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Exploiting Cheese Whey for Efficient Selection of Polyhydroxyalkanoates-Storing Bacteria

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Abstract: Agroindustrial by-products hold an enormous potential to be bioconverted into high-value-added products such as polyhydroxyalkanoates (PHA), a cost-effective alternative to conventional plastics. In this study, cheese whey, a highly abundant side stream of the cheese making process, was explored as a feasible substrate for the selection of a mixed culture highly enriched in PHA-storing bacteria using a sequencing batch reactor under an aerobic dynamic feeding regime. For that, the absence/presence of thiourea, magnesium and iron, as well as the application of two different organic loading rates (OLR), i.e., 60 and 80 CmM d^{-1}, were tested. The results showed an improved culture selection when thiourea, magnesium and iron were added to the culture medium as well as when the highest OLR was applied. Under these conditions, the biomass achieved a maximum PHA storage of 54% and a PHA production rate of 4.81 Cmmol-PHA L^{-1} h^{-1}. Additionally, the study of the microbial community showed that during this period of maximum productivity, the biomass was enriched in *Azoarcus* and *Amaricoccus* bacterial species. Conclusively, cheese whey can be considered a good feedstock to efficiently select a mixed culture with high potential to accumulate PHA and a good way to give this by-product added value.

Keywords: aerobic dynamic feeding; cheese whey; culture selection; microbial community; mixed microbial cultures; polyhydroxyalkanoates

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1. Introduction

Petroleum-based plastic can be considered one of the most important discoveries in the twentieth century to the point that its annual production has increased tremendously over the last 70 years, with an estimated 460 million tons produced worldwide in 2019 [1]. However, among their excellent properties, conventional plastics lack biodegradability, which causes their accumulation in the environment due to overuse and misuse, finally leading to polluted lands and seas.

In contrast to conventional plastics, biological and biodegradable polymers have gained increasing interest in recent decades as environmentally friendly alternatives. Polyhydroxyalkanoates (PHA), for example, are synthesized by a large number of different microorganisms in the form of cytoplasmic granules and, thanks to the contribution of the biotechnology community, they can be industrially produced as potential substitutes of a wide range of petroleum-based plastics. Nevertheless, the established PHA production involves the use of pure cultures, often recombinant microbes, as well as the utilization of synthetic substrates, finally leading to an increase in production costs [2].

In contrast, mixed microbial cultures (MMC) have been proposed as a cost-effective method for the production of PHA since they do not need sterile conditions and renewable carbon sources, such as agroindustrial by-products, can be used as substrates instead of synthetic ones [3]. To date, the process layout of the PHA production through MMC is usually based on a three-stage process that includes the acidogenic fermentation of surplus-based feedstocks, the selection of PHA-storing bacteria and the final production of PHA [4].

Among the three stages, whether the efficiency of the PHA production process is good or not depends mainly upon the selection stage and the operational conditions applied on it [5–9]. However, the nature of the feedstock also exerts a great influence. The volatile fatty acids (VFA) are considered the main precursors for synthesizing PHA in MMC, and a large number of different wastes have already been tested as suitable substrates for their bioconversion into VFA and subsequent PHA production [10–14]. However, some inhibitory compounds can persist in some agroindustrial by-products after acidification, affecting the selection stage and, therefore, the final PHA yield [15,16].

In that sense, cheese whey, a highly abundant by-product in Galicia, is characterized by the easy biodegradation rate of its components, and the feasibility of bioconverting this sugary by-product into VFA has previously been evidenced [17,18]. Hence, the present research has focused on studying the following two steps in the overall PHA producing process, the selection of PHA-storing bacteria and the final production of PHA. To achieve these goals, an MMC, fed fermented cheese whey, was subjected to a feast–famine regime for a long period of time in which some changes in the culture medium were applied. These modifications tended to achieve an improved PHA-accumulating culture selection performance. Moreover, taxonomic analyses were run in parallel to the selection stage in order to identify the main bacterial populations in charge of PHA accumulation.

2. Material and Methods

2.1. Selection of PHA-Accumulating Microorganisms

The selection stage was carried out in a 2 L working volume glass reactor inoculated with a sludge mixture from the aerobic tanks of different local wastewater treatment plants. In order to promote the selection of efficient PHA-storing bacteria, an aerobic dynamic feeding (ADF) process was imposed in a sequential batch reactor (SBR). Two 12 h-long cycles per day were applied, each one divided into four different phases: feeding (5 min), reaction (680 min), settling (30 min) and withdrawal (5 min).

A constant temperature of 30 °C was maintained throughout the whole selection, with the pH value adjusted in the range 8–8.5. The ADF process is a complete aerobic process. Thereby, the SBR was continuously aerated at a flow rate of 2 L min^{-1}, except in both the settling and withdrawal phases, in which aeration was switched off. A mechanical stirrer was installed in the reactor working at 500 rpm. A solid retention time (SRT) of 3 days was imposed via daily purge of one third of the SBR volume at the end of the famine phase. Additionally, during the withdrawal phase, half of the working volume was removed from the reactor resulting in a hydraulic retention time (HRT) of 1 day.

The culture medium was composed of both a carbon and a nutrient source. The carbon source was a VFA-rich stream coming from the previous acidification of cheese whey [17] and diluted with tap water to achieve the desired organic loading rate (OLR). On the other hand, the nutrient source was composed of a nitrogen source (NH_4Cl) and a phosphorus source (KH_2PO_4). The carbon to nitrogen (C/N) ratio was set at 7 and the carbon to phosphorus (C/P) ratio at 40. Both C/N and C/P ratios were chosen to ensure nitrogen and phosphorus availability throughout the whole cycle. During the feeding phase, 1 L fresh medium was introduced in the reactor, comprising 0.5 L carbon source and 0.5 L nutrient source.

