tests, and remained at 200 $Cmmol.L^{-1}.d^{-1}$ during the remaining operational periods. For each condition, the reactors were operated at least 30 days to reach pseudo-steady state. The same conditions were applied to both reactors except the withdrawal strategy, consisting of purging one reactor at the end of the cycle (R1) and the other reactor after the exhaustion of carbon source at the end of the feast phase (R2). The SBR cycle was comprised of three phases: reaction phase (11 h), settling (45 min) and supernatant withdrawal (15 min; 50 % volume exchange rate), except at the SRT of 0.75 d, in which there was no need of settling and supernatant withdrawal phases. The addition of the carbon source solution (2 min) occurred twice per SBR cycle, once at the beginning of the cycle (500 mL) and a second time after 1–2 h (500 mL). The purpose of feeding carbon source in two pulses was to avoid inhibition per substrate at high OLR. The feeding of the nitrogen source solution took place after exhaustion of the carbon source (varying between 150 to 420 min) to maximise growth of microorganisms that store PHA [10]. The biomass was withdrawn after 645 min (705 min at SRT of 0.75 d) in R1 and before the addition of the nitrogen source in R2.

The simulated fermented waste stream fed to the SBRs contained acetic acid (80 %Cmol) and propionic acid (20 %Cmol) as carbon source at a concentration obtained from the target OLR, a phosphate buffer composed of 0.1 M of KH_2PO_4 and 0.1 M K_2HPO_4 (to avoid high pH amplitude), 600 mg $MgSO_4{\cdot}7H_2O$, 70 mg $CaCl_2{\cdot}2H_2O$, 10 mg allyl-N thiourea (to prevent nitrification), 100 mg ethylene-diaminetetraacetic (EDTA), and 2 mL of a micronutrients solution described by [25]. The pH was adjusted to 7.2–7.3 with 5 M NaOH. The nitrogen source solution was composed of an NH_4Cl concentration corresponding to a C/N ratio of 100/6 (mol basis). Since biomass withdrawal in R2 resulted in a difference in reactor volume between the two reactors at the beginning of the famine phase, 25 mL of nitrogen source solution was added in R1 and 250 mL was added in R2, except at the SRT of 0.75 d, in which the volume was 25 mL for both reactors (concentrations were corrected).

The reactors were operated at 22 ± 1 °C and pH of 7.5 ± 0.2, controlled by the addition of 1 M HCl and 0.5 M NaOH, and stirred at 250 rpm with a double turbine stainless steel shaft. The dissolved oxygen (DO) was maintained above 2 $mg.L^{-1}$ by sparging compressed air. DO concentration and pH were measured online using analog sensors (Mettler Toledo, LLC, Columbus, Ohio, USA).

The reactors were closely monitored, and samples were regularly collected during the full length of the reaction phase, and analysed according to the analytical methods described below, to assess the performance of the MMC.

PHA accumulation assays

Fed-batch PHA accumulation assays were performed in duplicate to evaluate the potential for PHA accumulation of the cultures selected. 500 mL of withdrawn biomass were inoculated in a 1 L reactor with controlled airflow of 3.0 $L.min^{-1}$, agitation of 250 rpm and pH of 7.5 ± 0.2, controlled by a BioFlo®/CelliGen® 115 system (Eppendorf AG, Germany).

The composition of the fermented waste stream was the same as in the selection reactors, but the concentration was corrected to correspond to an initial pulse of 50 mL. The Food to Microorganism (F/M) ratio was maintained by adjusting the volume of the following pulses to the volume of samples taken along the tests. The assays were finalized when the reaction had slowed down significantly (DO levels reaching 80 % of saturation), varying from 3 to 11 h.

Analytical methods

Samples for the determination of TSS and VSS were treated according to standard methods [26]. Samples for the determination of VFA, ammonia and phosphate, and PHA were centrifuged at 9600 rcf for 3 min, the supernatant was filtered through 0.45 μm membrane filters and the pellets were lyophilized. VFA and ammonia and phosphate concentrations were determined by high-performance liquid chromatography and segmented continuous-flow analysis as described by [10].

PHA was quantified by gas chromatography with flame ionization detector according to the method described by [27]. Biomass samples were collected from the initial inoculum and the selection reactors and were analyzed by high-throughput sequencing, according to the method described by [28].

Calculations

Active biomass concentration (X_A, $g.L^{-1}$) and VFA and PHA concentration ($Cmmol.L^{-1}$) were obtained as defined by [16]. The feast to cycle length ratio (F/CL, $h.h^{-1}$) was calculated by dividing the length of the feast phase by the total duration of the SBR cycle.

The specific rates were calculated by applying linear regression to the experimental data over time and dividing its slope by the average X_A. The μ (h^{-1}) was estimated by the consumption of ammonium during the famine phase and converted to $Cmmol.L^{-1}$ of X_A based on the chemical formula of biomass $C_5H_7NO_2$. The yield of PHA per substrate ($Y_{PHA/S}$, $Cmol\text{-}PHA.Cmol\text{-}S^{-1}$) and the yield of biomass per PHA ($Y_{X/PHA}$, $Cmol\text{-}X_A.Cmol\text{-}PHA^{-1}$) were calculated as the quotient of the respective specific rates.

The volumetric biomass productivity (Prod-X_A, $g\text{-}X_A.L^{-1}.d^{-1}$) was calculated by dividing the average X_A ($g.L^{-1}$) by the SRT (d).

The PHA production during the accumulation assays was considered to be completed when the PHA content was 90 % of the total reached since accumulation tests tend to reach a plateau (a detailed description of the calculations done for the accumulation assays is given in the Supporting Information).

The global PHA productivity was calculated by multiplying the Prod-X_A by the cycle length and the PHA content obtained and divided by the total time elapsed.

Single-factor ANOVA was used to establish differences in means using Minitab software, with significance declared at a confidence level of 95 %.

Cost assessment

The calculations of the estimations of the cost and the assumptions used in the cost assessment are described in the Supporting Information (see also Table S1).

