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Polyhydroxyalkanoates production from syngas fermentation effluents: Effect of nitrogen availability

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ABSTRACT

Syngas fermentation is a promising technology for bioalcohols production even though some produced volatile fatty acids (VFA) may remain unconsumed in the effluent. The present research explored the production of polyhydroxyalkanoates (PHA) from the remaining VFA of the syngas fermentation effluents in fed-batch bioreactors so that bioalcohols and biopolymers could be obtained in a combined way. In order to perform these PHA accumulation tests, two different syngas fermentation effluents, composed mainly of a mixture of VFA and alcohols, were used as substrate. Those effluents were characterized by different nitrogen availabilities, one being N-rich and the other N-limited. A mixed microbial culture (MMC) with a maximum PHA storage ability of 53.6% was used as inoculum. The microorganisms of the MMC mainly consumed the VFA rather than the alcohols, allowing the latter to accumulate as end product, besides PHA. However, when the N-rich effluent was used as substrate, the consumption rate of alcohols was 20 times higher (0.040 Cmmol-Alcohol Cmmol-X^{-1} h^{-1}) compared to the N-limited effluent (0.002 Cmmol-Alcohol Cmmol-X^{-1} h^{-1}). Despite not observing large differences in the maximum amount of accumulated PHA (40.5 – 41.5%), there was a decrease in the PHA content after reaching its maximum peak when the N-rich effluent was used as substrate. This trend was not observed with the N-limited effluent, in which the maximum PHA peak exactly matched with the end of the experiment.

1. Introduction

Non-renewable fossil feedstocks are still being used as the major source of energy and also for the production of polymers, worldwide. From the awareness that the prolonged utilization of these resources will lead to adverse and irreversible environmental impacts, there has been an increasing interest over the last decades in biorefinery processes, which include the sustainable production of bioenergy as well as bio-based products [1]. One of the main strategies involving biorefineries is waste valorization, which provides the procurance of high value-added products from a large number of wastes.

Synthesis gas or syngas is a mixture of gases, usually including CO, CO_2 and H_2, obtained from the gasification of different possible feedstocks. To date, syngas components such as CO and CO_2 can also be considered an environmental problem because they are found in emissions of many industrial processes, among others those involving combustion [2]. Nevertheless, syngas can also be metabolized by anaerobic bacteria and converted into high value-added products such as bioalcohols [3], carboxylic acids [4], biomethane [5] or biopolymers [6]. Within the anaerobic consortia, *Clostridium spp* are the most used bacteria to lead the bioconversion of such C1 gases [7].

On the other hand, polyhydroxyalkanoates (PHA) are biodegradable and biobased polymers that have attracted much attention in recent years due to their consideration as potential substitutes of petroleum-based plastics [8]. The production of PHA is more environmentally friendly compared to conventional plastics, besides their biodegradable condition. However, the PHA biopolymers are industrially produced using pure cultures mainly, tending to increase their final price and consequently, becoming the major limitation for their commercialization [9]. To produce PHA in a cheaper way, the use of more economical carbon sources like agroindustrial by-products as well as the use of mixed microbial cultures (MMC) are attractive [10]. Within the agro-industrial sector, cheese whey (CW) has recently received increased interest as a suitable substrate for bioprocesses due to its easily fermentable condition. Particularly, CW is a quite interesting carbon source and suitable substrate for its anaerobic bioconversion into volatile fatty acids (VFA) either as ready-for-application products [11] or as intermediate metabolites for the synthesis of PHA [12].

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During syngas fermentation to bioalcohols, some VFA often remain in the culture medium as intermediate products without complete further bioconversion to alcohols. To the best of our knowledge, only one previous work, of our laboratory, explored the suitability of using these VFA as potential substrates for the synthesis of PHA as a novel solution to produce bioalcohols and biopolymers in a combined way, with promising results [13]. The present study went one step further in the understanding of this novel biorefinery process, and the effect of nitrogen on this specific PHA accumulation process was evaluated. To achieve this, PHA accumulation tests were run using two different syngas fermentation effluents as substrate, one N-rich and the other N-limited. An MMC, enriched in PHA accumulating microorganisms, fed fermented CW, was used as inoculum.

2. Material and methods

2.1. Experimental design

Fig. 1 provides a complete overview of the four different reactor set-ups that were somehow involved in the whole process. The first stage consisted in the production of VFA through the acidogenic fermentation of an agroindustrial by-product such as CW. The VFA-rich stream was then used as a carbon source to enrich a PHA-storing MMC. In parallel, syngas fermentation was carried out to obtain an alcohol-rich effluent, containing also VFA. Finally, in the last stage, PHA accumulation assays were carried out using the enriched MMC as inoculum and the effluent from the syngas fermentation as substrate, to obtain a PHA- and alcohol-rich stream as value-added end products.

2.2. Acidogenic fermentation

The acidogenic fermentation of CW for its bioconversion into a VFA-rich stream has recently been optimized in our research group and described elsewhere [14,15].

2.3. Enrichment of PHA accumulating bacteria

The MMC used to carry out the PHA accumulation assays using the syngas fermentation effluents as substrate was firstly enriched in PHA accumulating bacteria in a sequencing batch reactor (SBR) under the aerobic dynamic feeding (ADF) process for a long period of time. The enrichment took place in a 10-L working volume glass reactor and the initial inoculum came from a mixture of biomass from two other PHA producing reactors. Half of the inoculation volume came from a 1-L SBR fed fermented brewery wastewater [16]. The other half came from a 2-L SBR in which fermented CW was used as substrate (non-published data).

The reactor was operated under the feast-famine regime in order to ensure appropriate environmental conditions to provide a competitive advantage to PHA storing bacteria. The SBR was operated in a cyclic way with two cycles per day, lasting 12-h each and including four different phases: feeding (5 min), reaction (665 min), settling (40 min) and withdrawal (10 min). Aeration and stirring were provided throughout the entire cycle except during the settling and withdrawal phases. The reactor was cleaned once a week in order to remove the accumulated biofilm from all the sensors and the walls of the reactor.