At the beginning of the selection stage (Period I), the SBR was working as described above for the first 86 days of operation (or 172 cycles). Thiourea, which promotes the inhibition of any nitrifying activity, was not even added to the culture medium. After that, the reactor was subjected to gradual changes in the culture medium to determine their effect on the efficiency of the culture selection. All the changes were sequential and cumulative, as shown in Table 1. In addition, a 3-week period was used between each operational period to acclimatise the biomass to the new environment. The first change included the addition of thiourea in the culture medium as a fraction of the nutrient source, in a concentration of approximately 20 mg L^{-1}, in order to induce the inhibition of nitrifiers (Period II). The SBR had been running under these concrete conditions for 80 days

(160 cycles). Then, magnesium ($MgSO_4 \cdot 7\ H_2O$) and iron ($FeSO_4 \cdot 7\ H_2O$) were also added to the nutrient source, together with thiourea, in a second modification of the culture medium (Period III). The concentrations of those nutrients were 27 and 8 mg L^{-1} for magnesium and iron, respectively. The operational time under these conditions lasted for the next 50 days of operation (100 cycles). Finally, the OLR was increased from 60 to 80 CmM d^{-1} (Period IV). The SBR was working under these specific conditions for the last 77 days of operation (or 154 cycles).

Table 1. Summary of the operational conditions in the sequencing batch reactor (SBR) at each of the different periods in the culture selection stage.

Period	I	II	III	IV
Time (d)	86	80	50	77
Cycles (n°)	172	160	100	154
Temperature (°C)	30	30	30	30
pH	8–8.5	8–8.5	8–8.5	8–8.5
SRT (d)	3	3	3	3
HRT (d)	1	1	1	1
C/N ratio	7	7	7	7
C/P ratio	40	40	40	40
Substrate (%) [1]	26/9/57/8	33/7/56/4	40/14/40/6	42/12/41/5
Thiourea (mg L^{-1})	0	20	20	20
Mg^{2+}/Fe^{2+} (mg L^{-1})	0/0	0/0	27/8	27/8
OLR (CmM d^{-1})	60	60	60	80

[1] Percentage of each VFA: Acetate/Propionate/Butyrate/Valerate.

2.2. Maximization of PHA Production

Fed-batch bioreactors to evaluate the maximum ability of the MMC to intracellularly accumulate PHA were run when reaching steady state in each of the different periods (I–IV). All the PHA accumulation tests were carried out in a 2 L glass reactor with an initial biomass volume of 0.8 L. The biomass was harvested from the enrichment SBR at the end of the famine phase in order to minimize the amount of intracellular PHA as well as free dissolved ammonia. All the assays were run at the same pH, temperature, stirring and aeration conditions as in the selection stage. The biomass was exclusively fed fermented cheese whey, and therefore, no extra nutrients were externally added to the culture medium in order to limit N and P availability. In this way, bacterial growth was limited to the presence of the remaining amount of ammonia and phosphate from the preceding SBR cycle. The fermented cheese whey was manually introduced in the reactor following a pulse feeding strategy and the dissolved oxygen (DO) was the leading parameter indicating the end of the pulses.

2.3. Taxonomic Analysis

Biomass samples were withdrawn from the SBR at Period I, III and IV, when the reactor reached steady state. Denaturing gradient gel electrophoresis (DGGE) was the selected molecular technique to carry out the bacterial identification, and the followed procedure was the same described in a previous work of our research group [19]. In brief, the whole DNA of the mixed culture was first isolated, and then the V3–V5 region was amplified by PCR with specific primers. In the next stage of the process, the amplicons were run in a polyacrilamide gel with a denaturing gradient of 40–60%. The resulting bands after the running stage were excised from the gel and reamplified using the same specific primers. Finally, reamplified bands were sequenced and compared with the NCBI database.

2.4. Analytical Techniques

For both the culture selection and the PHA accumulation tests, the analytical methods included the determination of total suspended solids (TSS), volatile suspended solids (VSS), dissolved ammonia and phosphate, water soluble products (VFA) and the intracellularly

accumulated PHA. A detailed description of all of these procedures can be found elsewhere [17,20]. For the quantification of PHA through gas chromatography, n-heptadecane was used as internal standard.

2.5. Calculations

The total content of PHA accumulated in the biomass was calculated as the fraction of VSS in the form of PHA on a mass basis, following Equation (1).

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

The storage yield ($Y_{PHA/S}$) referred to the total production of PHA based on the consumed substrate. Similarly, the growth yield ($Y_{X/S}$) was calculated as the increase in the active biomass concentration related to the consumed substrate. Finally, the substrate uptake rate ($-q_S$) and the PHA production rate (q_{PHA}) were obtained from the linear regression of substrate consumption or PHA production vs. time and active biomass at that point of time.

3. Results and Discussion

3.1. Performance of the SBR Cycles

From day 1 after the inoculation, the microorganisms of the MMC were subjected to the abovementioned ADF process. The feast and famine phases were clearly visible from the first week of operation, and the DO concentration inside the reactor became the leading parameter to indicate the phase shift. Figure 1 shows an SBR cycle with both the feast and the famine phases at the beginning of the experiment (day 22). The evolution of VFA, ammonia, PHA and DO show the usual trend, also reported in the literature [15,21,22]. Once the carbon and the nutrient sources were pumped into the SBR, the bacteria started consuming all the VFA as well as the oxygen supplied to the reactor as electron acceptor. As long as the VFA were not fully consumed, the feast phase remained active, with DO at low values and the microorganisms filling their reservoirs of PHA. In parallel, some consumption of dissolved ammonia was also detected, associated with biomass growth. In accordance to the last observation, PHA accumulation and biomass growth took place simultaneously in the feast phase, which is quite usual when VFA and dissolved ammonia concur in the culture medium. A new alternative has been proposed in order to avoid biomass growth during the feast phase. This new selection method uncouples the carbon and nutrient sources by introducing ammonia at the beginning of the famine phase, when all the VFA were used up [5,23]. Although it has been demonstrated that this new methodology improved the selection performance, the conventional ADF process, in which ammonia availability is ensured in both feast and famine phases, was used in the present research. Once the microorganisms had taken up all the VFA, a sharp increase in the DO concentration was observed. This increase marked the end of the feast phase and the beginning of the famine phase. As no more carbon source was available, the PHA-storing bacteria could survive the famine phase thanks to the consumption of the PHA that has been stored during the feast phase together with the consumption of ammonia, explaining the decrease in both parameters detected during the famine phase. Ammonia availability throughout the famine phase is essential to enable the survival of PHA-accumulating microorganisms; otherwise, the enrichment process would not be good enough, as previously reported by Johnson et al. [24]. To promote nitrogen and phosphorus availability in the current work, low C/N and C/P ratios were applied.