Results

Selection reactors

Two selection reactors were operated for 300 days to evaluate the effect of increasing the OLR and decreasing the SRT in the case of conventional BW (R1) or FeBW (R2). The feast phase per cycle length (F/CL) during the operation of both reactors can be found in the supplementary information (Fig. S1).

During the periods of stable performance at each OLR and SRT, the stoichiometric and kinetic parameters of the culture were determined. The profiles of VFA, PHA, active biomass (X_A) and ammonia for R1 and R2 are shown as an example for a cycle operated under SRT of 2.6 d and OLR of 100 $Cmmol.L^{-1}.d^{-1}$ in Fig. 1. A typical cycle for each combination of OLR and SRT can be found in the supplementary information (Fig. S2). These figures demonstrate VFA consumption during the feast phase with PHA production, while PHA was used for biomass growth during the famine phase, consuming ammonia.

The effect of increasing the OLR in selection reactors under different BW strategies

Table 1 shows the kinetic and stoichiometric parameters (average ± standard deviation) obtained for reactors R1 and R2 at different conditions of OLR and SRT. As the OLR was increased, the X_A concentration increased from 2.2 ± 0.4 to 3.0 ± 0.2 $g.L^{-1}$ in R1 and from 1.6 ± 0.4 to 2.4 ± 0. 6 $g.L^{-1}$ in R2, while the F/CL ratio increased by ~ 40 % in R1 and by ~ 20 % in R2. The increase of the amount of carbon source available, and consequently the higher amount of PHA stored, led to an expected increase in X_A concentration. This relative increase was higher in R2, where the X_A concentration increased by 54 % as compared to a 33 % increase in the X_A of R1. This agreed well with a lower increase in the F/CL, which was closely correlated with the X_A concentration in both reactors.

Regarding the kinetic performance of the reactors, the specific substrate consumption rate ($-q_S$) and specific PHA production rate (q_{PHA}) of R2 were significantly lower at the OLR of 200 $Cmmol.L^{-1}.d^{-1}$ (0.29 ± 0.01 $Cmol\text{-}S.Cmol\text{-}X_A^{-1}.h^{-1}$ and 0.20 ± 0.03 $Cmol\text{-}PHA.Cmol\text{-}X_A^{-1}.h^{-1}$) than at 100 $Cmmol.L^{-1}.d^{-1}$ (0.48 ± 0.03 $Cmol\text{-}S.Cmol\text{-}X_A^{-1}.h^{-1}$ and 0.30 ± 0.04 $Cmol\text{-}PHA.Cmol\text{-}X_A^{-1}.h^{-1}$) at similar SRT. A similar observation was made for R1 (Table 1). The yield of PHA per substrate ($Y_{PHA/S}$) in R2 was maintained at a comparable level (0.69 ± 0.1 $Cmol\text{-}PHA.Cmol\text{-}S^{-1}$ at OLR of 200 $Cmmol.L^{-1}.d^{-1}$ vs 0.71 ± 0.03 $Cmol\text{-}PHA.Cmol\text{-}S^{-1}$ at OLR of 100 $Cmmol.L^{-1}.d^{-1}$), however, R1 presented a lower $Y_{PHA/S}$ (0.53 ± 0.00 $Cmol\text{-}PHA.Cmol\text{-}S^{-1}$) when the OLR was 200 $Cmmol.L^{-1}.d^{-1}$ as compared to 100 $Cmmol.L^{-1}.d^{-1}$ (0.79 ± 0.2 $Cmol\text{-}PHA.Cmol\text{-}S^{-1}$). These results were consistent with some literature studies, which predicted that an increase in OLR could reduce the storage response in MMC [12,18,9], as observed in R1.

The PHA content at the end of the feast phase was not affected by increasing the OLR, and no significant difference was found between the reactors (32.5 ± 6.2 wt% in R1 and 28.5 ± 6.7 wt% in R2). While the PHA concentration increased from 0.61 to 0.97 $g\text{-}PHA.L^{-1}$ in R1 and from 0.44 to 0.68 $g\text{-}PHA.L^{-1}$ in R2, these increases were balanced by a comparable increase in the X_A concentration, leading to a similar %PHA. Nevertheless, the HV fraction decreased in each reactor following the increase in OLR (Table 1), though the differences between R1 and R2 were not statistically significant at each OLR. This could be related to the change of the population's composition, shown in Table 2, since several PHA producing genera, including *Neomegalonema*, *Paracocccus* and *Zoogloea*, vary substantially with the OLR.

Regarding the famine phase, the $-q_{PHA}$ presented no statistically significant difference between the reactors during the OLR of 100 $Cmmol.L^{-1}.d^{-1}$ and 200 $Cmmol.L^{-1}.d^{-1}$. When the OLR changed from 100 to 200 $Cmmol.L^{-1}.d^{-1}$, μ increased about 3-fold in R1 and 2-fold in R2, which was probably related to the amount of organic carbon available for growth in each reactor, increasing the $Y_{X/PHA}$.

The effect of decreasing the SRT in selection reactors under different BW strategies

When reducing the SRT from 1.7 d to around 0.75 d, the ability to produce PHA was severely affected in both cultures, with the specific PHA production rate (q_{PHA}) reduced to 0.18 ± 0.002 $Cmol\text{-}PHA.Cmol\text{-}X_A^{-1}.h^{-1}$ in R1 and 0.19 ± 0.005 $Cmol\text{-}PHA.Cmol\text{-}X_A^{-1}.h^{-1}$ in R2, approximately half of that observed at an SRT of 1.7 d. The specific substrate consumption rate (q_S) was also reduced, by approximately 1/4, to 0.32 ± 0.05 $Cmol\text{-}S.Cmol\text{-}X_A^{-1}.h^{-1}$ and 0.35 ± 0.02 $Cmol\text{-}S.Cmol\text{-}X_A^{-1}.h^{-1}$, respectively at SRT of 0.75 d. Consequently, the PHA per substrate yield coefficient ($Y_{PHA/S}$) of R1 and R2 reduced to 0.58 ± 0.09 and 0.54 ± 0.04 $Cmol\text{-}PHA.Cmol\text{-}S^{-1}$, respectively.