The hydraulic retention time (HRT) and the solid retention time (SRT) were set at 1 and 4 days, respectively. The reactor was purged, daily, at the end of the famine phase by removing 2.5 L of the medium, in order to keep the suitable SRT. Oxygen was provided by an air compressor using a ceramic diffuser and a flow rate of 1 vvm (volume of gas per volume of medium and minute). A stirrer was introduced in the reactor, maintaining constant stirring at 150 rpm. A warm water heating jacket was used to maintain a constant mesophilic temperature of 30 °C. The pH was adjusted during the feeding phase, in order not to drop below 8, by using a NaOH (1 M) solution. The organic loading rate (OLR) was 64 Cmmol L^{-1} d^{-1}.

The culture medium consisted of a carbon source, a nutrient solution and dilution water. The carbon source was the VFA-rich stream from the acidogenic fermentation of CW (Section 2.2). This stream was mainly

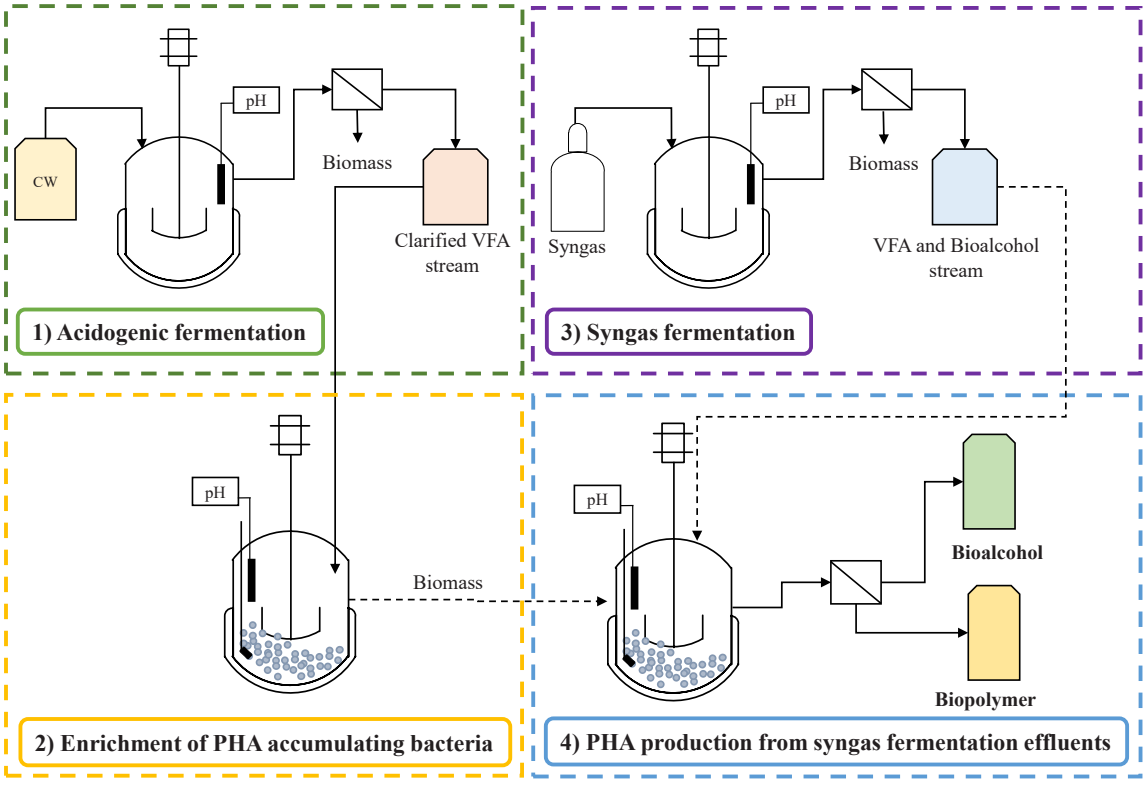

Fig. 1. Complete overview of the PHA production process from syngas fermentation effluents in order to obtain biopolymers and bioalcohols in a combined way.

composed of acetic and butyric acids, representing about 95% of the soluble chemical oxygen demand (COD). The other 5% was valeric acid. The synthetic nutrient solution was composed of 24 g NH_4Cl, 6.4 g KH_2PO_4, 14.3 g $MgSO_4.7H_2O$, 186 mg $FeSO_4.7H_2O$, and 1 g thiourea per litre distilled water. All the nutrients were assumed to be in excess as compared to the available carbon. The carbon to nitrogen (C/N) ratio was kept at 7. During the feeding phase, 0.8 L of the carbon source, 0.1 L of nutrient solution and 4.1 L of water were pumped into the reactor.

To evaluate the maximum PHA storage ability of the selected MMC, PHA accumulation assays were performed. These assays were run in fed-batch mode and carried out in a 10-L glass reactor with the same substrates and operational conditions as for the enrichment SBR.

2.4. Syngas fermentation

Two different effluents were collected from two different syngas fermentation experiments in order to perform the PHA accumulation assays in the present study. One of the effluents was obtained from syngas fermentation by *C. carboxidivorans*, with an initial composition of 20/20/10/50 ($CO/CO_2/H_2/N_2$) [17]. The other effluent came from the fermentation of syngas by *C. aceticum* with an initial composition of 30/5/15/50 ($CO/CO_2/H_2/N_2$) [18]. Both effluents were characterized before starting the assays (Table 1). Besides, some pre-treatments were also done before starting the experiments. In order to remove remaining cells from the syngas fermentation stage, the effluents were centrifuged at 8000 rpm for 5 min and then filtered through a 0.2 μm pore size filter.

2.5. PHA production from syngas fermentation effluents

The assays for PHA production were performed using the effluents from the syngas fermentation experiments as substrate and the MMC from the enrichment SBR as inoculum. These PHA accumulation assays were carried out in a 2-L glass reactor in fed-batch mode. To collect the biomass, a volume of 0.8 L was harvested from the SBR at the end of the famine phase in order to minimize the presence of any carbon source, dissolved ammonia or accumulated PHA. Then, the biomass was settled and re-suspended with the same volume of tap water. This way, cellular growth was limited by reducing the remaining nutrient availability of the end of the previous SBR cycle. Finally, the fed-batch reactor was inoculated with the 0.8 L of the re-suspended biomass. The operational conditions applied to the fed-batch reactor were the same as for the enrichment SBR in order to maximize the production of PHA. A pulse feeding strategy was used and the dissolved oxygen (DO) concentration was monitored using a DO probe. The different pulses of substrate were introduced with an initial concentration of around 20 – 40 Cmmol L^{-1}.