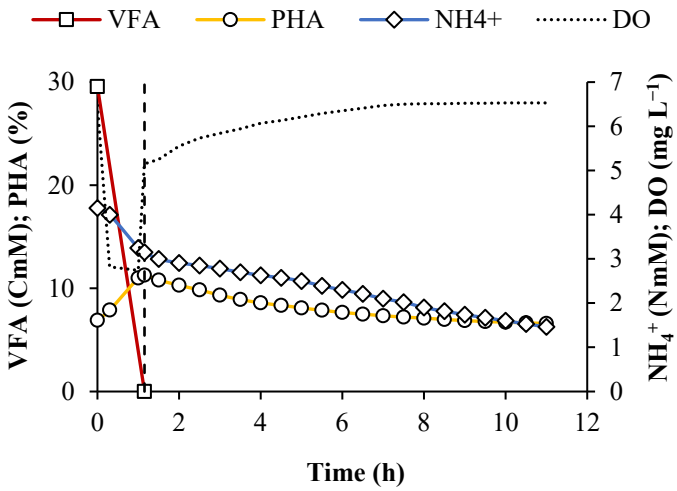

Figure 1. SBR cycle on day 22 (43rd cycle) of the reactor operation. It includes both the feast and the famine phases. The black and vertical dashed line shows feast boundary. The evolution of VFA (squares), PHA (circles), NH_4^+ (diamonds) and DO (black dotted line) are also represented.

3.2. Long-Term Operation of the PHA-Accumulating Culture Selection

One of the most influencing factors within the selection stage is the feast-to-famine (F/F) ratio. According to Dionisi et al. [25], to prevent biomass growth and to promote the selection of PHA-accumulating microorganisms, the F/F ratio should not exceed a value of around 0.25. Based on the experimental data of the present research, it could be concluded that the F/F ratio usually remained low and lay within the reported optimal range, regardless of the operational period (Figure 2). Therefore, the famine phase lasted for a long period of time, leading to a higher competitive disadvantage of the non-PHA-accumulating microorganisms, therefore favouring the enrichment process. The F/F ratio varied in the range 0.05–0.15, and only a few values at the beginning of the operation deviated from the considered optimal range. Fortunately, these values were occasional and not prolonged in time, resulting in a non-detrimental effect in the selection of PHA-storing bacteria.

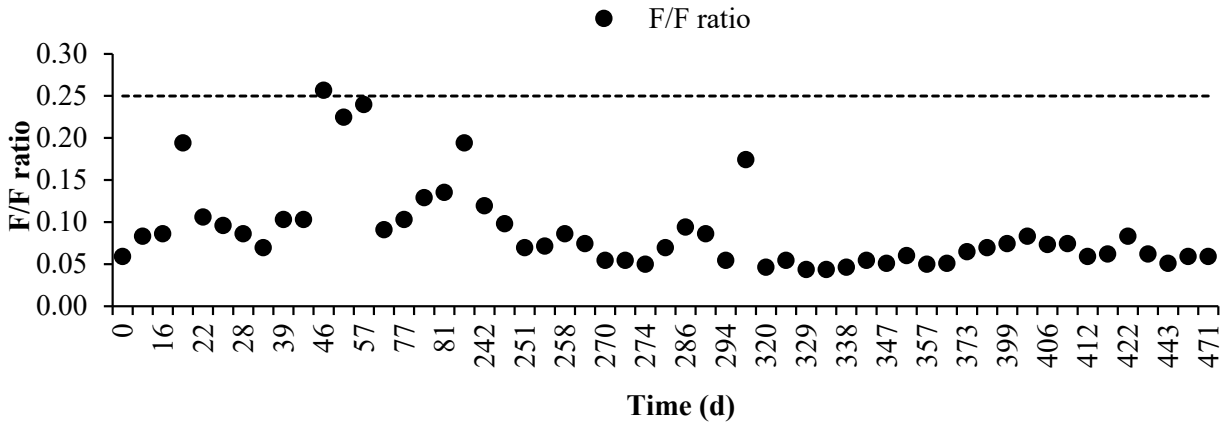

Figure 2. Evolution of the F/F ratio throughout the whole operation of the SBR culture selection.

Within the F/F ratio, there are relevant factors that have to be taken into consideration in achieving low values. One of them is feedstock complexity. As an example of that, Queiros et al. [15], who fed the MMC hardwood sulphite spent liquid, a carbon source characterized by the presence of a highly complex substrate, such as lignosulphonates, needed 261 days of operation to establish an F/F ratio below 0.25. Similarly, Oliveira et al. [26] observed that the increasing percentage of proteins in the VFA-rich stream resulted in higher F/F ratios. Conversely, the fermented cheese whey used in our study,

characterized by its easily biodegradable condition, mainly composed of VFA, has likely stimulated the fast adaptation of the MMC to the feast–famine regime. Thus, low F/F ratios were observed from day 1 of operation and were comparable to several others reported using similar substrates [5,26–28].

Another factor influencing the F/F ratio is the OLR. As a general rule, the higher the applied OLR, the higher the F/F ratio [29,30]. However, there was not a noticeable increase in the F/F ratio in the current work when the OLR was increased from Period III (mean value of 0.05 ± 0.01) to Period IV (mean value of 0.07 ± 0.01), meaning the OLR could have been further increased if considered.