Despite a reduction of the PHA producing capacity, the PHA content obtained at the end of the feast phase was still relatively high (33.7 ± 8.7 wt% in R1 and 41.7 ± 5.5 wt% in R2), and this effect was attributed to the X_A concentration in both reactors being roughly halved when the SRT decreased from 1.7 d to 0.75 d. By applying the same OLR of 200 $Cmmol.L^{-1}.d^{-1}$ to a culture with significantly less X_A concentration (comparing SRT 1.7 d with 0.75 d), more external carbon was available to each cell (i.e. provoking a higher food to microorganisms, or F/M ratio), so the amount of PHA that cells were able to store also increased. However, the feast phase length approximately doubled in both reactors, due to the decreasing of the substrate uptake and PHA storage

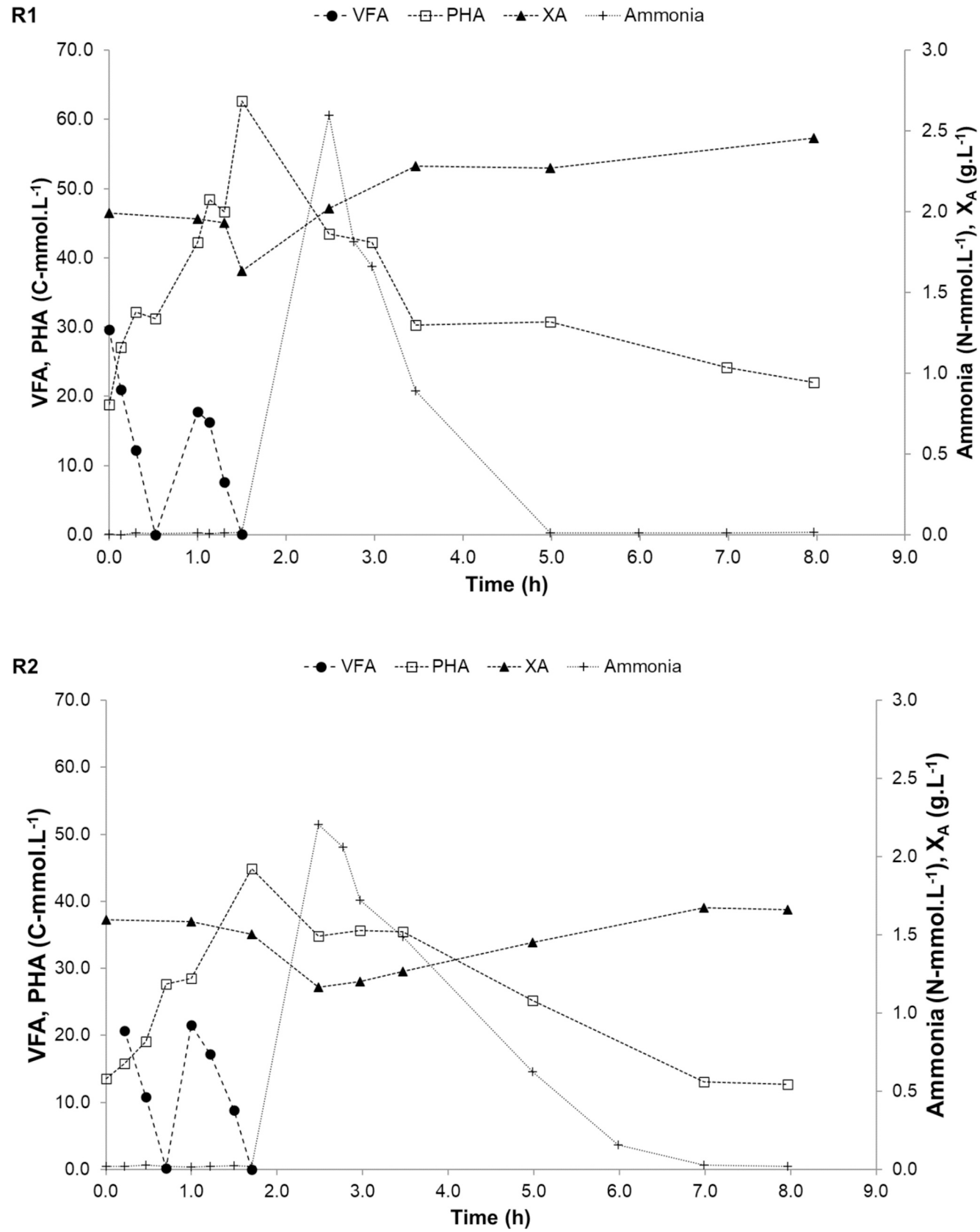

Fig. 1. Concentration profiles of volatile fatty acids (VFA), polyhydroxyalkanoate (PHA), active biomass (X_A) and ammonia during a typical cycle at pseudo-steady state of reactor 1 (R1-left) and reactor 2 (R2-right) at solids retention time (SRT) of 2.6 d and organic loading rate (OLR) of 100 Cmmol.L^{-1}.d^{-1}.

Table 1
Stoichiometric and kinetic parameters (mean ± standard deviation) in selection reactors R1 and R2 obtained from cycles assessed during a period of pseudo-steady state.

