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Influence of the total concentration and the profile of volatile fatty acids on polyhydroxyalkanoates (PHA) production by mixed microbial cultures

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Abstract

Polyhydroxyalkanoates (PHAs) production from lignocellulosic biomass using mixed microbial cultures (MMC) is a potential cheap alternative for reducing the use of petroleum-based plastics. In this study, an MMC adapted to acidogenic effluent from dark fermentation (DF) of exhausted sugar beet cossettes (ESBC) has been tested in order to determine its capability to produce PHAs from nine different synthetic mixtures of volatile fatty acids (VFAs). The tests consisted of mixtures of acetic, propionic, butyric, and valeric acids in the range of 1.5–9.0 g/L of total acidity and with three different valeric:butyric ratios (10:1, 1:1, and 1:10). Experimental results have shown a consistent preference of the MMC for the butyric and valeric acids as carbon source instead other shorter acids (propionic or acetic) in terms of PHA production yield (estimated in dry cell weight basis), with a maximum value of 23% w/w. Additionally, valeric-rich mixtures have demonstrated to carry out a fast degradation process but with poor final PHA production compared with high butyric mixtures. Finally, high initial butyric and valeric concentrations (1.1 g/L and 4.1 g/L) have demonstrated to be counterproductive to PHA production.

Keywords Polyhydroxyalkanoates · Bioplastic · Volatile fatty acids · Mixed microbial cultures

1 Introduction

Polyhydroxyalkanoates (PHAs) are bio-polyesters synthesized intracellularly by some microorganisms. Its main function is to serve as a carbon and energy reserve in stressful situations in which the growth of microorganisms is unfavorable (inadequate balance of nutrients, scarcity of carbon sources, heat stress, etc.) [1, 2]. Their main practical application is based on the fact that they present physical properties similar to certain plastics derived from petroleum. For this reason, there is a growing interest in the industrial sector for their production since they are precursors of plastics with excellent biocompatibility characteristics and biodegradability [3]. Moreover, these polymers are thermoplastic and/or elastomers, insoluble in water, and have a high polymerization degree. They are also pure compounds in relation to their enantiomeric structure; are non-toxic and biocompatible; have a high crystallinity degree (between 60 and

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80%); are optically active; and also exhibit piezoelectric properties. These properties make them especially interesting to replace some plastics derived from petroleum; more specifically, they agree in properties in a very relevant way with polypropylene [1, 4].

The main advantage of PHA polymers over petroleum-derived plastics is that they are biopolymers that can be produced from vegetal biomass and even from various kinds of organic wastes. Their chemical structure makes them easily biodegradable both in soils and in aqueous environments through aerobic or anaerobic degradation processes [5, 6]. All this implies that they are not materials harmful to the environment being, therefore, a sustainable product obtained from renewable materials that replaces plastics from fossil sources [7].

Despite the great advantages that PHAs have over petroleum-derived plastics, their current use is very limited due to their high production cost, which is largely attributed to the use of carbonaceous substrates of industrial interest. In addition, the most studied PHA production processes focus on the use of pure strains of microorganisms, such as *Cupriavidus necator*, that presents a high PHA accumulation yield in dry weight but increases the production costs due to the requirements to avoid the contamination with other microorganisms [8–11]. As a more economical alternative, some authors have proposed the use of wastewater, wastes, and residual biomass as cheap carbon sources for the process [7]. It is also possible to make the process cheaper by using mixed microbial cultures (MMC) instead of pure cultures, although PHA productivity declines notably [1, 12–14]. In addition, the diversity of microorganisms in MMC could lead to biopolymers with non-defined properties since each microorganism can produce different PHA monomers [15].

Volatile fatty acids (VFAs) are excellent substrates for the production of PHA by MMC [16]. Therefore, the utilization of the dark fermentation (DF) process to obtain VFAs from different biomass waste is a very interesting option for the aerobic production of PHAs [7, 17, 18]. Thus, acidogenic anaerobic digestion (AAD) or commonly DF is a variation of the classic biological process of anaerobic digestion in which the stages that involve methanogenesis do not occur since operating conditions are used to prevent it. Thus, the microorganisms involved in DF transform organic matter into short-chain volatile fatty acids (VFAs) and produce biogas rich in carbon dioxide and mainly hydrogen [19, 20].

With regard to the VFAs profile, the proportion between the different acids has a great influence on the mechanical properties of the final plastics and their subsequent industrial application. In the PHA production with MMC, acetic and butyric acids favor the production of 3-hydroxybutyrate (3HB) while propionic and valeric acids promote the synthesis of 3-hydroxyvalerate (3HV) [16, 21]. Poly(3-hydroxybutyrate) (P (3HB)) is brittle and rigid and therefore has limited industrial applications. However, the incorporation of 3HV into P(3HB) leads to the formation of the copolymer P (3HB-co-3HV), which is more flexible and harder. In addition, the latter is less permeable to oxygen compared to polyethylene and commercial polypropylene, making it a suitable material for food packaging.

Among the various types of residual biomass that can be used for the biosynthesis of PHAs, exhausted sugar beet cossettes (ESBC) have been chosen in this study to develop an inoculum enriched in PHA producers. The ESBC is generated during the sugar extraction process from sugar beet and it is an agrifood byproduct with a low lignin content [22]. In this context, the objective of this work is to study the influence of the VFAs profile and the total acidity concentration on the PHA yield using a MMC as inoculum coming from a sequential process coupling DF and aerobic PHA production that used ESBC as substrate. To study the influence of these variables on the PHA production, synthetic mixtures of VFAs were used.

This study reveals the existence of some counterproductive limits to PHA production by MMC linked to the presence of high concentrations of valeric and butyric acids. Likewise, the study shows as the ratio between butyric and valeric acids affects PHA yield.

2 Materials and methods

To address the proposed objective, three sets of batch tests have been carried out with 1L-bioreactors fed with a mixture of synthetic acids, simulating the VFAs profile typical of the DF of ESBC but modifying the proportions of valeric and butyric acids. The batch tests have been carried out in duplicate. Nine different conditions were studied modifying the valeric:butyric ratio and the total volatile acidity as it is showed in Table 1.

Table 1 Nomenclature applied to identifying assays and experimental conditions

Code	Total VFAs concentration (g HAc/L)	Valeric:butyric ratio
1.5B	1.5	1:10
2.5B	2.5	1:10
3.5B	3.5	1:10
3.0 N	3.0	1:1
3.5 N	3.5	1:1
5.0 N	5.0	1:1
5.0 V	5.0	10:1
7.0 V	7.0	10:1
9.0 V	9.0	10:1

2.1 Obtaining the PHA-producing inoculum

The inoculum used in the batch tests comes from a sequential system of two semi-continuous reactors coupled in series.

The first reactor is a 5L-anaerobic and thermophilic stirred tank reactor (55 °C) which has been fed with ESBC that produces hydrogen-rich biogas and a VFA-rich effluent (acetic, propionic, butyric, valeric, etc.). The reactor was started from an anaerobic reactor that operated under methanogenic anaerobic digestion conditions. In this reactor, to produce the complete washing of the methanogenic microbiota in 21 days, a strategy of reducing the hydraulic retention time (HRT), from 18 to 6 days, and the pH until 5.5 as set point was applied. This reactor was in stable operation for more than 250 days with an average production of biogas (hydrogen and carbon dioxide) at the moment to perform the batch tests over 1.5–2.0 $L/L_{reactor}$·day and with an average hydrogen percentage of 30–40% v/v in the biogas.