Finally, the $-q_S$ also play an important role in the achievement of low F/F ratios. This parameter is dependent on both substrate complexity and applied OLR, but it is also dependent on the bacterial populations [31]. In this way, the $-q_S$ contributed to the low F/F ratios thank to their high values throughout the whole experiment, ranging from 0.261 to 0.591 Cmmol-VFA Cmmol-X^{-1} h^{-1} (Table 2). It was interesting, however, to observe some substrate uptake preferences in the microorganisms regarding the consumption of the different VFA that composed the fermented cheese whey. In this way, among the most abundant VFA in fermented cheese whey, bacteria preferably consumed butyrate rather than acetate (Table 2). This observation has already been reported by others, and the preference for butyrate is supposed to also benefit the PHA storage yield [32]. Accordingly, the modification of some operational conditions during the first stage in the 3-stage process for PHA production, i.e., the acidogenic fermentation, in order to promote butyric acid fermentation, such as the pH [33] or the SRT [17], could be interesting options thinking about improving the overall PHA production performance. Furthermore, the evolution of $-q_S$ was linked to the evolution of q_{PHA}. The faster the microorganisms took all the VFA up from the culture medium, the faster they diverted them to form PHA (Table 2).

Table 2. Average performance in the SBR operation to select a high PHA-storing MMC. Average values are given with standard deviation in brackets.

Period	$-q_{Hac}$ [1]	$-q_{HBu}$ [2]	$-q_S$ [3]	q_{PHA} [4]	$Y_{X/S}$ [5]	$Y_{PHA/S}$ [6]
I	0.061 (±0.026)	0.152 (±0.083)	0.261 (±0.142)	0.066 (±0.032)	0.140 (±0.044)	0.275 (±0.066)
II	0.181 (±0.053)	0.316 (±0.118)	0.553 (±0.176)	0.186 (±0.075)	0.075 (±0.067)	0.327 (±0.057)
III	0.238 (±0.030)	0.233 (±0.031)	0.591 (±0.081)	0.235 (±0.046)	0.070 (±0.040)	0.396 (±0.050)
IV	0.198 (±0.036)	0.204 (±0.057)	0.479 (±0.093)	0.219 (±0.050)	0.049 (±0.026)	0.458 (±0.061)

[1] Acetic acid uptake rate (in Cmmol-Acetate Cmmol-X^{-1} h^{-1}). [2] Butyric acid uptake rate (in Cmmol-Butyrate Cmmol-X^{-1} h^{-1}). [3] In Cmmol-VFA Cmmol-X^{-1} h^{-1}. [4] In Cmmol-PHA Cmmol-X^{-1} h^{-1}. [5] In Cmmol-X Cmmol-VFA^{-1}. [6] In Cmmol-PHA Cmmol-VFA^{-1}.

Although the F/F ratio is a determinant factor in the efficient selection of PHA-storing bacteria, it does not ensure the success of selecting microorganisms with high ability to accumulate PHA. In such a way, growth and storage yields are the most reliable factors to define whether the selection is effective or not. As shown in Figure 3, there was a continuous differentiation along the operational time of the culture selection regarding both $Y_{PHA/S}$ and $Y_{X/S}$. Since the increase in $Y_{PHA/S}$ and the decrease in $Y_{X/S}$ took place chronologically, it could be discussed that biomass acclimation was in relation to these achieved results. However, in order to reduce the effect of biomass acclimation, a 3-week intermediate period (SRT × 7) was imposed on the MMC when introducing the new conditions to the culture medium. In consequence, we strongly believe that the changes applied to the culture medium positively affected the culture selection, and therefore, acclimation as the sole explanation is unlikely.

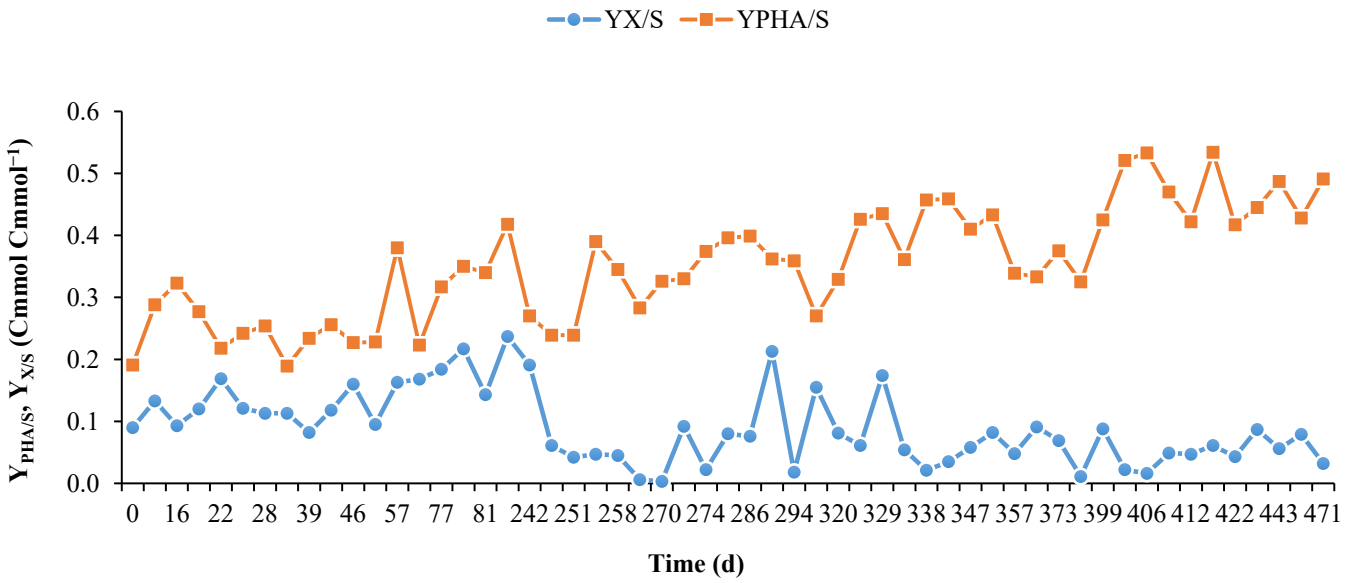

Figure 3. Monitoring of both the growth and the storage yields along the operation of the SBR culture selection.