Reactor	R1	R2	R1	R2	R1	R2	R1	R2
OLR (Cmmol.L^{-1}.d^{-1})	100		200		200		200	
SRT (d)	2.86 ± 0.33	2.36 ± 0.67	2.41 ± 0.34	2.15 ± 0.43	1.65 ± 0.07	1.72 ± 0.25	0.71 ± 0.05	0.82 ± 0.16
F/CL(h.h^{-1})	0.15 ± 0.05	0.17 ± 0.05	0.21 ± 0.04	0.20 ± 0.03	0.18 ± 0.06	0.24 ± 0.05	0.45 ± 0.15	0.44 ± 0.16
X_A(g.L^{-1})	2.2 ± 0.4	1.6 ± 0.4	3.0 ± 0.2	2.4 ± 0.6	3.3 ± 0.6	2.2 ± 0.2	1.9 ± 0.1	1.0 ± 0.3
Feast phase								
$-q_S$(Cmol-S.Cmol-X_A^{-1}.h^{-1})	0.49 ± 0.01	0.48 ± 0.03	0.33 ± 0.06	0.29 ± 0.01	0.45 ± 0.09	0.46 ± 0.02	0.32 ± 0.05	0.35 ± 0.02
q_{PHA}(Cmol-PHA.Cmol-X_A^{-1}.h^{-1})	0.38 ± 0.09	0.30 ± 0.04	0.18 ± 0.03	0.20 ± 0.03	0.35 ± 0.05	0.44 ± 0.01	0.18 ± 0.002	0.19 ± 0.01
$Y_{PHA/S}$(Cmol-PHA.Cmol-S^{-1})	0.79 ± 0.2	0.71 ± 0.03	0.53 ± 0.00	0.69 ± 0.1	0.75 ± 0.04	0.95 ± 0.01	0.58 ± 0.09	0.54 ± 0.04
PHA^{max}(wt.%)	27.2 ± 3.8	28.2 ± 8.2	32.5 ± 6.2	28.5 ± 6.7	36.7 ± 3.7	35.7 ± 4.6	33.7 ± 8.7	41.7 ± 5.5
HV(Cmol%)	46.4 ± 4.2	50.1 ± 4.2	33.0 ± 1.8	33.4 ± 5.4	36.8 ± 8.3	46.6 ± 2.8	23.4 ± 4.2	30.8 ± 1.7
Famine phase								
$-q_{PHA}$(Cmol-PHA.Cmol-X_A^{-1}.h^{-1})	0.07 ± 0.02	0.07 ± 0.02	0.11 ± 0.02	0.07 ± 0.006	0.13 ± 0.01	0.09 ± 0.01	0.16 ± 0.02	0.27 ± 0.1
μ(Cmol- X_A.Cmol-X_A^{-1}.h^{-1})	0.03 ± 0.01	0.03 ± 0.01	0.10 ± 0.03	0.06 ± 0.01	0.10 ± 0.01	0.04 ± 0.01	0.14 ± 0.01	0.13 ± 0.03
$Y_{X/PHA}$(Cmol-X.Cmol-PHA^{-1})	0.55 ± 0.2	0.50 ± 0.05	0.94 ± 0.06	0.81 ± 0.1	0.77 ± 0.08	0.42 ± 0.08	0.75 ± 0.1	0.51 ± 0.1

Table 2
Relative abundance (>0.1 %) of bacteria identified by high-throughput sequencing in the two selection reactors under a stable period of operation at the different values of OLR and SRT tested. Genera in bold text contain species known to exhibit PHA production potential.

		Inoculum	R1	R2	R1	R2	R1	R2	R1	R2
	OLR (Cmmol.L^{-1}.d^{-1})		100		200		200		200	
	SRT (d)		2.9 ± 0.3	2.4 ± 0.7	2.4 ± 0.3	2.2 ± 0.4	1.7 ± 0.1	1.7 ± 0.3	0.71 ± 0.1	0.82 ± 0.2
Class	**Genus**									
Alphaproteobacteria	**Neomegalonema**	5.1	22.8	**35.6**	**49.5**	**46.4**	1.3	36.5	0.6	20.0
Alphaproteobacteria	Rhizobiales_OTU_6 *	0.0	0.3	6.8	18.6	4.2	**51.2**	6.5	16.8	**29.0**
Alphaproteobacteria	**Paracocccus**	1.3	**47.4**	27.4	2.6	0.0	7.3	**43.7**	0.0	0.2
Betaproteobacteria	**Zoogloea**	0.0	0.0	0.3	7.1	24.9	0.0	0.0	8.8	17.8
Alphaproteobacteria	**Amaricoccus**	0.0	1.2	8.5	6.8	15.2	12.2	3.3	0.0	1.5
Alphaproteobacteria	Kaistia	0.0	0.0	0.1	0.4	0.1	0.0	0.0	**44.0**	1.9
Flavobacteria	Flavobacterium	0.1	0.8	1.6	3.1	0.6	0.4	1.0	10.4	7.5
Alphaproteobacteria	**Rhodobacter**	23.2	0.0	0.0	0.7	0.0	0.0	0.0	0.2	0.2
Gammaproteobacteria	**Thiothrix**	5.7	0.0	0.5	0.5	0.0	16.6	0.4	0.3	0.0
Alphaproteobacteria	**Brevundimonas**	0.1	2.3	4.1	0.5	1.7	1.8	2.2	1.7	0.2
Alphaproteobacteria	Rhizobiales Incertae Sedis_OTU_71 **	0.0	0.0	0.2	2.6	0.9	1.4	0.2	1.9	6.1
Alphaproteobacteria	Aminobacter	0.2	7.8	1.1	0.0	0.0	2.4	0.0	0.0	0.0
Alphaproteobacteria	Hyphomonadaceae_OTU_29 **	0.0	0.0	0.0	1.4	0.6	0.0	0.0	2.7	5.9
Betaproteobacteria	Acidovorax	10.4	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Alphaproteobacteria	Rhodobacteraceae_OTU_200 **	0.0	0.1	0.1	0.9	0.5	0.3	1.5	4.1	2.8
Gammaproteobacteria	**Plasticicumulans**	0.0	7.8	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Alphaproteobacteria	FukuS110	7.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Actinobacteria	Tetrasphaera	6.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Betaproteobacteria	Comamonadaceae_OTU_25 **	5.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gammaproteobacteria	188up	5.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*Identified to the order level; **Identified to the family level.

rates. In addition, the composition of the polymer was affected by the reduction of SRT also, since the %3HV monomers decreased along with SRT (Table 1). This may have also been related to the microbial community selection (Table 2).

In the famine phase at 0.75 d of SRT, the specific rate of PHA consumption ($-q_{PHA}$) and the specific biomass growth rate (μ) increased in both reactors, but this increase was most dramatic in R2, increasing about 3-fold when compared to 1.7d of SRT. The performance of both reactors was very similar at 0.75 d of SRT, however, R1 was still able to produce more biomass than R2, resulting in an active biomass (X_A) concentration 2 times higher in R1 than in R2. This effect was also observed in previous work at an SRT of 4 d [16], so it appears that R1 maintained a higher X_A concentration than R2 at all SRTs tested, likely due to the higher mass of PHA present in the famine phase in R1, where biomass withdrawal takes place only at the end of the famine phase. Also, the $Y_{X/PHA}$ was significantly higher in R1 than in R2 at SRT 1.7 d and 0.75 d.