The liquid fraction of the acidogenic effluent from this first reactor was used to feed a second reactor destined for the PHA production, with a stable population of microorganisms selected from mixed sludge from a wastewater treatment plant (WWTP). A solution of nutrients recommended in the literature was added to this liquid fraction to enhance the PHA production [23]. The total volatile acidity concentration in the acidogenic effluent was maintained in the range of 10–20 g HAc/L.

In the second 5L-reactor for the PHA production, a microbiota capable of producing PHAs was selected, applying a strategy based on alternating oxic and anoxic periods of 24 h, coinciding with periods of scarcity and abundance of the substrate (famine-feast cycles). During the oxic periods, the soluble oxygen concentration was kept between 6 and 8 mg/L. The operating HRT, which was initially set at 12.5 days, was reduced to 9 days once the selection of the PHA-producing microbiota was made to increase the rate of the process. Throughout this process to obtain a stable PHA-producing inoculum, the PHA production yields were among 20–40 mg PHA/L, with values close to 60 mg PHA/L (13% dry cell weight) when it was used for the inoculation of batch reactors. The reduction of organic matter, in terms of soluble chemical oxygen demand (COD), was around 90–95% with an organic loading rate of 2.5 g COD/$L_{reactor}$·day at the moment in which it was used as inoculum for the start-up of the batch tests.

2.2 Study of the effect of total acidity and the VFA profile on the PHA production

To carry out this study, nine duplicated tests were carried out operating in batch mode and using 1-L reactors with a 50:50 (v/v) substrate to inoculum ratio. The inoculum used comes from the inoculation PHA reactor described in the previous section. The substrate was composed of a synthetic mixture of acetic, propionic, butyric, and valeric acids in a nutrient solution previously indicated. All the trials were performed by combining these conditions according to Table 1. Albuquerque et al. [24] have reported that no significant variations were observed in terms of PHA yield, maximum PHA content, and PHA composition between the synthetic and fermented feedstocks.

At the inoculation time, in the effluent from the acidogenic reactor, the average proportions of acids were 83.2:1.0:43.9:2.0 in terms of acetic:propionic:butyric:valeric, respectively. In all the synthetic mixtures, concentrations of acetic and propionic acids were incorporated in the same proportion of the acidogenic effluent obtained from the DF reactor but modifying the proportions between butyric and valeric acids, with respect to their original proportion with acetic acid, in order to obtain the valeric:butyric ratios to be tested (10:1; 1:1; and 1:10).

Given the results obtained for the first set of assays with high butyric acid proportion, it was decided to modify the initially planned experimental set up in the study. Thus, it was decided to check, in the second set of assays (neutral ratio assays), a range of total acidity concentrations higher than the previous ones (1.5–3.5 g/L) but maintaining the butyric acid concentration lower than 1 g HBu/L.

Finally, for the third set of assays (high valeric assays), it was considered appropriate to continue increasing the total volatile acidity concentration but limiting the presence of butyric acid in order to analyze the independent influence of valeric acid on the PHA production in a more evident way.

Each set of tests consisted of six reactors (three conditions by duplicate) with synthetic mixtures. The tests have been carried out at 25 °C and with a constant aeration rate of 20 $L_{air}/L_{reactor}$·h in order to maintain an oxic environment and to promote the PHA production. The system used the forced aeration itself for homogenizing the content of the reactors.

2.3 Analytical techniques

The analytical determinations were carried out according to the standard methods for wastewater [25] adapted to high organic content waste. For the monitoring of the reactors, the following parameters were analyzed: pH (4500-H^+), dissolved oxygen (DO) (4500-O), volatile suspended solids (VSS) (2540-B/E), COD (5220-C), and dissolved organic carbon (DOC) (5310-B).

For the determination of DO, an oximeter (Labprocess® OXY 70) was used based on an optical luminescence cell. The DOC was determined by means of a carbon analyzer (Analytik Jena® multi N/C 3100) with samples previously filtered by 0.47 μm.

Additionally, the quantification of the total PHA concentration was performed by UV spectrophotometry at 235 nm

after an extraction process according to the procedure described in the literature [26, 27].

The individual VFAs were measured by gas chromatography in a Shimadzu® GC-2010 equipped with a flame ionization detector (FID) and a fixed phase column of the Nukol® type (polyethylene glycol modified with terephthalic acid). Hydrogen was used as carrier gas at a flow rate of 50 mL/min. The temperature of the injection port and the detector were 200 °C and 250 °C, respectively [28].

The biogas generated by the acidogenic anaerobic reactor was collected in Tedlar® gas bags (SKC® 232 series) and it was quantified using a wet rotary drum gas flow meter (Ritter® TG5) connected to a Laboport gas suction pump (KNF®, model KT18) with a capacity of 5.5 L/min under standard conditions of pressure and temperature. The biogas composition from the acidogenic anaerobic reactor was determined by a gas analyzer EMERSON® X-Stream.

2.4 Inoculum

PHA-producers bacteria have been identified in the effluent of the PHA inoculum reactor. For that, the sample was serially diluted and plated on LB (Luria–Bertani) and VKM culture medium. VKM medium contained acidogenic effluent:nutrient solution:distilled water, 3:4:3 ratio, supplemented with yeast extract (1 g/L) and agar (25 g/L). The plates were incubated at 25 °C for 7 days. For each medium, several representative types of colonies were identified and purified after several passages on the same type of medium.

The most representative colonies were isolated and genomic DNA was extracted following the protocol described by Saba et al. [29]. The isolates were typed by sequencing the 16S rRNA gene that had been amplified by PCR from genomic DNA [30]. Similarity searches were performed with the Basic Local Alignment Search Tool (BLAST) [31] on the DNA sequences with the Genbank® database.

3 Results and discussion

3.1 Verification of the presence of PHA producers in the inoculum

A total of 20 bacteria were isolated from the PHA production bioreactor. The 16S ribosomal RNA sequences of the isolates were matched with the contents of the databank by Nucleotide BLAST. At a percent identity score of ≥ 97%, a total of 8 different bacteria genera were identified: *Agrococcus* (98.4%), *Corynebacterium* (97.2%), *Cupriavidus* (97.5%), *Enterobacter* (98.9%), *Microbacterium* (98.9%), *Salana* (97.1%), *Stenotrophomonas* (99.7%), and *Pseudoclavibacter* (99.1%). *Cupriavidus* (n = 6 isolates tested)

was the most common genus in the inoculum, followed by *Corynebacterium* (n = 5) and *Enterobacter* (n = 4), while *Salana*, *Pseudoclavibacter*, *Agrococcus*, *Stenotrophomonas*, and *Microbacterium* were identified in one isolate. Of these, the genera *Enterobacter* [32], *Corynebacterium* [33], *Cupriavidus* [34, 35], and *Microbacterium* [36, 37] have been previously described as PHA producers. In addition, *Cupriavidus necator* is the most widely used Gram-negative bacterium due to its high PHA production capacity [8].