During Period I, both yields reached the closest values of the entire experiment (Table 2), indicating that the biomass almost used the same amount of VFA for growth as well as for PHA storage. The mean value was 0.275 (±0.066) Cmmol-PHA Cmmol-VFA^{-1} for $Y_{PHA/S}$ and 0.140 (±0.044) Cmmol-X Cmmol-VFA^{-1} for $Y_{X/S}$. However, when the culture medium was modified for the first time, i.e., in Period II, both yields considerably diverged. There was no high improvement in $Y_{PHA/S}$ (0.327 ± 0.057 Cmmol-PHA Cmmol-VFA^{-1}) when thiourea was added to the culture medium, but the fraction of VFA directed towards cell growth exhibited a remarkable reduction to almost half of its value (0.075 ± 0.067 Cmmol-X Cmmol-VFA^{-1}) (Table 2). A reasonable explanation could be associated with an ammonia competition. During Period I, the absence of thiourea led both autotrophs and heterotrophs to coexist in the SBR, competing for the consumption of the same resource, ammonia. Therefore, the PHA-accumulating bacteria tended to divert more VFA for growth purposes. Conversely, when thiourea was added to the culture medium, autotrophic bacteria were suppressed, and heterotrophs did not need to compete anymore. Fra-Vázquez et al. [21] also studied the influence of thiourea, but without noticing any appreciable difference in the performance of the PHA-accumulating ability when working both with or without the addition of this chemical reagent. However, there was an inverse experimental design in the mentioned work that could have affected the final results compared to the present study, as the authors first enriched the MMC with the addition of thiourea to then suppress its supply.

The sole addition of thiourea was not enough to select a high PHA-accumulating MMC, as can be seen in Section 3.4. In this way, inductively coupled plasma mass spectrometry (ICP-MS) analyses were performed on the SBR feed (i.e., fermented cheese whey) as well as on the SBR effluent in order to determine a lack of some essential nutrients for the microorganisms, which could ultimately lead to a detrimental impact on the production of PHA. Among all the measured nutrients, magnesium, iron, zinc and boron showed a more limiting condition (Table 3), although only magnesium and iron were chosen to be synthetically included as part of the nutrient source (Period III). As a consequence of the external addition of these nutrients, a positive impact on PHA storage ability was observed (Table 2; Figure 3). The amount of VFA used for biomass growth was quite similar in comparison to the previous period (0.070 ± 0.040 Cmmol-X Cmmol-VFA^{-1}), but the microorganisms used a larger amount of VFA to synthesize PHA (0.396 ± 0.050 Cmmol-PHA Cmmol-VFA^{-1}). Thus, the competitive advantage of PHA-storing bacteria improved. Rarely have published research works referred to the influence of these nutrients in relation

to the PHA accumulation ability. Albuquerque et al. [29] observed that magnesium-limiting conditions due to struvite precipitation prevented biomass growth and therefore caused a cascade effect by increasing the F/F ratio, with the subsequent loss in the competitive advantage of the PHA-storing bacteria. On the other hand, Mohan and Reddy [34] tested the effect of many different factors, one of which was iron availability, in order to enhance the production of PHA. From their experiment, it turned out that the presence of iron in the culture medium benefited the production of PHA, although it was not the most influencing factor of all tested. It can be concluded, therefore, that magnesium and iron appeared to be somehow important factors in the selection of the PHA-storing bacteria, even though their role in the cells remains uncertain to date, and further research is needed.

Table 3. ICP-MS analyses for SBR feed (fermented cheese whey) and SBR effluent. The effluent was taken during withdrawal phase at the end of the famine phase.

	Sodium (mg L^{-1})	**Potassium (mg L^{-1})**	**Calcium (mg L^{-1})**	**Magnesium (mg L^{-1})**	**Iron (µg L^{-1})**	**Zinc (µg L^{-1})**	**Boron (µg L^{-1})**
SBR_{Feed}	1029	423	48.1	11.8	63	140	71
$SBR_{Effluent}$	873	38.5	6.62	<1	5.4	<10	5

Although the PHA-storing ability of the MMC was improved from the beginning of the experiment up to here, analogous experiments using fermented cheese whey as substrate suggested that higher $Y_{PHA/S}$ than achieved were feasible [5,27,28,35]. For this reason, the OLR was increased from 60 up to 80 CmM d^{-1} (Period IV). The OLR is one of the most important and, therefore, most studied parameters in PHA-accumulating systems. It has already been demonstrated that the higher the OLR, the better the performance in terms of PHA storage, but up to a certain point, beyond which biomass is negatively affected [29,30]. Hence, the optimal OLR is considered the highest possible that still turn out into a stable operation of the SBR. In our case, applying a higher OLR (80 CmM d^{-1}) led to an improvement in the PHA accumulation ability of the MMC by increasing and reducing even more $Y_{PHA/S}$ and $Y_{X/S}$, respectively (Figure 3). In that specific period, $Y_{PHA/S}$ reached its highest mean value of 0.458 (±0.061) Cmmol-PHA Cmmol-VFA^{-1} with a maximum peak of 0.534 Cmmol-PHA Cmmol-VFA^{-1}. Although this value was not in the same range as in the abovementioned research yet, indicating the MMC enrichment could be further optimized by changing some other operational conditions, it can be considered a good result, comparable to several other reported [16,21,23]. According to a better performance in the PHA accumulation ability, $Y_{X/S}$ reached its lowest mean value throughout the 471 days of operation (0.049 ± 0.026 Cmmol-X Cmmol-VFA^{-1}). Based on these results, which were in agreement with the previous literature, the OLR could be further increased since nor the F/F ratio was widely disturbed (Figure 2) neither oxygen-limiting conditions were achieved in the SBR cycles (data not shown).