One of the factors that impact the overall productivity of a PHA production process the most is the productivity of biomass [9]. Fig. 2 shows the values of the biomass productivity (Prod-X_A) and X_A concentrations for both reactors under the different operational conditions tested. Prod-X_A increases as the SRT decreases in R1, reaching 2.60 ± 0.14 g-X_A.L^{-1}.d^{-1} at an SRT of 0.75 d, however, in R2, Prod-X_A does not follow the same tendency and reaches a steady value of around 1.2 g-X_A.L^{-1}.d^{-1} at a SRT of 2.3 d and below. R2 has less capacity to improve the Prod-X_A by lowering the SRT than R1. At an SRT of 2.6 d and OLR 100 Cmmol.L^{-1}.d^{-1}, no significant difference in the Prod-X_A was observed between reactors. Increasing the OLR from 100 to 200 Cmmol.L^{-1}.d^{-1} seemed to have a positive effect in the Prod-X_A of both reactors, but the increase was higher in R1 than in R2 (172 % vs 88 %). This is likely due to the greater availability of organic carbon for PHA storage and subsequent growth in the famine phase in each system, whereby the biomass wastage prior to the growth phase in R2 would have lessened the impact of the OLR increase on biomass growth.

Accumulation assays

PHA accumulation assays were conducted to determine the maximum % of PHA accumulated by the cultures selected in the selection reactors. The profiles of the PHA content obtained for each OLR and

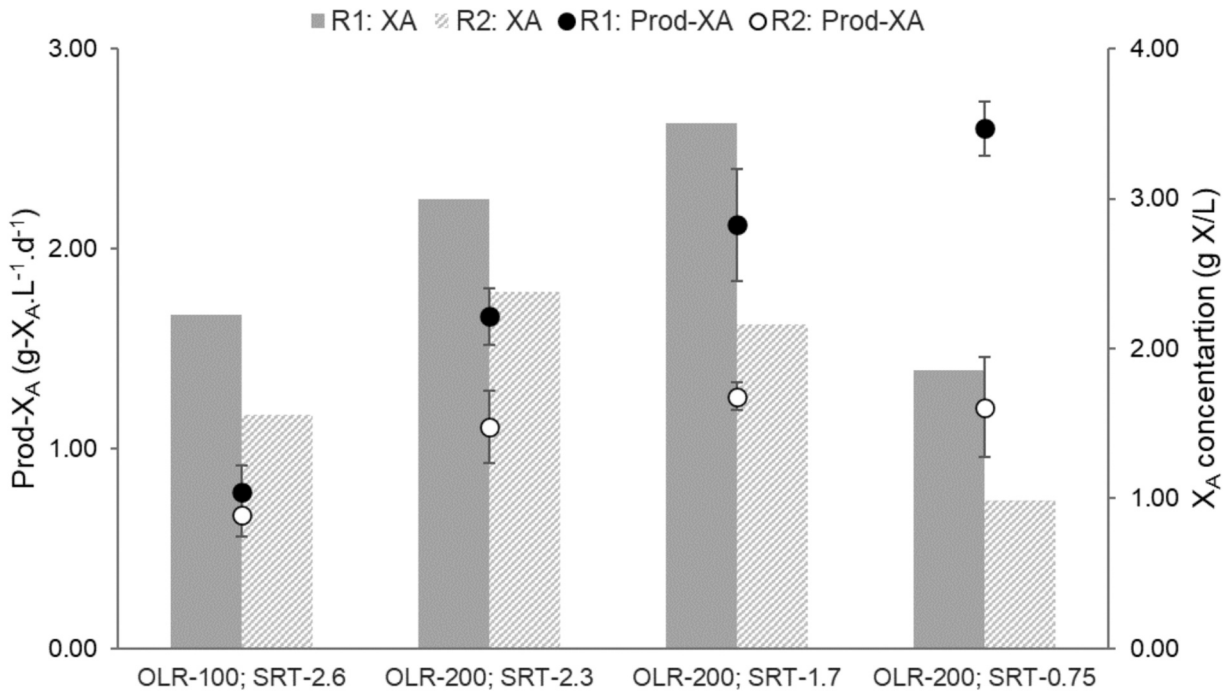

Fig. 2. Productivity and concentration of active biomass (X_A) for both reactors during the periods of different conditions tested.

SRT can be found in Fig. S3 in the Supporting Information. Kinetic and stoichiometric parameters were calculated for the accumulation tests and are presented in Table 3. The initial PHA content of the biomass used in the reactor 2 accumulation (R2-Ac) tests was significantly higher than that of the reactor 1 accumulation (R1-Ac) tests, since biomass for R2-Ac was collected at the end of the feast phase while biomass used in R1-Ac was collected at the end of the famine phase.

The maximum PHA content (PHA^{max}) was significantly affected by the reduction of the SRT, decreasing from 53.9 wt% to 32.9 wt% in R1-Ac at SRT of 1.65 d and 0.71 d and from 46.8 wt% to 34.0 wt% in R2-Ac at SRT of 2.0 d and 0.82 d, respectively (Table 3). At the lowest SRT, there was no significant difference between the %PHA obtained in the accumulation assays when compared with the corresponding selection reactors in both cases. These results may be explained by the loss of capacity of the culture to produce PHA, demonstrated by the reduction of the specific PHA production rate (q_{PHA}) and specific substrate consumption rate ($-q_S$) concomitantly with the decrease in SRT. Consequently, the duration of R1-Ac increased substantially from 3.7 h at SRT 1.65 d to 5.7 h at SRT 0.75 d. In R2-Ac, on the other hand, the inverse tendency occurred. Since the biomass used in the inoculation of the accumulation assays was already containing a high %PHA, it was much closer to the culture's maximum accumulation capacity at the beginning of the accumulation assays than R1-Ac. Accumulation assays at SRT of 0.75 d for both R1-Ac and R2-Ac did not result in a higher PHA content than at the end of the feast phase of the respective selection reactors, suggesting that an accumulation stage would not be beneficial at this SRT. As observed in the selection reactors, there was a dramatic decrease in the HV content in PHA at SRT of 0.75 d, possibly related to the change in the population composition along the study (see Table 2).