3.2 pH and oxygen

Except on day 0 (start-up) when some reactors had pH below 7, which was immediately corrected, the pH values always remained in a very stable range between 8 and 9, with a slight trend to increase. This range of values agrees with data reported by Chua et al. [38] using batch reactors fed with urban wastewaters.

On the other hand, DO concentration showed the opposite behavior to the PHA accumulation, decreasing gradually as the VFAs were consumed (see Sect. 3.3). Thus, the lowest values were reached when PHA production peaked while the initial DO level was recovered once the microorganisms begin to consume the PHAs formed due to a shortage of other available carbon sources. Similar results were obtained by Mannina et al. [13] working with acetic- and acetic-propionic-based feedings.

In order to illustrate this behavior, the evolution of pH, DO oxygen, and PHA production in the assay called 1.5B has been selected to be shown in Fig. 1. The rest of the tests have similar evolutions of the mentioned parameters.

3.3 Organic matter removal

This section presents the evolution of COD, DOC, and total volatile acidity as representative parameters of the organic matter available for the PHA production in the batch tests.
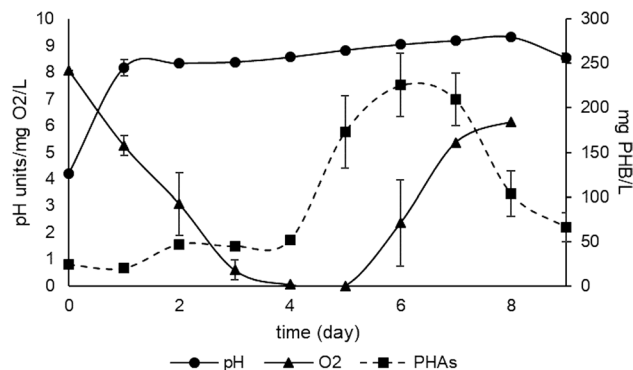
Fig. 1 pH in pH units, DO in mg O$_2$/L, and PHA production expressed as mg PHB/L evolutions for the assay 1.5B

Figure 2 shows the evolution of the COD in the reactors grouped by the valeric:butyric ratio. All evolutions show a clear decreasing trend except for the assay named 3.5B where the decrease was slow and weak and, hence, as far as the trial progresses, the amount of organic matter is lower due to the degradative activity of the microorganisms.

However, the diminishing does not occur in the same way for tests that have the same valeric:butyric ratio but different total volatile acidity concentration. In general, the decline is rapid (reaching the final levels in 6–8 days) except for test

Fig. 2 Total COD evolution expressed in mg O_2/L. **a** Assays with 1:10 valeric:butyric ratio; **b** assays with 1:1 valeric:butyric ratio; **c** assays with 10:1 valeric:butyric ratio

3.5B where the high butyric acid concentration seems to be a problem for COD reduction. The literature [39] indicates that if *C. necator* has optimal growth conditions (including enough carbon and nutrient sources), the acetyl-CoA is massively incorporated into the Krebs cycle. This metabolic process involves great CoA production. Consequently, the excess of CoA inhibits the actuation of 3-ketothiolase (the enzyme related to acetoacetyl-CoA production which is the first step in the PHA production pathway). Therefore, in optimal condition, the PHA production could be stopped in the first step [39]. An increase in substrate concentration could derive in a PHA production inhibited by the substrate as some authors have confirmed [40–43]. While the other assays with the same ratio valeric:butyric (1.5B and 2.5B) reduce their concentrations to less than half in 4 days, in the 3.5B trial, the degradation is weak and delayed. Nevertheless, when the proportion of valeric acid is highest (5.0 V, 7.0 V, and 9.0 V), the degradation is still fast and very uniform even when the total volatile acidity increases. This fact shows that the system presents a lower sensitivity to high valeric acid concentrations (up to 4 g/L in assay 9.0 V) as it will be seen in the following sections.

The other two parameters related to available organic matter that have been analyzed are DOC and total volatile acidity. Their evolutions are shown in Figs. 3 and 4, respectively. As for the previous COD graphs, the results have also been grouped by their valeric:butyric ratio.

In a much more evident way than in the case of COD, a gradual decrease in DOC concentration has been observed. It should be noted that the final levels after 8–9 days are very similar regardless of the initial concentration of total volatile acidity (419–453 mg C/L and 183–227 mg C/L in assays V, and N, respectively), except for the highest butyric concentration assay (503 mg C/L in 3.5B versus 93–179 mg C/L in 1.5B, and 2.5B, respectively). This test, as it was previously noted in the COD evolution, seems to have different behavior and it could have developed an incomplete substrate degradation for the duration of the test. Moreover, the 3.5B test also showed an initial period of 4–5 days with a notably lower COD consumption rate than the other tests.

In the case of the total volatile acidity, determined as the weighted sum of the concentrations of individual VFAs (C2-C7) and expressed in units of mg HAc/L, decreasing is gradual and clear. VFAs are the ultimate substrate that would lead to the PHA production.

It could be observed that high valeric acid concentration assays (Fig. 4c) have shown a sharper and more continuous VFAs degradation rate than high butyric (Fig. 4a) and neutral ratio assays (Fig. 4b). Thus, a much more uniform trend can be observed for the degradation of total volatile acidity in the high valeric acid content assays.