Concerning the monomeric composition of the final biopolymer, no big differences were found throughout the whole PHA-storing culture selection. The microorganisms only synthesized hydroxybutyrate (HB) and hydroxyvalerate (HV) as PHA monomers and in quite constant percentages. The HB was the most synthesized monomer by far with 70–80% of total PHA. Conversely, the remaining 20–30% was HV. No other hydroxyalkanoate monomer was detected, and therefore, the final biopolymer was a copolymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3-HB-co-3-HV)). The introduction of 20–30% HV in the final monomeric composition of the biopolymer is reported to improve the physicochemical properties compared to polyhydroxybutyrate homopolymers [36]. This also leads to an increase in commercial interest of the PHA biopolymers derived from cheese whey.

3.3. Impact of Operational Changes on Microbial Community

In order to obtain a deeper knowledge about the selection stage, microbial analyses were run in parallel to the SBR performance. To achieve this, biomass samples were taken from the SBR under steady state in Periods I, III and IV and analysed through DGGE.

Once the microbial analysis was finished, a typical banding pattern was observed. All the bands were excised from the gel and sent to sequencing, even though not all of them yielded explicit sequences (Figure 4). In Period I, three main bacterial populations dominated the microbial community. Bands A1–A4 had a percentage of similarity of 89–93% to *Hydrogenophaga temperata* from the *Comamonadaceae* family. Band A5 fitted 98% to *Brevundimonas faecalis* and band A6 shared 99% of its sequence with *Millisia brevis*. In Period III, only one single band was properly identified. Band B1 fitted 99% to the partial sequence of *Thauera linaloolentis*. Finally, in Period IV, the microbial community was mainly enriched in two bacterial populations. Band C1 showed 93% similarity with *Azoarcus tolulyticus*, while bands C2 and C3 shared 95 and 99% with the partial sequence of *Amaricoccus kaplicensis*.

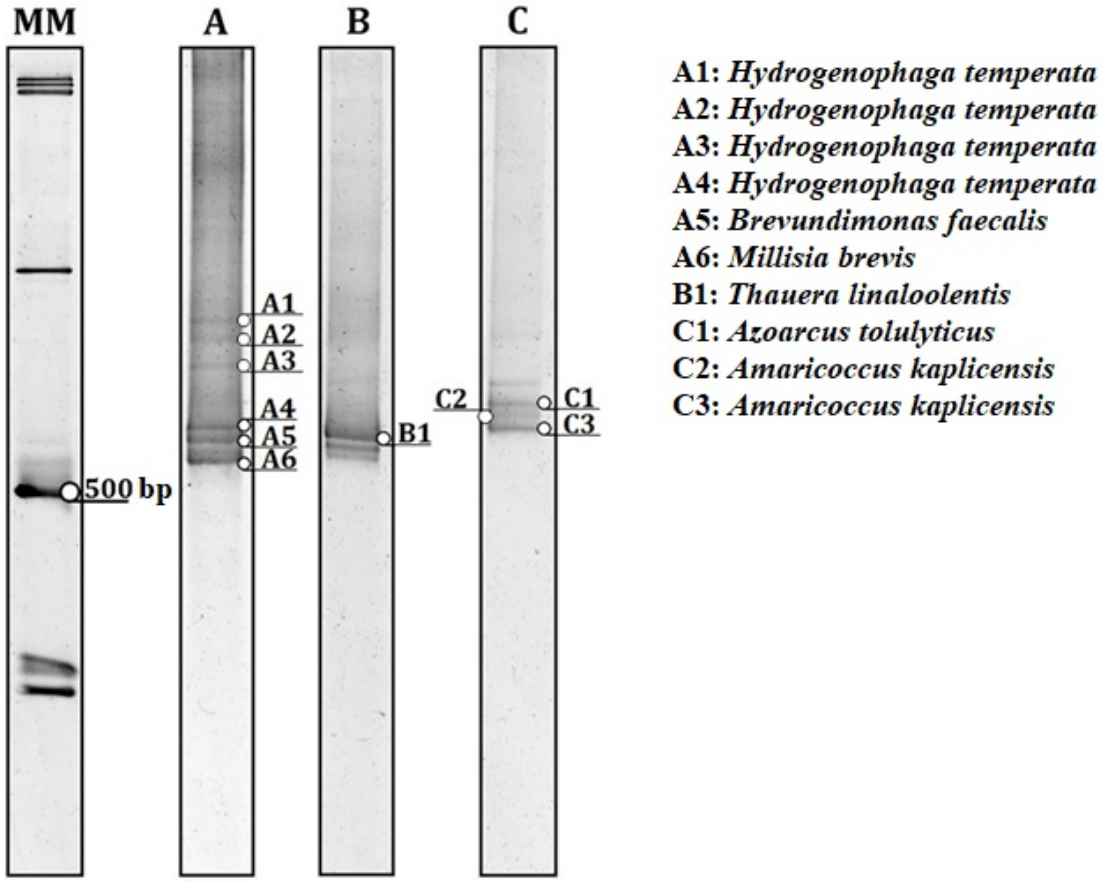

Figure 4. DGGE gel of the PCR amplicons obtained from the enrichment SBR at Period I (lane **A**), Period III (lane **B**) and Period IV (lane **C**). A molecular ladder was also run, and it is shown on the left-hand (lane **MM**).

Based on the DGGE observations, it can be concluded that the operational conditions applied to the SBR did strongly influence the microbial community composition. No one single organism was indeed able to withstand the shift in the operational conditions. One of the factors that might have contributed to the observed microbial fluctuations amongst the different operational periods could be the low SRT. Applying a SRT of 3 days might have promoted a quick wash-out of bacteria non-adapted to the new environmental conditions, and therefore, the bacteria were limited only to those who had a competitive advantage. The first sequencing point (Period I) revealed that the simple application of the ADF process was

enough to enrich the microbial community mainly in PHA-accumulating bacteria despite not adding thiourea. The feast-to-famine regime did indeed create a proper selective pressure on the non-PHA-accumulating bacteria, and therefore, it favoured the prevalence of microorganisms with PHA-storing ability. In fact, the presence of *Hydrogenophaga* (or other members of the *Comamonadaceae* family) as well as *Brevundimonas* has been extensively reported in the literature as PHA-accumulating microorganisms selected from sewage sludge [5,13,37,38]. No references to the ability of the third identified taxon, *Millisia brevis*, to synthesize PHA were found, and it could be a non-PHA-storing microorganism. Thiourea has been demonstrated to be an effective inhibitor of nitrifying activity by chelating the copper of the ammonia monooxygenase active site [39]. Therefore, the lack of thiourea in the culture medium could have promoted a competition between the heterotrophic and autotrophic populations, but no group from the latter has been identified as the dominant group. Nevertheless, this does not confirm the complete absence of ammonia-oxidizing bacteria (AOB) in the microbial community and, consequently, the absence of nitrifying activity. In that sense, Fra-Vázquez et al. [21] observed that AOB only represented a 10% of the whole microbial community despite monitoring a great nitrifying activity in the performance of their SBR without any addition of this chemical.