Table 1
Physicochemical properties of the syngas fermentation effluents used as substrate in the PHA accumulation experiments.

Syngas effluent	N-rich	N-limited
Strain	*C. carboxidivorans*	*C. aceticum*
COD (g L^{-1})	11.73	12.48
% VFA[1]	73.46	72.10
% Alcohol[2]	12.76	7.12
NH_4^+ (mg L^{-1})	387.00	0.00
PO_4^{3-} (mg L^{-1})	40.00	1701
C/N ratio	30	NA[3]
C/P ratio	293	7
pH	4.75	7.90
Composition (%)[4]	Acetate (32) Butyrate (27) Caproate (14) Ethanol (7) Butanol (6)	Acetate (72) Ethanol (7)

[1] Proportion of the soluble COD in the form of VFA.
[2] Proportion of the soluble COD in the form of alcohol.
[3] NA: not applicable.
[4] Percentage of the soluble COD.

2.6. Microbial analysis

Prior to the PHA production from syngas fermentation effluents, the MMC from the enrichment SBR was subjected to a taxonomic characterization in order to determine the main bacterial populations involved in PHA storage. For that purpose, a denaturing gradient gel electrophoresis (DGGE) analysis was performed. The microbial analysis started with DNA isolation. Then, V3 – V5 region of the 16S rRNA gene was amplified using the primers F-357GC and R-907, as described by Sass et al. [19]. The amplicons were run in a 7.5% polyacrylamide gel with a denaturing gradient of 40 – 60%, according to Valentino et al. [20]. Once the running process was finalized, all the bands were excised, re-amplified and sent for sequencing to an external commercial company (STAB VIDA, Portugal). Finally, the sequences were compared with the NCBI database.

2.7. Analytical methods

For the characterization of the syngas fermentation effluents as well as for the monitoring of the different bioreactors (SBR and fed-batch), HPLC analyses were performed in order to quantify the total amount of alcohols and organic acids, following the method described in Bermúdez-Penabad et al. [21]. PHA were determined by gas chromatography with a FID, according to Lagoa-Costa et al. [13]. Dissolved ammonia and phosphate concentrations were determined by a colorimetric method, at 635 and 690 nm, respectively. Finally, COD and solids determinations were performed using Standard Methods [22].

2.8. Calculations

The intracellular PHA content (wt%) was calculated as the percentage of PHA, on mass basis, by the measured volatile suspended solids (VSS), following Eq. (1).

$$\mathrm{PHA}(\%) = \frac{\mathrm{PHA\ (g)}}{\mathrm{VSS\ (g)}} \times 100 \quad (1)$$

The storage yield ($Y_{PHA/S}$) was calculated as the total amount of produced PHA in relation to the amount of consumed substrate. The growth yield ($Y_{X/S}$) was calculated as the increase in active biomass due to substrate consumption. All the rates, substrate uptake rate ($-q_S$) and PHA production rate (q_{PHA}), were calculated in a similar way. The $-q_S$ was calculated as a linear regression of substrate consumption along time and biomass concentration at that point of time. The same procedure was followed for q_{PHA} replacing substrate consumption by PHA production.

Apart from their assimilation, bioalcohols may undergo certain evaporation under aerobic circumstances. Therefore, the evaporation rate of both bioalcohols, ethanol and butanol, was determined in an abiotic short-term experiment with the same aeration, stirring, pH and temperature conditions as for the fed-batch assays, but using synthetic ethanol and butanol as the sole carbon source. The reactor was sampled periodically for 2 h and the evaporation rate was calculated as the linear regression of the decrease of alcohol concentration along time.

3. Results and discussion

3.1. Enrichment of PHA accumulating bacteria

From day 1 of operation, the MMC got well adapted to the feast-famine (F/F) regime, as the biomass came from another enrichment SBR. However, the feast length, and therefore the F/F ratio, showed a greater variability within the first 115 days of operation (Fig. 2), even though it always lied within the considered optimal range for a good selection of PHA accumulating microorganisms [23]. It was after 136

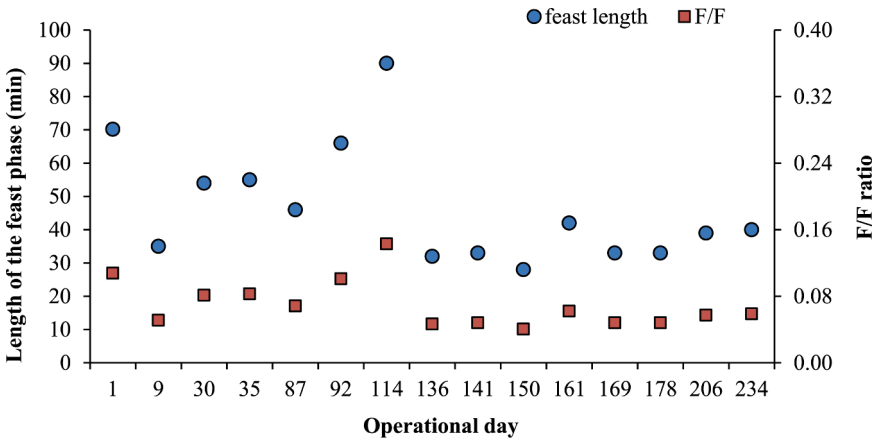

Fig. 2. Length of the feast phase and F/F ratio during the enrichment SBR operation.

days when a more stable operation was reached and steady state was assumed (Fig. 2). This stability was also reflected in the DO profile (data not shown). From day 136, it was observed that DO reached lower concentrations during the feast phase and higher concentrations during the famine phase, which have been proven to benefit the selection of PHA-storing bacteria [24,25]. Changes in the DO concentration, regardless of the operational day, correlated quite well with the supply and depletion of VFA in the culture medium, confirming the use of VFA as the sole carbon source in the VFA-rich stream. Therefore, the DO became the leading parameter to determine the boundary between feast and famine phases in the SBR cycles.