Influence of the OLR and SRT on the PHA global productivity

Fig. 3 shows the global PHA productivity calculated based on the accumulation assays and the culture selected in the different operational conditions of OLR and SRT. Additionally, the PHA productivity was also calculated based on the PHA content obtained at the end of the feast phase of R2, so a comparison would be drawn between the operation of the alternative biomass withdrawal strategy in the case of operation without the accumulation stage.

The global PHA productivity was, in all scenarios studied, higher in

Table 3
Summary of the performance parameters calculated based on the replicated PHA accumulation assays performed with biomass selected from reactor 1 (R1-Ac) and reactor 2 (R2-Ac) at different conditions of SRT and OLR tested.

Reactor	R1-Ac	R2-Ac	R1-Ac	R2-Ac	R1-Ac	R2-Ac	R1-Ac	R2-Ac
OLR ($Cmmol.L^{-1}.d^{-1}$)	100		200		200		200	
SRT (d)	2.86 ± 0.33	2.36 ± 0.67	1.10 ± 0.08	2.01 ± 0.09	1.65 ± 0.07	1.72 ± 0.25	0.71 ± 0.05	0.82 ± 0.16
X_A ($g.L^{-1}$)	2.6 ± 0.2	2.1 ± 0.2	3.1 ± 0.1	2.5 ± 0.5	3.6 ± 0.5	1.6 ± 0.1	1.2 ± 0.1	0.9 ± 0.5
Maximum biomass activity*								
Time (h)	1.4 ± 0.06	1.9 ± 0.04	1.8 ± 0.05	1.9 ± 0.3	1.4 ± 0.02	2.3 ± 0.2	5.2 ± 0.3	2.0 ± 0.0
$-q_S$ ($Cmol\text{-}S.Cmol\text{-}X_A^1.h^{-1}$)	0.66 ± 0.1	0.55 ± 0.02	0.41 ± 0.01	0.46 ± 0.07	0.43 ± 0.04	0.30 ± 0.03	0.27 ± 0.10	0.32 ± 0.3
q_{PHA} ($Cmol\text{-}PHA.Cmol\text{-}X_A^1.h^{-1}$)	0.62 ± 0.08	0.39 ± 0.05	0.23 ± 0.01	0.38 ± 0.06	0.38 ± 0.02	0.18 ± 0.03	0.09 ± 0.03	0.18 ± 0.1
$Y_{PHA/S}$ ($Cmol\text{-}PHA.Cmol\text{-}S^{-1}$)	1.0 ± 0.05	0.7 ± 0.07	0.56 ± 0.03	0.84 ± 0.001	0.89 ± 0.04	0.60 ± 0.2	0.33 ± 0.003	0.63 ± 0.1
Overall accumulation performance								
Time (h)	2.9	3.1	5.2	2.2	3.7	2.4	5.7	0.84
PHA^{max} (wt.%)	61.8	57.4	43.9	46.8	53.9	43.4	32.9	34.0
HV(Cmol%)	43.7	43.7	34.6	36.0	41.3	46.4	18.9	23.5
r_{PHA} ($g\text{-}PHA.L^{-1}.h^{-1}$)	1.2 ± 0.1	0.80 ± 0.07	0.39 ± 0.01	0.89 ± 0.2	0.96 ± 0.1	0.51 ± 0.02	0.11 ± 0.01	0.58 ± 0.3

* The maximum rates and yield were calculated for these tests.