There was a considerable change in the microbial community composition in Period III, and *Hydrogenophaga* and *Brevundimonas* genera were displaced by *Thauera* species. Many of the different species belonging to the *Thauera* genus have a close relation to PHA production processes, and their ability to synthesize the biopolymer has already been reported [40–43]. The presence of thiourea, magnesium and iron as part of the culture medium were the only differences regarding Period I. Since thiourea has already been observed to only influence the autotrophic populations [21], these results suggested that both magnesium and iron had an active role in the modification of the PHA-accumulating populations, even though a deeper knowledge is needed.

Finally, the OLR also showed to influence the microbial community composition. As had happened before, the new change in the operational conditions completely modified the main bacterial populations, and *Thauera* was displaced by *Azoarcus* and *Amaricoccus*. Species from both genera have already been identified as PHA-storing microorganisms in previous works [44,45]. The OLR is a widely studied parameter for its effect on the selection stage. However, research on its influence in reference to the microbial community composition is not so widespread. However, from a few of these studies, quite similar results to those obtained in the current research were found, and the applied OLR seemed to clearly affect the development of one or another bacteria [29,31]. Moreover, Carvalho et al. [31] also reported that *Azoarcus* prevailed when applying an OLR of 90 Cmmol L^{-1} d^{-1}, while *Thauera* was more abundant at an OLR of 60 Cmmol L^{-1} d^{-1}. In addition, the aforementioned reference has also pointed out that *Azoarcus* diverted more VFA to PHA storage compared to *Thauera*, and therefore, a higher storage yield was reached. This fact was also observed here. When *Azoarcus* and *Amaricoccus* genera dominated the microbial community, a higher $Y_{PHA/S}$ was achieved.

3.4. Determination of the Maximum PHA-Storage Ability

Fed-batch assays allowed us to test the maximum ability of the MMC to accumulate PHA in each of the four different operational periods applied during the SBR operation. In addition, these experiments were designed when steady state was reached, at the end of each operational period. In consequence, it was a reliable way of evaluating whether the improvement achieved during the selection stage had a real influence on PHA accumulation or not. The DO became a key factor within the pulse feeding strategy that has been followed for the introduction of new pulses of substrate to the culture medium, as described previously [22]. Figure 5 shows the PHA evolution monitored throughout the entire fed-batch assays. In the first two hours of experiment, faster PHA production kinetics was observed except for the fed-batch assay of Period I in which the PHA concentration increased more gradually throughout the experiment. After that, the PHA production rate

progressively decreased at increasing PHA content, a typical response in fed-batch assays due to biomass saturation [22,46,47]. Based on these results, it was possible to confirm that the improvement observed in the selection stage did indeed influence the maximum ability of the MMC to produce PHA. In fact, the maximum accumulation more than doubled from Period I (24%) to Period IV (54%). The PHA content in Period II and III was in an intermediate position with an intracellular PHA content of 40 and 45%, respectively. The maximum percentage of accumulated PHA that has been reached in the present study (54%) can be considered a very good result and near the average reached by other researchers who used fermented cheese whey as substrate [26,35]. Nevertheless, it is still far from those obtained by Colombo et al. [27], Albuquerque et al. [29] or Jiang et al. [47]. From a future perspective, the implementation of new approaches to improve the selection stage and, consequently, the maximum accumulation potential may be required.

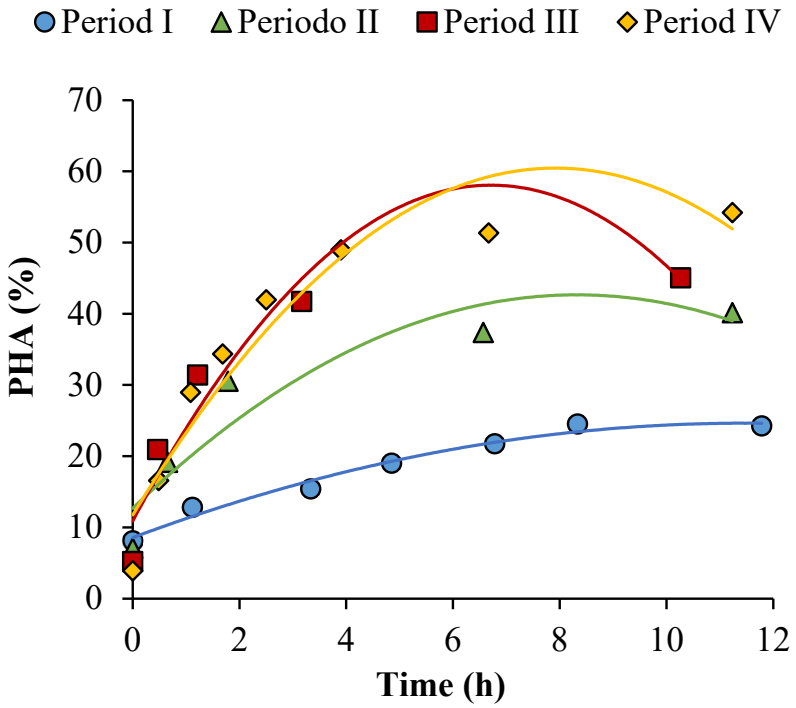

Figure 5. Accumulation tests to evaluate the maximum ability of the MMC to produce PHA at each of the four different operational periods.