As it was observed for COD and DOC evolutions, the behavior of the 3.5B test is also differentiated. In fact, VFAs
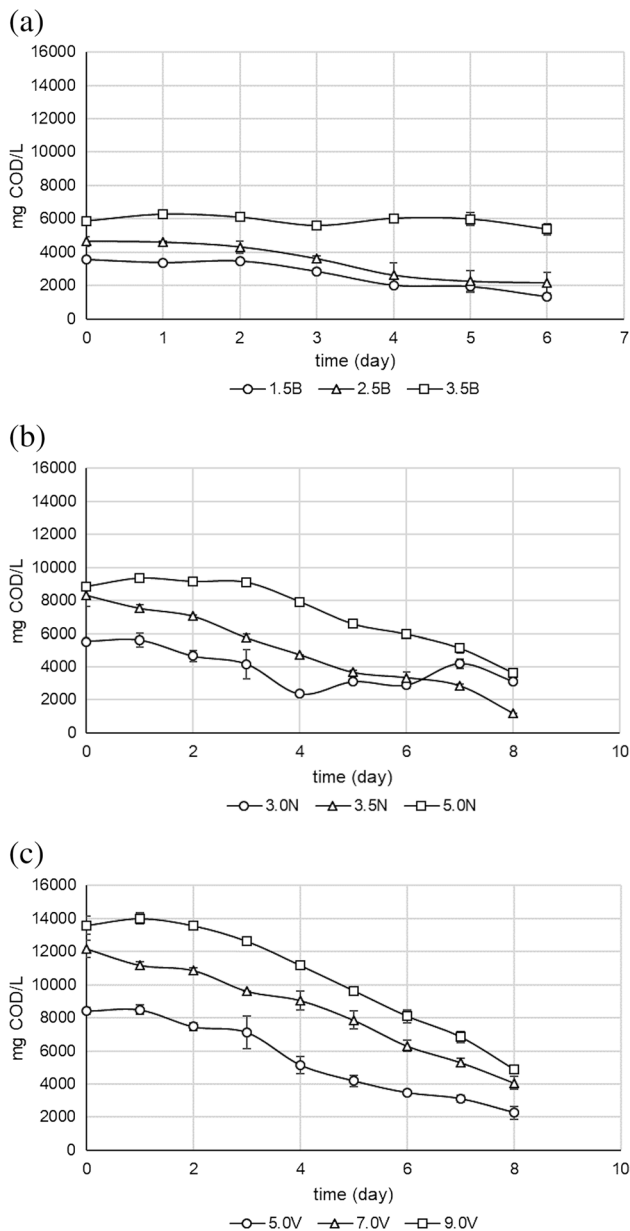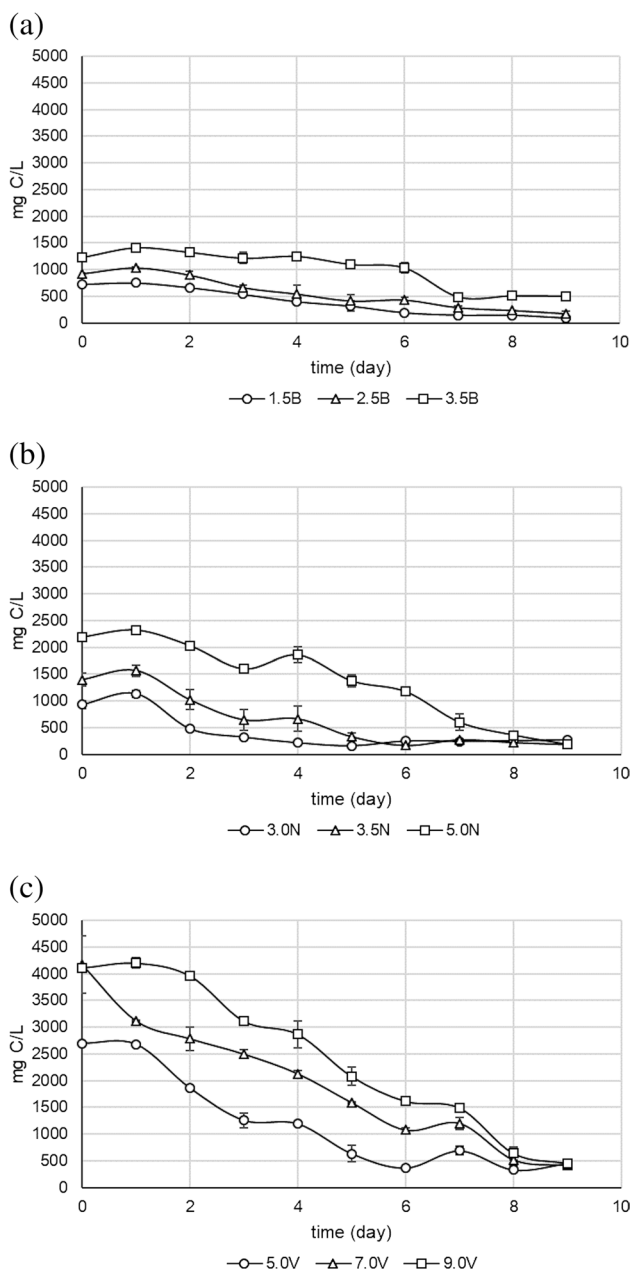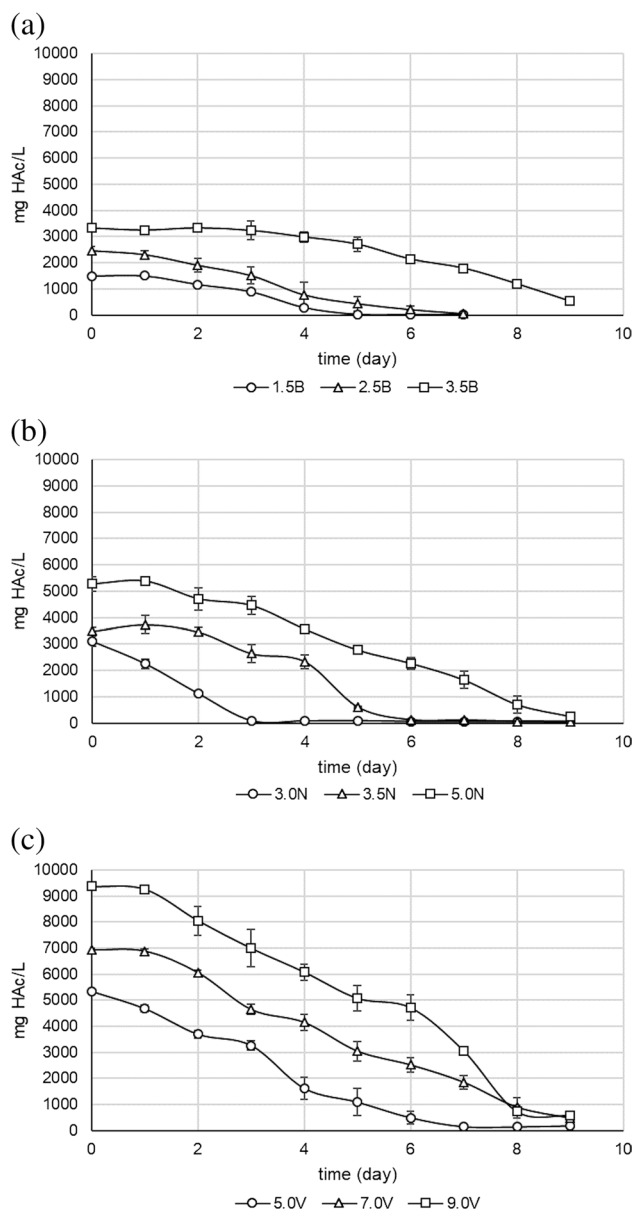
(a)

(b)

(c)

Fig. 3 DOC evolution expressed in mg C/L. **a** Assays with 1:10 valeric:butyric ratio; **b** assays with 1:1 valeric:butyric ratio; **c** assays with 10:1 valeric:butyric ratio

(a)

(b)

(c)

Fig. 4 Total volatile acidity evolution expressed in mg HAc/L. **a** Assays with 1:10 valeric:butyric ratio; **b** assays with 1:1 valeric:butyric ratio; **c** assays with 10:1 valeric:butyric ratio

3.4 PHA production coupled to VFA consumption

The analysis of the individual evolution of the four VFAs used in the synthetic feed allows observing how their consumption is coupled to the PHA production. For each condition, a graph has been built in order to analyze how the total volatile acidity and valeric:butyric ratio influence the PHA production (see Fig. 5).

degradation was slower and delayed in 3.5B compared with 1.5B and 2.5B.

In short, the analysis of DOC, COD, and total volatile acidity denotes that all the tested conditions, except 3.5B which corresponds to the highest butyric acid concentration, have been successful in removing a high proportion of organic matter in a very short time.

3.4.1 High butyric assays (1.5B, 2.5B, and 3.5B and Fig. 5a, 5b, and 5c)

Acetic and butyric are major acids at the beginning of the assays. Both acids have been gradually degraded until PHAs, reaching a maximum PHA production around day 6 for the tests with the lowest total volatile acidity (1.5B and 2.5B). From day 6, the microorganisms in the absence of a carbon source started to degrade the formed PHA for their cellular maintenance and catabolic needs [2].