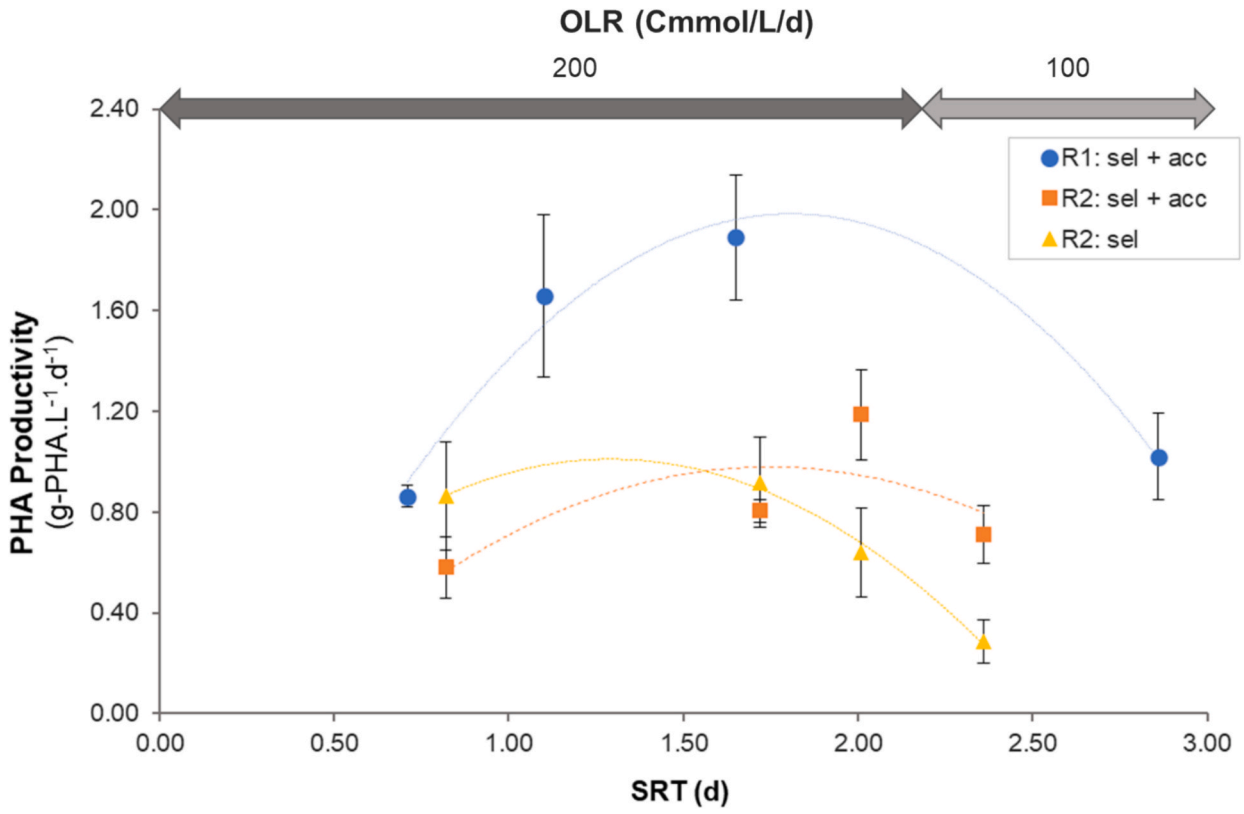

Fig. 3. Global PHA productivity in reactors operated under different biomass withdrawal strategies and different values of SRT and OLR. An alternative in which the selection reactor R2 is used alone is also considered.

the 2-reactor system (culture selection and PHA accumulation reactors) operating with the conventional BW than with the FeBW. The best performance regarding PHA productivity was achieved at an SRT of 1.7 d and OLR of 200 Cmmol.L^{-1}.d^{-1} in R1, corresponding to 1.9 ± 0.2 g-PHA.L^{-1}.d^{-1}, and at the SRT of 2.0 d and OLR of 200 Cmmol.L^{-1}.d^{-1} in R2, corresponding to 1.2 ± 0.2 g-PHA.L^{-1}.d^{-1}. R1 demonstrated a bigger amplitude in PHA productivity for the conditions tested. The lowest value 0.86 ± 0.05 g-PHA.L^{-1}.d^{-1}, was obtained for SRT 0.7d. It can also be noted that the difference between the global productivity of both systems was smaller at the lowest SRT, showing that, at very short SRT, the systems tend to present similar performance. Additionally, when considering the system composed only by the selection reactor R2, the PHA productivity increased as the SRT decreased, due to reaching a stable level of Prod-X_A combined with high PHA content in biomass at the end of the feast phase. This effect resulted in similar PHA productivity when using only the selection reactor R2 and when using an accumulation reactor at an SRT of 1.7 d (0.92 ± 0.2 g-PHA.L^{-1}.d^{-1} and 0.80 ± 0.04 g-PHA.L^{-1}.d^{-1}, respectively) and 0.8 d (0.86 ± 0.2 g-PHA.L^{-1}.d^{-1} and 0.58 ± 0.1 g-PHA.L^{-1}.d^{-1}, respectively). Thus, the utilization of an accumulation stage using the FeBW strategy did not result in a gain in PHA productivity in R2 at the two lowest SRT tested. In addition, the PHA productivity of the selection reactor R2 at the SRT of 0.8 d was the same as that of the system composed by the selection reactor R1 operated with conventional BW and an accumulation reactor at a similar SRT (Fig. 3). These results indicate that at low SRT, the PHA process can be run in a single reactor operated with the FeBW strategy, which could significantly reduce the costs of the PHA production process by bypassing the accumulation stage and redirect the biomass withdrawn directly to downstream processing.

Discussion

Economic assessment

Relevant parameters used for the cost assessment are presented in Table 4. The different SRTs studied above were considered in this analysis. Based on the specific parameters for each configuration, the size of the accumulation reactors (in the conventional BW cases) was calculated for a 500 m^3 selection reactor. The total capital costs (TCC) was calculated based on the bioreactor's costs, since they account for the major part of the capital cost [29]. Although the purpose of this cost assessment is to compare two systems operating with different BW strategies, and thus, with different equipment necessities, the cost of auxiliary equipment is always related to the scale of the bioreactor, so the most important factor to estimate the equipment costs is the scale of the bioreactor. Operating costs were based on the performance of reactors R1 and R2 and their respective accumulation assays. The results obtained for each category are presented in Table 5, including the total

Table 4
Parameters from the systems studied for PHA production.

Parameter	Culture selection + Accumulation stages (Conventional BW)			Selection stage (Feast phase BW)		
SRT (d)	**0.7**	**1.1**	**1.7**	**0.8**	**1.7**	**2.0**
HRT (d)	1	1	1	1	1	1
Cycle length (d)	0.5	0.5	0.5	0.5	0.5	0.5
Biomass productivity (g-X_A.L^{-1}.d^{-1})	2.60	3.03	2.12	1.21	1.26	1.60
PHA Productivity (g-PHA.L^{-1}.d^{-1})	0.9	1.7	1.9	0.86	0.92	0.64
PHA (wt%), final*	32.9	43.9	53.9	41.7	42.1	28.5

* Conventional BW: end of the accumulation stage; Feast phase BW: end of the feast phase of the culture selection stage.

Table 5
Cost assessment of the PHA production process considering the two operational strategies studied.

	Culture selection + Accumulation stages (Conventional BW)			Selection stage (Fe BW)		
SRT (d)	**0.7**	**1.1**	**1.7**	**0.8**	**1.7**	**2.0**
Reactors (Vol, m^3)						
Culture selection	500	500	500	500	500	500
PHA accumulation	710	460	300	n/a	n/a	n/a
Costs (€/yr)						
Capital (annualised)	63 982	54 315	47 335	27 973	27 973	27 973
Utilities	150 704	166 105	147 095	134 068	134 068	134 068
Material	1 047 690	931 180	814 715	546 377	546 377	546 377
Maintenance	18 164	15 420	13 438	7 941	7 941	7 941
Labor	100 000	100 000	100 000	100 000	100 000	100 000
TAC	1 380 540	1 267 021	1 122 583	816 359	816 359	816 359
Price (€/kg)						
Break-even price (BEP)	2.22	2.20	3.22	1.88	3.79	4.34

annual costs (TAC) and the break-even price (BEP).