Fig. 3A shows a typical behaviour of a 12 h feast-famine cycle

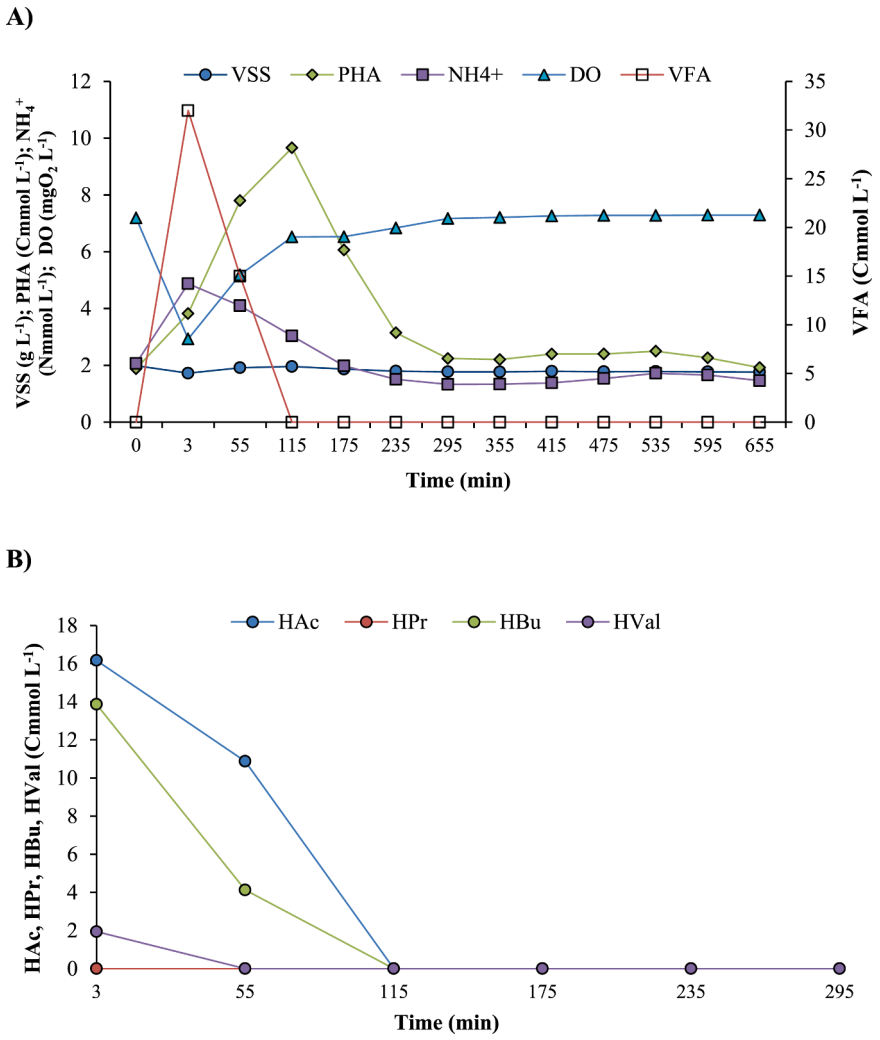

Fig. 3. Typical SBR 12-h cycle including (A) the evolution of VFA, PHA, dissolved oxygen, biomass and ammonium throughout the entire cycle and (B) detailed consumption of individual VFA during the feast phase.

obtained after almost 170 days of continuous operation under the ADF process. The cycle started with the introduction of both the carbon (fermented CW) and the nutrient solutions. The DO concentration instantly decreased right after substrate addition to values of around 2 mg O_2 L^{-1}. This fact marked the beginning of the feast phase. As long as all the VFA were not fully consumed, the DO was kept at low concentrations; but once all the substrate had been used up by the microorganisms, the DO did sharply increase up to a value of around 6 – 7 mg O_2 L^{-1}, and it remained roughly constant throughout the famine phase. During the feast phase, the VFA were converted into PHA, with the corresponding increase of PHA storage by the microorganisms. However, dissolved ammonia was consumed in parallel to the substrate uptake causing a simultaneous growth and storage response, which is usual when VFA and ammonia are present in the culture medium. New strategies are being considered by several authors in order to minimize growth during feast [26,27]. It was recently reported that uncoupling the carbon and the nitrogen sources results in a more successful strategy to enrich PHA accumulating MMC in either synthetic or real wastewaters [28]. Despite a possible competitive advantage of this new strategy for PHA storing bacteria, the conventional ADF process was applied in the present research. Additionally, ammonia was consumed faster during feast rather than famine phases. During the feast phase, both PHA and non-PHA accumulating bacteria, could grow simultaneously, while only PHA accumulating bacteria could grow during the famine phase, thus consuming less ammonia [29]. It can be assumed that, in the famine phase, stored PHA was mainly used for cell maintenance.

Although all the VFA were depleted, a preferable consumption of butyrate and valerate was observed, compared to acetate (Fig. 3 B). In particular, acetate uptake started slowly, though its assimilation increased when butyrate and valerate were partially consumed, as also reported by Marang et al. [30] for an SBR fed a mixture of butyrate and acetate. A reasonable explanation for the preferable consumption of longer chain VFA lies in the lower energy requirements for their transformation into PHA compared to short chain VFA [31], even though DO concentration [25] and microbial community [32] have also their own influence.

Throughout the PHA accumulating culture selection, a maximum $Y_{PHA/S}$ of 0.374 ± 0.045 Cmmol-PHA Cmmol-VFA^{-1} was reached with a maximum intracellular PHA content of 19 ± 2%. These results are in line with other studies that reported similar biopolymer contents and analogous behaviour of the MMC. For example, Oliveira et al. [33] obtained similar PHA production values using fermented CW as substrate. The monomeric composition of the final biopolymer averaged 77% hydroxybutyrate (HB) and 23% hydroxyvalerate (HV). Thereby, a copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P (3HB-co-3HV) was obtained, which is one of the widely documented copolymers when using real wastewaters in SBR as substrate [26,29,34].