However, the test 3.5B has shown a differentiated behavior. In this case, there are evident difficulties to begin to degrade the butyric acid, which remains above the level of 1100 mg/L for 4 days. As consequence, a delay in the PHA production takes place. Once the degradation of butyric acid starts, PHA production increases and reaches a maximum value around day 8.

Before day 4, PHA production seems to be coupled almost exclusively to acetic acid consumption. It seems evident that the presence of high butyric acid concentrations has partially inhibited the PHA production by slowing down the whole process. In this sense, Wang et al. [43] had already established that under high initial concentrations of short-chain VFAs (C2-C4), the growth of *C. necator* was markedly impaired. In their kinetic analysis, they established the onset of inhibition at 125 Cmmol of total volatile acidity, using a synthetic mixture with a proportion of 11:9:4 for acetic:propionic:butyric. Therefore, in the inhibitory threshold of 125 Cmmol total volatile acidity, the concentration of each acid was 3.4 g acetic/L, 3.5 g propionic/L, and 1.8 g butyric/L, slightly higher than the concentration of butyric acid used in this study.

In addition, the final maximum PHA concentration reached in 3.5B test is half of that achieved in the lower concentration tests (1.5B and 2.5B), indicating that PHA accumulation is not favored at high butyric acid concentrations. The maximum PHA concentrations were 225, 215, and 104 mg/L for 1.5B, 2.5B, and 3.5B, respectively.

3.4.2 Balanced valeric:butyric ratio assays (3.0 N, 3.5 N, and 5.0 N and Fig. 5d, 5e, and 5f)

For balanced valeric:butyric ratio, a very different behavior is observed in each assay. The 3.0 N assay immediately started the degradation of all the VFAs (acetic, propionic, butyric, and valeric) at very high rate, reaching the maximum PHA production after only 3 days. These acids were depleted in reverse order of their initial concentrations. Furthermore, in 3.0 N and 3.5 N test, valeric and butyric acids were simultaneously consumed at the same time at a coincident rate.

However, in the assay 3.5 N, the maximum PHA level was reached at day 5 and does not match with the complete consumption of all VFAs. As it can be seen in Fig. 5e, the maximum PHA production occurs when butyric and valeric acids were depleted on day 5, although there is still a residual acetic acid concentration. This time delay to reach maximum PHA production versus 3.0 N test is due to the apparition of an initial latency periods of 2 days for butyric and valeric consumption and 4 days for acetic acid. From day 4, once the valeric and butyric acid concentrations were almost depleted, a sharp consumption of acetic acid occurred. However, its contribution to increasing the maximum of PHA production was minor.

In this sense, Wang et al. [23] have found that the use of valeric and butyric acids is preferential to the use of acetic and propionic acids even at low DO concentrations. In addition, Marang et al. [44] reported that butyric acid seems to be the preferred carbon source over acetic acid for PHB production in a study with sequential biological reactors (SBR) operated under feast-famine conditions where *Plasticicumulans acidovorans* was the predominant microorganism. The theoretical PHA production from butyric acid has a stoichiometric yield of 0.94 Cmol PHB/Cmol HBu, which is 40% higher than the yield associated with acetic acid [45].

In summary, for balanced valeric:butyric ratio, the results have shown a delay in the maximum PHA production and the appearance of lag periods. These facts seem to indicate that the change in valeric:butyric proportion (high butyric to neutral balance) has made the system more sensitive to the increase of butyric acid concentration. This distortion could be observed in 3.5 N where initial butyric acid concentration (568 mg HBu/L) was comprised between the butyric acid concentrations in 1.5B (488 mg HBu/L) and 2.5B (824 mg HBu/L) and clearly under the butyric acid level in 3.5B (1130 mg HBu/L). Therefore, the presence of important concentrations of valeric acid has negative effect on the PHA production.

Finally, about the effect of total acidity concentration on the PHA production, once again, when the acidity is increased up to 5 g/L (5.0 N), the consumption of acetic acid slows down with respect to the consumption of valeric and butyric acid, which again occurred simultaneously. As consequence, the final acetic consumption contributed to the delay of the PHA production. As it occurred in 3.5B, high total acidity concentrations induce a PHA production slower and minor. In this way, the increase in total acidity concentration has impaired the process, reaching 155, 127, and 48 mg/L of maximum PHA production for 3.0 N, 3.5 N, and 5.0 N tests, respectively.

Although the initial butyric acid concentration is lower in 5.0 N (920 mg/L) than in 3.5B (1130 mg/L), the cumulative effect of the presence of a high initial total volatile acidity concentration in 5.0 N has reduced the maximum PHA production to one third. This observation indicates that as the total volatile acidity increases the metabolism of PHA
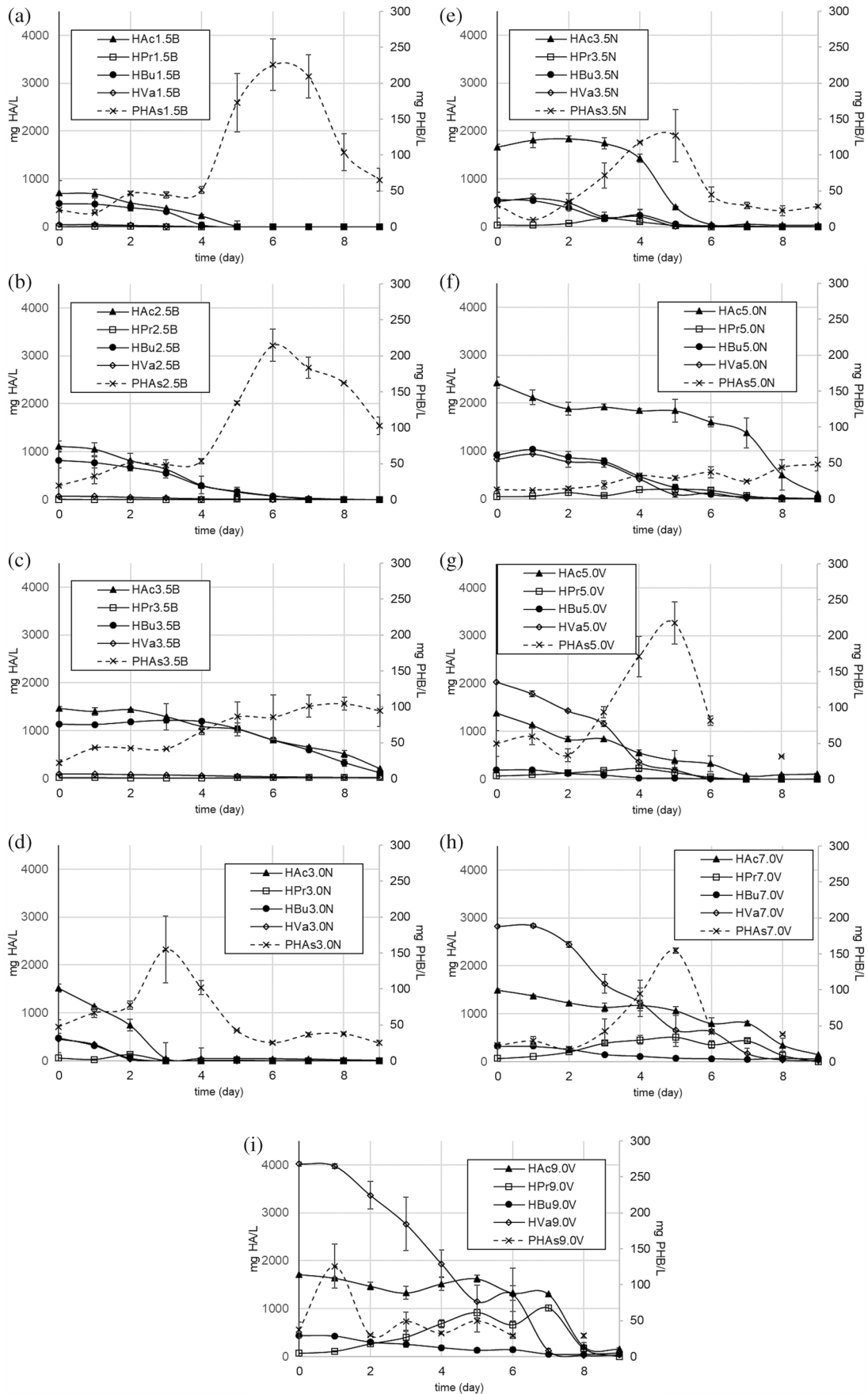
◀**Fig. 5** Evolutions of the individual VFAs (acetic, propionic, butyric, and valeric) expressed in mg/L and PHA production expressed in mg PHB/L. **a** Assay 1.5B; **b** assay 2.5B; **c** assay 3.5B; **d** assay 3.0 N; **e** assay 3.5 N; **f** assay 5.0 N; **g** assay 5.0 V; **h** assay 7.0 V; **i** assay 9.0 V