The costs of production were consistently higher in all categories in the process configuration using culture selection and accumulation stages than in the configuration using only the selection reactor (Table 5). An extra reactor increases the equipment costs, and consequently the AC, but also the utilities increased, due to the energy spent aerating and mixing the accumulation reactor and the carbon substrate used for PHA production in the accumulation stage. The size of the accumulation reactor was considered to be double of the withdrawn biomass, thus the size of the bioreactor is intrinsically related to the SRT applied to the system. The lower the SRT, the higher the volume of biomass available for the accumulation stage, and so, the higher the volume of the bioreactor needed to accommodate the biomass withdrawn from the selection reactor plus the substrate pulses fed during the assay. The utilities costs are related to the energy spent on the process. The increased energy spent during the system operation with conventional BW is related to the operation of the accumulation reactor, and it varies between the different parameters used, because it is expected that the accumulation stage has different duration at different SRT (from 3.7 h at a SRT of 1.7d and 5.7 h at a SRT of 0.7 d). The material costs of the configuration operating with the conventional BW strategy was nearly double that of the configuration using feast phase BW strategy. The conventional BW strategy has a much higher demand for substrate, since carbon substrate entering the culture selection is utilized only to produce biomass, while in the feast phase BW strategy, the carbon substrate is utilized to produce biomass and PHA. Therefore, additional carbon must be supplied during the PHA accumulation stage to produce PHA in the conventional approach. Maintenance costs were directly estimated from the TCC, and therefore are higher in the conventional process configuration due to the higher equipment investment. Labor was considered the same in both approaches, but the reduced complexity of operation of the system operating under the feast phase BW strategy could reduce the labor costs. Consequently, the TAC was higher in all conditions tested for the conventional BW strategy than for the feast phase strategy. The TAC for each condition tested for the feast phase strategy was the same, because the system and mode of operation used are also the same.

The break-even price (BEP), in terms of Euro per kg of crude PHA, was calculated to better compare the different process configurations and conditions tested (Table 5). The strategy that resulted in a lower BEP was the configuration with only a selection reactor with feast phase BW at a SRT of 0.8 d (1.88 €/kg), followed by the configuration with selection and accumulation reactors operated under conventional BW at a SRT of 1.1 d (2.20 €/kg) and 0.7 d (2.22 €/kg). Both strategies resulted in a BEP higher than 3€/kg PHA-crude when the SRT was $\geq$ 1.7 d, where the conventional BW strategy substantially outperformed the feast phase BW strategy in these cases. It appears that the potential success of the FeBW strategy hinges upon its implementation at low SRT. These results support that lower SRT is economically beneficial due to the higher production of PHA-producing biomass. [29] found that the minimum estimated production cost of the raw PHA were between 1.26 and 2.26 US$/kg PHA-crude (1.22 and 2.19 €/kg PHA-crude) for a PHA production process implemented in an existing wastewater treatment plant (WWTP). The BEPs calculated in this work are in the same range as those previously referred, even when considering that the carbon substrate was a synthetic mixture of VFA. It is expected that when using fermented residues, the material costs could be significantly reduced (the highest cost fraction in this work). It should be noted that this process also consumes a substantial quantity of water (500–1200 m^3/d), resulting from the need to prepare the substrate and nutrient solutions to be fed to the reactors. Therefore, it is proposed to implement a strategy to recover and reuse, at least partially, the water used in this process. To improve the costs and environmental impact of the process, wastewater streams rich in ammonium and phosphate could be used to replace the commercial nutrient substrates considered in this analysis. There are also some studies that propose to implement the PHA production system directly in a WWTP. Besides significantly reducing the costs of the substrate, it would increase the cash flow of the WWTP, as the price of PHA is considerably higher than that of biogas [29,23]. PHA production helps to close the material loops in WWTPs according to the circular economy model, recovering waste carbon and using it for a productive purpose.

Another important aspect of the process is the content of PHA in the biomass at the end of the production stage. PHA content impacts directly on extraction costs [30]. Usually, a minimum of 40 % is required for downstream economic recovery of the polymer [29]. Due to their lowest break-even prices (Table 5) as well as their high PHA content of $>$ 40 % (Table 4), the operation of a selection reactor with the feast phase BW strategy at a SRT of 0.8 d appears to be the most advantageous process configuration, despite not displaying the highest PHA productivity. Furthermore, if the OLR and the PHA content in the biomass could be further increased while operating the selection reactor with feast phase BW, the costs of extraction could be reduced, since they are related to the PHA content [30]. It should be acknowledged, however, that utilization of a system composed of culture selection and accumulation reactors with conventional BW strategy at a SRT of 1.1 d was also a potentially favourable combination of conditions, with a low BEP and high PHA productivity. Depending upon the revenue that could be generated from the crude PHA product, this combination of conditions would also be worth considering to help maximise profit from a mixed microbial culture approach to PHA production.

There are still other opportunities for the improvement of the economics of PHA in MMCs. Nonetheless, as this study shows, maximizing PHA productivity does not necessarily result in the most favorable process from an economic standpoint and economic analyses should be considered in PHA production optimisation studies.

Conclusions

The findings of this study showed that the highest PHA productivity was achieved by the conventional BW strategy at an SRT of $\sim$ 1.7d, however, the lowest break-even price was the FeBW strategy at an SRT of 0.8d. At very low SRT ($<$1 d), the PHA productivity of systems operated with either BW strategy became very similar, and the productivity of the culture selection reactor operated with FeBW was similar to that of the 2-reactor systems. In this case, biomass with 30–40 wt% PHA content could be harvested and directed to downstream

processing, by-passing the accumulation stage. This mode of operation of the PHA production process with only 2 stages (fermentation and culture selection reactor) results in a significant reduction of the initial investment costs (mainly lower equipment costs) and savings in energy and feedstock costs, a critical factor to improve the competitiveness of PHA in the plastic industry. Also, using a SRT of 1 d could significantly reduce the complexity of operating the system, since an HRT of 1 day is often used and, in this situation, the hydraulic and solids withdrawal can be accomplished in the same step, reducing the need for a sedimentation phase, resulting in a longer reaction phase. This would also render irrelevant any need for biomass sedimentation, thus de-risking any loss of suspended solids in the effluent.

CRediT authorship contribution statement

Rafaela A.P. Cruz: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Adrian Oehmen:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Maria A.M. Reis:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiec.2025.08.030.

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