3.1.1. Determining the maximum PHA storage capacity of the selected MMC.

The maximum PHA accumulating capacity of the MMC was first evaluated, before the biomass from the SBR was harvested for the assays with syngas fermentation effluents. This also served as control to later compare the results when using the syngas fermentation effluents. Thus, two different PHA accumulation experiments were conducted using the VFA-rich stream from the acidogenic fermentation of CW as substrate. Besides, no nutrients were added, in order to avoid biomass growth. Experiment-1 was scheduled on day 101 of the SBR operation, before reaching the considered steady state of the reactor. Experiment-2 took place on day 185 of the SBR operation, when the reactor showed a better performance in terms of PHA accumulation. In both cases, the feedstock composition was quite similar. The VFA profile was 73% acetate, 26% butyrate and 1% valerate for Experiment-1, and 68% acetate, 28% butyrate and 4% valerate for Experiment-2. Although no nutrients were added to the culture medium, a remaining amount of dissolved ammonia from the previous SBR cycle was detected in Experiment-1. This remaining amount was only found within the first two hours of the experiment, which should not have a significant impact on the maximum PHA storing ability [35]. Nevertheless, in order to limit more the possibility of bacterial growth, the biomass was re-suspended in tap water before starting Experiment-2. This way, all the remaining free ammonia was successfully removed from the culture medium.

As shown in Table 2, a higher content of intracellular PHA accumulated in Experiment-2 (53.6%) compared to Experiment-1 (39.4%). Therefore, these results confirmed that by the time Experiment-2 took place, the selected microorganisms had a higher PHA accumulating ability. The maximum stored PHA content in Experiment-2 was in agreement to other PHA contents reported in the literature. For example, Colombo et al. [36] reached 55% polymer content using a similar fermented dairy by-product. Besides the maximum PHA content, the kinetic parameters also indicated the greater ability of the MMC to accumulate PHA on day 185 of operation compared to day 101. Thus, the maximums -q_S and q_{PHA} were considerably higher in Experiment-2 (Table 2). Furthermore, some VFA were diverted towards bacterial growth during Experiment-1, according to the nitrogen availability in the culture medium, as previously mentioned. In contrast, $Y_{X/S}$ was zero in Experiment-2. Although all the parameters showed a better performance in the second experiment, the microorganisms invested a slightly lower amount of VFA in PHA synthesis (Table 2).

Finally, there was no difference in the monomeric composition of the biopolymer, regardless of the PHA accumulation test. The biopolymer was mainly composed of HB, representing 80% of the total PHA. The HV monomer comprised the remaining 20%. No other PHA monomer was detected (Table 2). Therefore, the microorganisms of the MMC synthesized a copolymer P(3HB-co-3HV) according to what was previously observed in the PHA accumulating culture selection. These results could benefit the production of PHA in the subsequent PHA accumulation experiments using the syngas fermentation effluents as substrate since their carbon source is mainly composed of even chain VFA.

3.1.2. Identifying the PHA accumulating bacteria

A taxonomic analysis of the microorganisms was performed before starting the PHA accumulation tests with the syngas fermentation effluents. This microbial analysis aimed at the identification of the main groups of bacteria involved in the production of PHA. Therefore, a DGGE analysis was performed, as this molecular technique has already been widely used in previous research [20,30,37]. The biomass was collected from the enrichment SBR right before the beginning of the fed-batch assays with the syngas fermentation effluents. Fig. 4 shows a typical DGGE profile in which two different bands are well defined (lane A). In parallel, a molecular ladder was also run to check bands length (lane MM). It seemed that both bands were a bit longer than 500 pb, which matched quite well with the amplicons obtained from the PCR when using the primers detailed in Section 2.6. Although DGGE is not a good technique for the identification of all bacterial groups in the microbial community, the presence of only two bands suggests that the SBR was highly enriched in these two different taxa.

Both bands were excised from the gel and sent to sequencing. Band A1 fits 98% the identity with partial sequence of *Thauera linaloolentis*. On the other hand, band A2 has 93% identity with partial sequence of *Pseudoxanthomonas mexicana*. Taxa from each genus were previously

Table 2
Overview of the PHA accumulation assays using the fermented CW as substrate to determine the maximum capacity of PHA storage of the selected MMC.

	Experiment-1	Experiment-2
% PHA (wt%)	39.4	53.6
PHA composition (wt%HB/wt%HV)	82/18	79/21
Y_{PHA} (Cmol-PHA Cmmol-VFA^{-1})	0.39	0.26
q_{PHAmax} (Cmol-PHA Cmmol-X^{-1} h^{-1})	0.130	0.358
q_{Smax} (Cmol-VFA Cmmol-X^{-1} h^{-1})	0.182	0.954

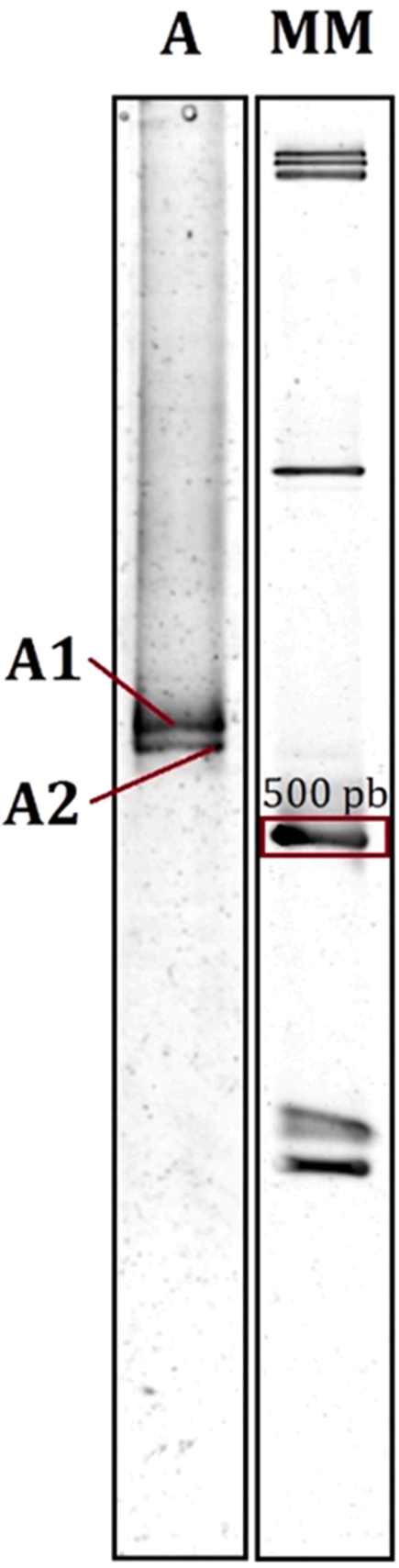

Fig. 4. DGGE profile. Lane A shows the enrichment SBR sample and lane MM shows the molecular ladder.