production is less favored [40–43]. However, is not clear the relative magnitude of the imparaing effect of valeric and butyric acids.

3.4.3 High valeric assays (5.0 V, 7.0 V, and 9.0 V and Fig. 5g, 5h, and 5i)

In this case, the tests were less sensitive to high volatile acidity concentrations. In 5.0 V test, which contained up to 2 g valeric/L, acetic and valeric acids were degraded rapidly, reaching the maximum PHA production on day 5. Nevertheless, this maximum is ahead of the total consumption of both acids.

This could be similar to previously discussed for the 3.5 N test, where the final acetic acid consumption was delayed from the maximum PHA production. Hence, the maximum PHA production seems to be decoupled to the complete consumption of both acids. In fact, it is remarkable that only in these high valeric concentrations, propionic acid concentrations reached 511 mg/L on day 5 for the 7.0 V test and 405–1015 mg/L in the period day 3 to day 7 in the 9.0 V test. In fact, this effect has been previously observed in 3.5 N and 5.0 N although with minor intensity.

It must be considered that the acidogenic effluent used to feed the inoculation reactor of PHA-producers microorganisms contained high butyric and acetic concentrations. The high butyric acid concentration required an adaptation period of the PHA-producing microbiota but, once adapted, the degradation of butyric acid occurs. However, the degradation of valeric acid seems to have resulted in lower PHA productivities. Figure 6 shows the degradation percentages of each individual acid after 6 days, showing the formation of propionic acid in those tests with a high initial proportion of valeric acid.

The formation of propionic acid, especially in high valeric:butyric ratio assays, shows the coexistence of a degradative metabolism of valeric acid non-related to the PHA production. The high concentration of valeric acid has allowed the development of this acidogenic degradative activity by means of β-oxidations that have subtracted carbon sources for the maximum PHA production. In fact, the maximum PHA production in the 9.0 V assay occurs after 24 h and, from this point on, the system started to degrade PHAs while metabolizing the most important fraction of valeric and acetic acids. The maximum PHA productions in V assays were 217, 154, and 126 mg/L.

In summary, butyric acid appears to be the most easily metabolized VFAs by PHA producers according to the maximum PHA concentrations reached in each test, although

dosing at concentrations below 1.1 g HBu/L is preferable. Acetic acid is completely degraded once the degradation of valeric and butyric acids has concluded, especially for tests with high concentrations of valeric acid. On the other hand, the presence of high concentrations of valeric acid seems to promote a parallel degradative pathway for propionic acid, leading to a diminishing of the PHA production capacity of the process.

3.5 PHA yield

The PHA yield should not be measured in terms of concentration, as the biomass concentration changes throughout the process due to cell growth. Therefore, the PHA production yields have been calculated by dividing the total PHA obtained by the VSS. In this case, the VSS were assumed to represent the amount of cell biomass in the assays.

Furthermore, this ratio (g PHA/g VSS) is a key factor for the PHA extraction stage since if its value is high the recovery of PHA (downstream industrial processes) is easier and cheaper [13]. Figure 7 shows the evolution of the PHA yield estimated in this way.

Table 2 shows the maximum values of the PHA yield (expressed as percentage) with an indication of the day on which they have been obtained. The average PHA yield values are also given for the period from the start of the test to the day when the maximum PHA production yield was reached. Additionally, Table 2 includes the values of the average daily rate (ADR) for PHA production. ADR represents the ratio between the maximum PHA yield of the process and the time required to reach it.

The ADR is a parameter that allows comparing the different conditions tested in order to select that leading to maximum PHA production in the minor time, which could be of industrial interest. Results of ADR in Table 2 show that better conditions are corresponding to the minor total volatile acidity concentration in each set of assays: 3.0 N, 1.5B, and 5.0 V.
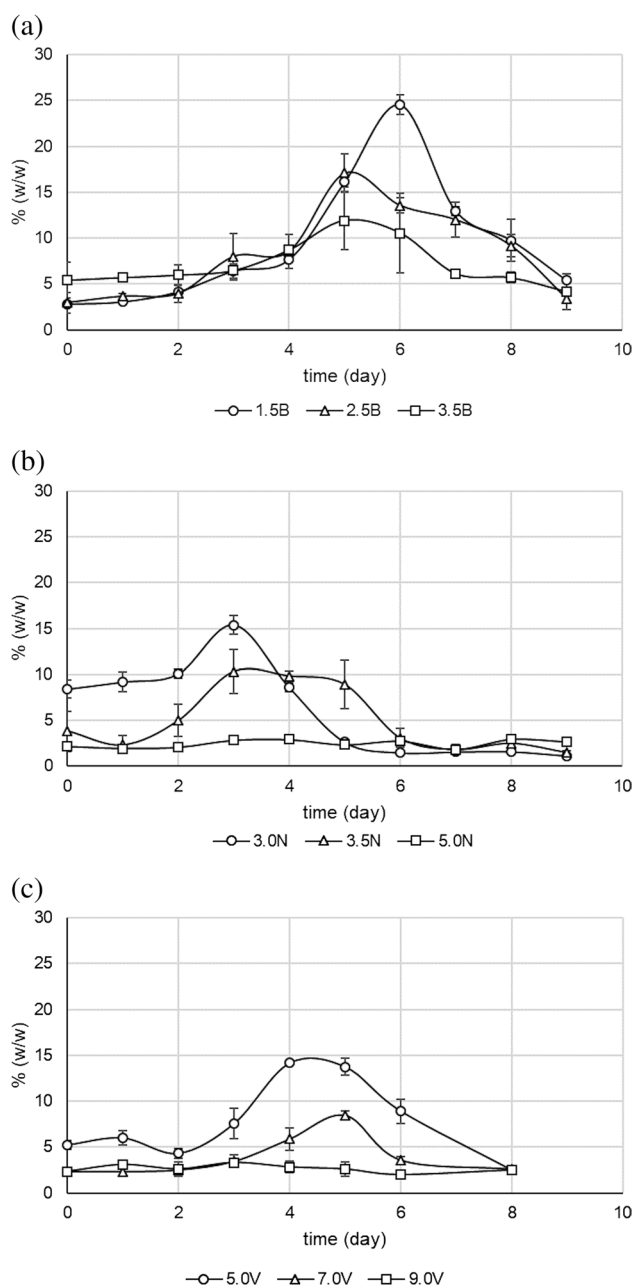
Clearly, the lower acid concentration assays (1.5B, 3.0 N, and 5.0 V) offered the best yields within each valeric:butyric ratio. As the acid concentration increases in each ratio, the MMC were less capable of directing carbon consumption towards PHA production. It is noticeably evident (assays 3.5B, 5.0 N, and 9.0 V) that the ADR of PHA production is visibly affected by the high butyric and valeric concentration. However, the sensitivity to each acid is not the same. The high butyric acid assays at low concentrations of total volatile acidity (1.5B and 2.5B tests) gave better PHA yields (23.0 and 17.1%, respectively) than the equivalent tests with high valeric:butyric ratio. Thus, in the last case (5.0 V and 7.0 V tests), the maximum PHA yield reached was 14.2 and 8.5% despite the butyric acid concentration was similar or even lower than in 1.5 B.
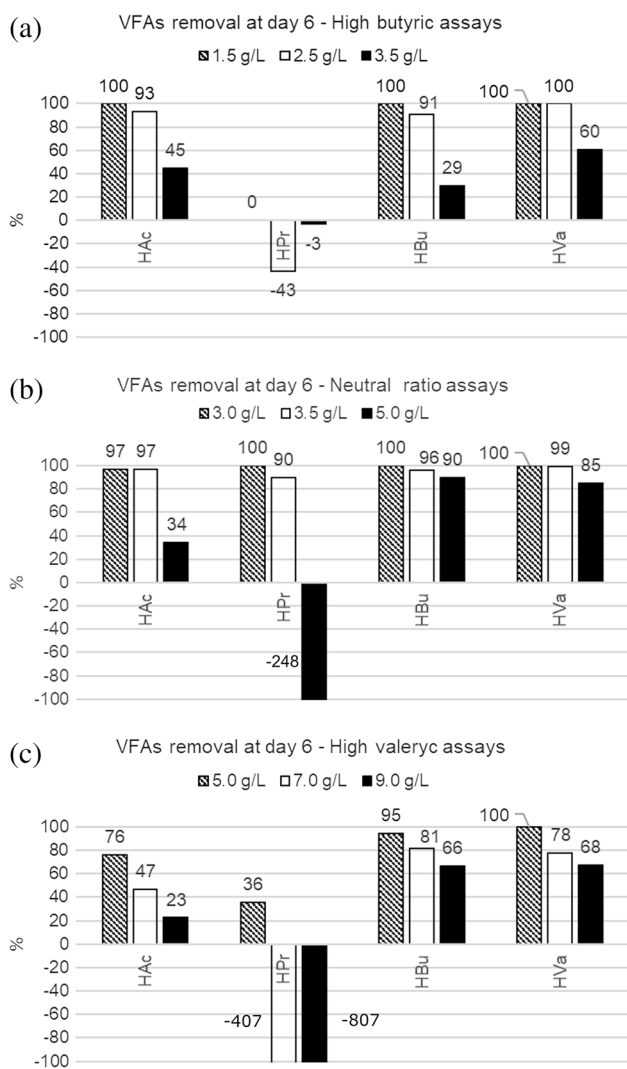
Fig. 6 Individual VFAs removal percentage at day 6. **a** Assays with 1:10 valeric:butyric ratio; **b** assays with 1:1 valeric:butyric ratio; **c** assays with 10:1 valeric:butyric ratio

Fig. 7 Evolution of the PHA yield at dry basis expressed as percentage. **a** Assays with 1:10 valeric:butyric ratio; **b** assays with 1:1 valeric:butyric ratio; **c** assays with 10:1 valeric:butyric ratio

Table 3 shows data from different authors of the maximum accumulation capacity for PHA production, using VFAs, by MMC. As it can be seen, the PHA yield values are between 23 and 53%. The results obtained in this work are in the lower range, although they are practically the same as those obtained by other authors who have worked with VFAs with MMC.

PHA titer values are highly dependent on the operational conditions and configuration of the process for PHA production from VFAs by MMC. Ciesielski and Przybylek [51] observed PHA titers between 227.8 (acetic as feeding) and 673 mg/L (propionic as feeding) in batch reactors inoculated with activated sludge from wastewater treatment plant. In the present study, propionic was always under 70 mg/L in feeding while acetic acid was fed between 704 and 2426 mg/L

and the PHA titer was over 200 mg/L just in 1.5B and 2.5B assays where acetic was in lower levels but higher butyric concentrations where supplied. It is should be remarked that butyric acid follows the same metabolic pathway than acetic acid for PHA production [52]. However, PHA titer from MMC could be considerably higher under most favorable conditions as it can be seen in the study of Pérez-Zabaleta et al. [16].

Table 2 Maximum PHA yield (expressed as percentage), time to reach the maximum PHA yield, and ADR for PHA production

Assay	Maximum PHA yield (%)	Day	ADR for PHA production (%/day)[1]
1.5B	23.0	6	4.08
2.5B	17.1	5	3.42
3.5B	11.9	5	2.38
3.0 N	14.0	3	5.13
3.5 N	10.3	3	3.43
5.0 N	3.0	8	0.38
5.0 V	14.2	4	3.55
7.0 V	8.5	5	1.70
9.0 V	3.3	3	1.10

[1]ADR has been calculated as the ratio between the maximum PHA yield (%) and the number of days that have been needed to reach the maximum for PHA production

3.6 Effect of total volatile acidity and valeric:butyric ratio on maximum PHA yield

The results obtained for the maximum PHA production yield indicated in Table 2 can be related to the two main variables analyzed in this study: the concentration of total volatile acidity fed to the system and the valeric:butyric ratio.

Thus, representing the maximum values of PHA production obtained in each test versus the initial total acidity (expressed as acetic acid), Fig. 8a is obtained. In this figure, it can be seen that the results corresponding to the tests with a ratio of butyric:valeric of 10:1 (B) and 1:1 (N) have the same trend, while the tests corresponding to the ratio 1:10 (V) show a differentiated behavior. In all the cases, the trends observed are fitted to linear correlations. In Fig. 8b and 8c, the maximum PHA yields are plotted versus the initial concentrations of butyric and valeric acids, respectively. These last two graphs clearly show that the data also cluster in linear distributions for each of the butyric:valeric ratios tested.