classified as PHA producing bacteria in previous publications. The genus *Thauera* is highly related to wastewater treatment plants. Consequently, its presence in systems with PHA accumulating MMC is quite common [38–41]. All of the afore cited works have identified *Thauera* as one of the main bacterial groups during the culture selection of 3-stage PHA production processes. This means that the members of this genus were capable to survive to the selective pressure imposed by the feast-famine regime thanks to their ability to accumulate PHA. Carvalho et al. [42] observed that the members belonging to *Thauera* genus used a major proportion of the substrate for biomass growth instead of PHA accumulation when compared to other PHA storing bacteria, for example *Azoarcus*. Despite such limitation, *Thauera* species can achieve a maximum PHA content of up to 81% of the cell dry weight [37], even though this content is usually lower when real wastewaters are used [23, 38,43]. Albuquerque et al. [43] studied the link between *Thauera* and its substrate uptake preference. Based on their results, *Thauera* showed a strong preference for the consumption of butyrate, which matches quite well with what was observed in the culture selection of the present research. However, these results do not mean that *Thauera* members are not able to synthesize PHA from other carbon sources. Actually, the members of this genus are able to grow in a wide range of organic acids such as acetate [44] and lactate [37]. Thereby, *Thauera* could also have contributed to the exhaustion of the remaining VFA once butyrate was depleted from the culture medium in the current work. Unlike *Thauera*, the PHA storing ability of *Pseudoxanthomonas* genus has not been so well documented in the literature yet. To the best of our knowledge, only Oliveira et al. [26] detected an undefined member of the *Xanthomonadaceae* family as PHA accumulating bacteria. As in our study, the abovementioned work also enriched the MMC on fermented CW, which may have had some influence on the development of this taxon.

From the taxonomic characterization of the MMC, it could be concluded that its main bacterial groups should have the ability to accumulate PHA. These results, besides the determination of the high PHA storing potential of the MMC, were important to ensure a good response of the microorganisms during the PHA accumulation experiments using the syngas fermentation effluents in terms of PHA storage.

3.2. Syngas fermentation effluents as substrate for PHA production

The ability of the MMC to accumulate PHA from syngas fermentation effluents was evaluated in PHA accumulation experiments under fed-batch conditions. They were performed during the considered steady state of the enrichment SBR. The experiment using the effluent from the syngas fermentation of *C. aceticum* took place on day 214 of the enrichment SBR. The experiment using the effluent from the syngas fermentation of *C. carboxidivorans* took place on day 232 of the enrichment SBR.

3.2.1. Impact of nitrogen availability on PHA storage

As explained in Section 2.5, the accumulation experiments were designed following a pulse feeding strategy to avoid biomass inhibition due to high substrate concentration inside the reactor [45]. Consequently, the DO became the leading parameter indicating the possibility of adding the new pulses of substrate in the reactor [29]. However, the evolution of the DO showed a reversal trend for both effluents, despite the experimental design was the same (Fig. 5 A). When the effluent from the syngas fermentation of *C. carboxidivorans* was used as substrate, the biomass did not consume much of the supplied oxygen during the first pulse of the assay and 4.54 mg L^{-1} of DO were still available in the culture medium (Fig. 5 A). This oxygen consumption was lower than expected and could be related to the foam observed in the reactor. In our opinion, foam emerged as a consequence of the low pH of the effluent as well as the presence of high buffer capacity chemicals in the initial composition of the syngas fermentation medium [17]. Thereby, pH control was hampered and turned into a sharp decrease of its value down to 5 – 6, negatively affecting the biomass and creating foam. To avoid

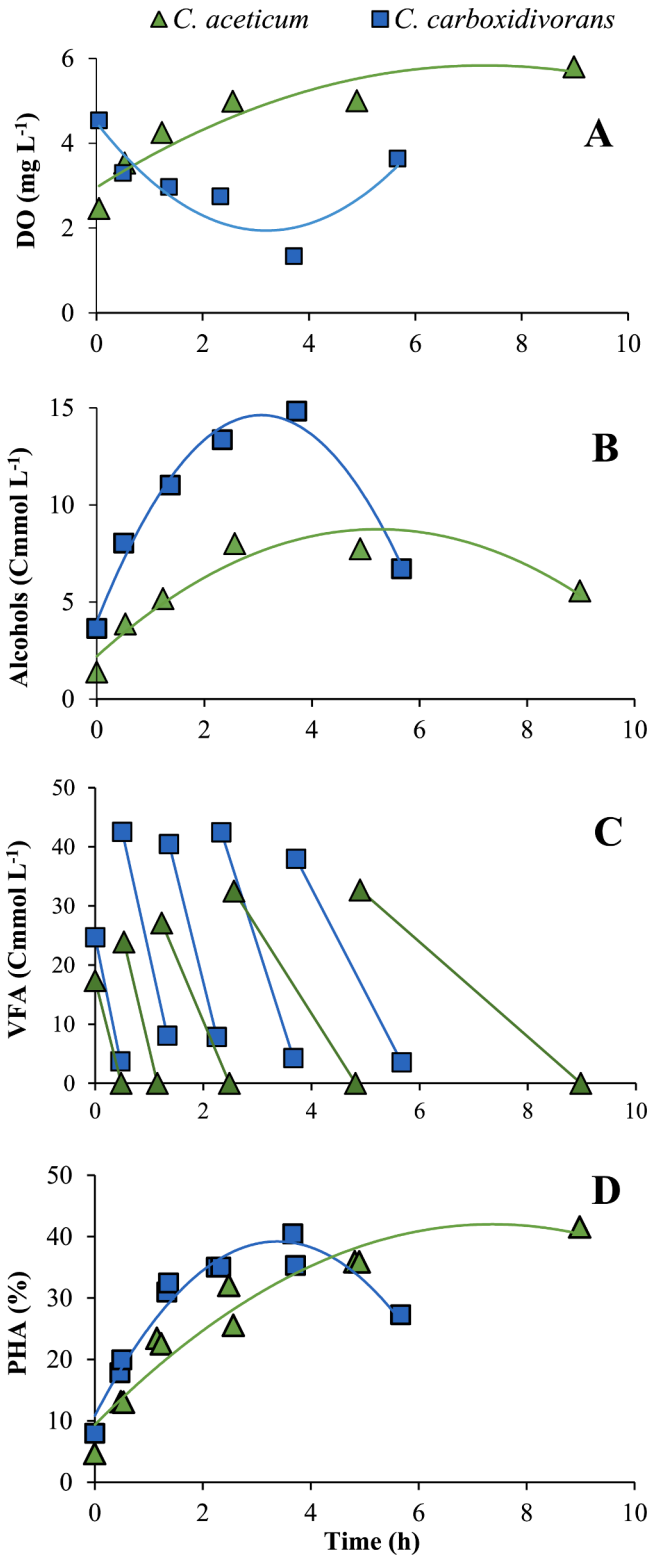

Fig. 5. Evolution of (A) the concentration of DO, (B) the concentration of alcohols (C) the consumption of VFA and (D) the accumulation of PHA during the fed-batch experiments using the syngas fermentation effluents from *C. carvoxidivorans* (blue squares) and *C. aceticum* (green triangles). The graph of the DO concentration shows the minimum concentration of DO reached at each of the five pulses; the sixth dot displays the concentration of DO when the experiment was stopped.

new pH shocks, the input flow rate of the substrate was reduced in subsequent pulses. This change allowed better pH control and consequently, foam formation was reduced. Despite the foam issue, the pulses went forward and the end of each pulse was indicated by a sudden increase of DO, a typical performance parameter when following the pulse feeding strategy (data not shown). New pulses of substrate were introduced into the reactor, based on the observed DO increase. As the PHA accumulation assay progressed, no signs of biomass saturation were detected. Actually, the entrance of fresh medium caused the biomass to gradually increase the oxygen demand until reaching the maximum consumption at the fifth pulse of the experiment, with only 1.34 mg L^{-1} of DO available in the culture medium (Fig. 5 A). Right after the addition of the fifth pulse, the biomass entered in a continuous feast phase with high oxygen demand. This response was quite similar to what was observed in our previous work [13]. This feast phase was only interrupted when the experiment was stopped, two hours later, and the available DO being only 3.64 mg L^{-1}, meaning the biomass was still metabolically active (Fig. 5 A). When the effluent of *C. aceticum* was used as substrate, the pattern of DO consumption was the opposite. There was no foam formation at all in the reactor. The pH value of this effluent remained near neutral and the feed of fresh medium did not disturb the pH value at all. Thus, the biomass did not suffer any pH shock and the oxygen consumption showed a more typical response. During the first pulse, the microorganisms consumed 70% of the oxygen supplied to the reactor and only 2.46 mg L^{-1} of DO were available in the culture medium (Fig. 5 A). Similarly as observed with the effluent of *C. carboxidivorans*, the end of each pulse corresponded to a sudden increase of the DO concentration (data not shown). However, oxygen consumption became milder as new pulses of fresh medium were introduced in the reactor, in contrast to what had been observed with the effluent of *C. carboxidivorans* and therefore, the available DO in the culture medium was progressively increased (Fig. 5 A). This suggested biomass saturation in terms of PHA storage, the most typical response in PHA accumulation assays [29].

In general, PHA accumulating bacteria are characterized by their high VFA uptake rates. Therefore, VFA should preferably be consumed rather than alcohols, causing the latter to accumulate in the culture medium (Fig. 5 B). Besides, the assimilation of higher alcohols, e.g., butanol, is rather unusual. This behaviour has been described before [46,47] and the same response has also been observed in our previous research with different bacteria and media [13]. However, when the effluent of *C. carboxidivorans* was used as substrate, some differences in the substrate uptake preference have also been observed for the different VFA. A small amount of non-consumed VFA remained in the medium at the end of each pulse (Fig. 5 C). This amount was exclusively associated to caproate, meanwhile acetate and butyrate were completely exhausted. Therefore, it could be concluded that acetate and butyrate were preferably consumed rather than caproate. The lower consumption rate of caproate had its explanation in the culture selection of the MMC. During the enrichment process, the MMC was fed fermented CW, mainly composed of acetate and butyrate but no caproate. This turned into an MMC highly specialized in the consumption of acetate and butyrate. There was no such observation when the effluent of *C. aceticum* was used as substrate. Indeed, acetate, which is the only acid produced by that strain (Table 1), was fully consumed at the end of each pulse (Fig. 5 C). However, the different ammonia availabilities in each effluent affected the performance of the PHA accumulation experiments differently. The high concentration of dissolved ammonia in the effluent of *C. carboxidivorans* caused the biomass to enter into a continuous feast phase that largely affected the consumption of alcohols. During this feast phase, acetate and butyrate were consumed, but also some caproate and, especially, the alcohols (Fig. 5 B and C). In fact, ethanol was observed to be used by the microorganisms from the third pulse onwards, at a rate of 0.014 Cmmol-Ethanol Cmmol-X^{-1} h^{-1}, which doubled at the last pulse of the assay (0.032 Cmmol-Ethanol Cmmol-X^{-1} h^{-1}). Besides, butanol consumption was also detected in the last pulse of the experiment (0.008

Cmmol-Butanol Cmmol-X^{-1} h^{-1}). The role of dissolved ammonia in a mixture of VFA and alcohols has been previously studied [47] and an increase in the methanol uptake rate when an N-excess condition was applied to the culture medium was observed. Based on the latter results, the presence of dissolved ammonia in high amounts allowed other microbial populations apart from PHA accumulating bacteria to grow. According to that, a remarkable increase in the $Y_{X/S}$ was observed throughout the whole experiment in the current work, to the point of reaching a maximum value of 0.622 Cmmol-X Cmmol-S^{-1}. Conversely, the absence of dissolved ammonia in the effluent of *C. aceticum* prevented the consumption of ethanol. In fact, ethanol uptake only took place in the last two pulses of this experiment and at a very low rate (0.002 Cmmol-Ethanol Cmmol-X^{-1} h^{-1}). Due to this non-consumption, ethanol remained mainly unused in the culture medium (Fig. 5 B). Additionally, the absence of dissolved ammonia resulted in no active biomass growth throughout the whole experiment and thus the $Y_{X/S}$ was zero.