As it can be seen in Fig. 8, the evolutions of the maximum PHA production yield adopt a distribution with respect to the initial acid concentrations that clearly differentiate the set of tests carried out with each butyric:valeric ratio. In all the cases, the observed evolution indicates that the maximum PHA production yield decreases as the initial concentration of the considered acid increases. This implies that the VFAs, although they are the substrate used by the microorganisms in the generation of PHA, have an inhibitory effect on the process. This effect has been observed by other authors [40–43, 53].

Concretely, Tamang et al. [53] have estimated the inhibitory effect of VFAs in some industrial wastewaters between 7.1 and 82.1 mM by means of measuring the diminishing in the specific oxygen uptake rate (SOUR) over diluted samples of wastewaters. With dairy wastewaters, the SOUR decrease by 83% when the total volatile acidity concentration was incremented from 4 to 15.7 mM.

As it can be seen in Fig. 8, the different sets of data have been fitted by linear regression. Table 4 shows the representative parameters of each linear adjustment. Letters B, N, and V in the column named as ratio indicate the set of points used for fitting according to the nomenclature defined in Table 1. The slope is related to the inhibitory effect of each acid, as it was previously commented. The y-interception corresponds to the maximum PHA yield value achievable under the operating conditions. These values are in the same range as described by other authors [24, 54–56] for the maximum PHA accumulation in the cell (expressed in %) when using MMC and carbon sources corresponding to wastes and not pure compounds and well-defined media.

Table 3 Comparison of the obtained results in this study with the literature data for PHA production from VFAs by MMC in terms of maximum accumulation capacity, PHA titer, and PHA yield based on VFA fed

Carbon source		Maximum PHA yield (%)	Maximum PHA titer (g/L)	Ref
VFAs from acidogenic fermentation (30% food waste + 70% primary sewage sludge)		44	2.4	[16]
Municipal wastewater supplemented with acetic acid		30	-	[38]
Acetic acid		40	-	[46]
VFAs from the acidogenic fermentation of paper mill wastewater		48	-	[47]
VFAs from the acidogenic fermentation of food waste		24	-	[48]
Synthetic waste (VFAs)		53	-	[49]
VFAs from heat pretreated sludge		24	-	[50]
Synthetic waste	Acetic acid	31	0.23	[51]
	Propionic acid	30	0.67	
Synthetic waste (VFAs)		23	0.23	This work

(a)

(b)

(c)
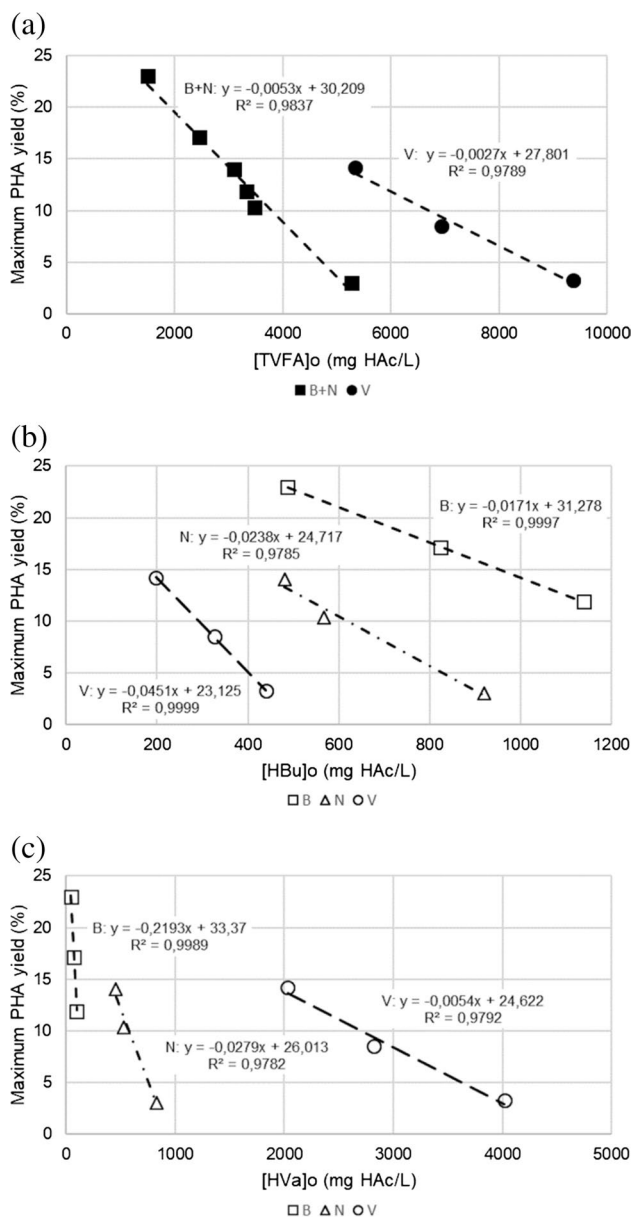
Fig. 8 Maximum PHA yield at dry basis expressed in percentage versus initial concentration of **a** total volatile acidity in mg HAc/L, **b** butyric acid in mg HBu/L, and **c** valeric acid in mg HVa/L. The lines represent the linear regression fittings

4 Conclusions

Based on the results obtained in this study and the discussion carried out in the previous section, the following conclusions can be drawn:

The PHA producers were capable to metabolize all the mixtures of VFAs tested, but the PHA production yield was clearly affected by the initial total volatile acidity concentration, as well as by feeding composition in terms of butyric:valeric ratio.

As the total volatile acidity concentration increases, the system is less able to direct carbon consumption towards PHA production. High butyric acid assays at low total volatile acidity concentrations give better PHA yields than equivalent assays with high valeric acid concentrations.

For assays with high concentrations of butyric and valeric acids, it is required the previous degradation of them to the beginning of the acetic acid degradation. Moreover, the presence of high concentrations of valeric acid seems to promote a parallel degradative pathway towards propionic acid that would decrease the PHA yield.

The maximum PHA yield reached in this study was 23% (w/w) corresponding to the test carried out with an initial total volatile acidity concentration of 1.5 g HAc/L and a butyric:valeric ratio of 10:1. However, the ADR for PHA production indicates that the best conditions correspond to the test with 3.0 g HAc/L and butyric:valeric ratio of 1:1.

The linear regression of the maximum PHA production yield achieved in the different tests performed against the initial acid concentration used allows determining that the threshold value of the maximum PHA in the system is in the range 23–33% (w/w).

Table 4 Parameters from the linear regression of maximum PHA yield versus the initial acid concentration (total volatile acidity, butyric, and valeric)

Fitting	Ratio	Y-intercept (%)	Slope (%/mg/L)	R^2
PHA(%) vs initial total VFAs	B + N	30.209	− 0.0053	0.9837
	V	27.801	− 0.0027	0.9789
PHA(%) vs initial HBu	B	31.278	− 0.0171	0.9997
	N	24.717	− 0.0238	0.9785
	V	23.125	− 0.0451	0.9999
PHA(%) vs initial HVa	B	33.370	− 0.2193	0.9989
	N	26.013	− 0.0279	0.9782
	V	24.622	− 0.0054	0.9792

Availability of data and material Supported by authors declaration.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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