Therefore, it can be concluded that the presence of dissolved ammonia had a large impact on the consumption of the alcohols. Bioalcohols produced from syngas fermentation are considered high valuable products as both chemicals and fuels. Therefore, their consumption in the PHA accumulation assays may not be desired from a biotechnological point of view. In order to reduce the consumption of bioalcohols, their recovery from the culture medium, as the bacteria use up all the VFA, would be an interesting alternative. Such strategy was evaluated by Korkakaki et al. [46] who induced the biomass to selective periods of settling to remove the fraction of the remaining alcohols, although for different purposes. Nonetheless, if the integrated production of biopolymers and bioalcohols is considered to have a great future potential as technology, the syngas fermentation process should also be designed to avoid the later consumption of the bioalcohols in the fed-batch bioreactors (i.e., reducing the free ammonia concentration). The reduction of the dissolved ammonia availability in syngas fermentation has also been studied to improve alcohol production [48,49]. Furthermore the presence of dissolved ammonia has also a strong impact on the final PHA accumulation. At the beginning of the experiments, the intracellular PHA content had a similar and quick up-growing trend in both effluents. Then, the PHA production rate slowed down until the microorganisms reached their maximum peak of PHA (Fig. 5 D). However, there was a remarkable difference in the evolution of the accumulated PHA regarding the dissolved ammonia availability of the syngas fermentation effluents. When the N-rich effluent was used as substrate, the PHA content decreased after reaching its maximum peak (Fig. 5 D). This evolution was not observed for the N-limited effluent. The reduction of the PHA content after reaching its maximum peak under N-excess conditions has been previously reported [47,50]. It seems that this reduction is more related to a dilution of the PHA content due to biomass growth rather than a consumption of the accumulated PHA. Nonetheless, the maximum PHA content was similar using both N-rich (40.5%) and N-limited (41.5%) effluents despite a higher growth response when using the effluent from *C. carboxidivorans*. A reasonable explanation of the unchanged results could be related to the different VFA profiles of the effluents. Acetate was the sole VFA in the effluent from *C. aceticum* while a mixture of acetate, caproate, but specially butyrate, which has been proven to reach better yields for PHA production [30], were present in the effluent of *C. carboxidivorans*. In any case, the maximum PHA content almost doubled the one obtained in our previous work [13], but was still below the 53.6% maximum ability of the selected MMC. Since the physicochemical properties of the syngas fermentation effluents were different from those wherein the MMC was enriched, some reduction of the maximum PHA content was expected.

3.2.2. *Effect of the type of syngas fermentation effluent on the polymer composition*

Regardless of the effluent, the VFA were mostly transformed into one single PHA monomer, i.e., HB. The presence of mainly acetate and butyrate led the synthesis of PHA to this monomer. No HV was synthesized during the whole experiments due to a lack of its precursors, propionate and valerate. However, the HV monomer has been quantified as up to 3% of the total PHA content at the end of the experiments. This HV was associated to a residual amount of the previous SBR cycle. Interestingly, a low amount of hydroxyhexanoate (HHx) monomer was also synthesized apart from the HB. It comprised 1% of the total PHA content and it was exclusively synthesized when the effluent from the syngas fermentation of *C. carboxidivorans* was used as substrate. For its synthesis, the bacteria would most probably have used caproate as its precursor [51].

From a commercial point of view, the polyhydroxybutyrate (PHB) homopolymer is considered a brittle and stiff material [52]. Copolymers are more interesting than homopolymers because of their better physicochemical properties. For that reason, alternatives to increase the proportion of other monomers are demanded. The incorporation of HV monomers up to a 30% of the total PHA content would suppose an improvement in the mechanical properties of the biopolymer and it would reduce its crystallinity [53]. However, the MMC would need the odd chain VFA as precursors to synthesize HV (i.e., propionate and valerate). The use of another different *Clostridium* strain in the syngas fermentation process may result in the production of these VFA. For example, it has been reported that *C. ragsdalei* can produce acetic, propionic, butyric and valeric acids from syngas [54]. Another interesting alternative is the incorporation of the HHx monomer to the PHB since it increases the elongation to break between 6% and 850% [55]. Thus, the final biopolymer would become much more flexible. In this particular case, there would be no need to change the biocatalyst. Caproate produced during syngas fermentation with *C. carboxidivorans* would be enough for the MMC to synthesize this monomer. However, only 1% was reached in the current work, which is a low proportion to considerably improve the physicochemical properties of the resulting PHA. To increase the percentage of the HHx monomer, a more efficient MMC in the synthesis of this medium chain PHA would be needed.

After the PHA accumulation experiments finalized, both products, PHA and bioalcohols, should be subjected to separation and purification processes. One of the most attractive aspects of this novel biorefinery process is that each bioproduct accumulates in different phases. While the alcohols are dissolved in the culture medium, PHA are store inside the cells. This finally turns into an easy separation process, e.g., by centrifuging. Then, the purification method depends on each component. While the downstream processes for bioalcohol recovery are more focused on distillation [56], PHA are often recovered either through digestion or direct extraction methods [57]. However, the downstream processing is still considered to be one of the main challenges in both cases and future research would be useful.

4. Conclusions

The present study confirmed the possibility of producing PHA from syngas fermentation effluents and therefore, obtaining two high value-added products, bioalcohols and biopolymers. It was observed that the microorganisms preferably consumed VFA rather than the alcohols for the synthesis of PHA. In fact, the alcohols remained largely unused in the culture medium, which may facilitate their recovery. Besides, the PHA accumulation tests did also confirm the key role of dissolved ammonia and its concentration in the culture medium for PHA production. Although the minimum PHA content to profitably recover the polymer is set at 50%, the innovative process described in the current work has the potential for further improvement and the maximum PHA content was already highly promising (41.5%). An MMC with the ability to better adapt to the physicochemical properties of the effluents as well as nutrient limiting conditions in the syngas fermentation effluents could be important factors to improve this biorefinery process.

CRediT author statement

BL and AP performed the experiments; BL, AP, MCV, CK analyzed the data and contributed to the writing of the manuscript. MCV supervised the work and proposed the original research project and concept. All authors read and approved the final manuscript.

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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