4. Conclusions

The results obtained in this study demonstrated that PHA production from cheese whey is feasible using MMC. Although the selection of PHA-storing biomass was poor at the beginning, it greatly improved when thiourea, magnesium and iron were included in the culture medium, as well as when the OLR was increased. In fact, the maximum ability of the biomass to accumulate PHA more than doubled (from 21 up to 54%). Along with the increase in the storage ability, there also was a change in the microbial community. *Amaricoccus* and *Azoarcus* dominated the microbial community when the highest PHA-storing potential was reached. The next steps should involve PHA production at pilot and at industrial scales as well as the development of an efficient and greener technology for the extraction of the intracellularly accumulated PHA, which appears to be the major bottleneck.

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References

1. OECD. *Global Plastics Outlook: Economic Drivers, Environmental Impacts and Policy Options*; OECD: Paris, France, 2022. [CrossRef]
2. Kourmentza, C.; Plácido, J.; Venetsaneas, N.; Burniol-Figols, A.; Varrone, C.; Gavala, H.N.; Reis, M.A.M. Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production. *Bioengineering* **2017**, *4*, 55. [CrossRef] [PubMed]
3. Fernández-Dacosta, C.; Posada, J.A.; Kleerebezem, R.; Cuellar, M.C.; Ramirez, A. Microbial Community-Based Polyhydroxyalkanoates (PHAs) Production from Wastewater: Techno-Economic Analysis and Ex-Ante Environmental Assessment. *Bioresour. Technol.* **2015**, *185*, 368–377. [CrossRef] [PubMed]
4. Matos, M.; Cruz, R.A.P.; Cardoso, P.; Silva, F.; Freitas, E.B.; Carvalho, G.; Reis, M.A.M. Combined Strategies to Boost Polyhydroxyalkanoate Production from Fruit Waste in a Three-Stage Pilot Plant. *ACS Sustain. Chem. Eng.* **2021**, *9*, 8270–8279. [CrossRef]
5. Oliveira, C.S.S.; Silva, C.E.; Carvalho, G.; Reis, M.A. Strategies for Efficiently Selecting PHA Producing Mixed Microbial Cultures Using Complex Feedstocks: Feast and Famine Regime and Uncoupled Carbon and Nitrogen Availabilities. *New Biotechnol.* **2017**, *37*, 69–79. [CrossRef]
6. Moretto, G.; Lorini, L.; Pavan, P.; Crognale, S.; Tonanzi, B.; Rossetti, S.; Majone, M.; Valentino, F. Biopolymers from Urban Organic Waste: Influence of the Solid Retention Time to Cycle Length Ratio in the Enrichment of a Mixed Microbial Culture (MMC). *ACS Sustain. Chem. Eng.* **2020**, *8*, 14531–14539. [CrossRef]
7. Valentino, F.; Lorini, L.; Gottardo, M.; Pavan, P.; Majone, M. Effect of the Temperature in a Mixed Culture Pilot Scale Aerobic Process for Food Waste and Sewage Sludge Conversion into Polyhydroxyalkanoates. *J. Biotechnol.* **2020**, *323*, 54–61. [CrossRef]
8. de Oliveira, G.H.D.; Niz, M.Y.K.; Zaiat, M.; Rodrigues, J.A.D. Effects of the Organic Loading Rate on Polyhydroxyalkanoate Production from Sugarcane Stillage by Mixed Microbial Cultures. *Appl. Biochem. Biotechnol.* **2019**, *189*, 1039–1055. [CrossRef]
9. Montiel-Jarillo, G.; Carrera, J.; Suárez-Ojeda, M.E. Enrichment of a Mixed Microbial Culture for Polyhydroxyalkanoates Production: Effect of PH and N and P Concentrations. *Sci. Total Environ.* **2017**, *583*, 300–307. [CrossRef]
10. Ben, M.; Kennes, C.; Veiga, M.C. Optimization of Polyhydroxyalkanoate Storage Using Mixed Cultures and Brewery Wastewater. *J. Chem. Technol. Biotechnol.* **2016**, *91*, 2817–2826. [CrossRef]
11. Campanari, S.; Augelletti, F.; Rossetti, S.; Sciubba, F.; Villano, M.; Majone, M. Enhancing a Multi-Stage Process for Olive Oil Mill Wastewater Valorization towards Polyhydroxyalkanoates and Biogas Production. *Chem. Eng. J.* **2017**, *317*, 280–289. [CrossRef]
12. Moretto, G.; Russo, I.; Bolzonella, D.; Pavan, P.; Majone, M.; Valentino, F. An Urban Biorefinery for Food Waste and Biological Sludge Conversion into Polyhydroxyalkanoates and Biogas. *Water Res.* **2020**, *170*, 115371. [CrossRef]
13. Pereira, J.; Queirós, D.; Lemos, P.C.; Rossetti, S.; Serafim, L.S. Enrichment of a Mixed Microbial Culture of PHA-Storing Microorganisms by Using Fermented Hardwood Spent Sulfite Liquor. *New Biotechnol.* **2020**, *56*, 79–86. [CrossRef]
14. Tomas-Pejo, E.; Gonzalez-Fernandez, C.; Greses, S.; Kennes, C.; Otero-Logilde, N.; Veiga, M.C.; Bolzonella, D.; Muller, B.; Passoth, V. Production of short-chain fatty acids (SCFAs) as chemicals or substrates for microbes to obtain biochemicals. *Biotechnol. Biofuels Bioprod.* **2023**, *16*, 96. [CrossRef]
15. Queirós, D.; Fonseca, A.; Lemos, P.C.; Serafim, L.S. Long-Term Operation of a Two-Stage Polyhydroxyalkanoates Production Process from Hardwood Sulphite Spent Liquor. *J. Chem. Technol. Biotechnol.* **2016**, *91*, 2480–2487. [CrossRef]
16. Palmeiro-Sánchez, T.; Fra-Vázquez, A.; Rey-Martínez, N.; Campos, J.L.; Mosquera-Corral, A. Transient Concentrations of NaCl Affect the PHA Accumulation in Mixed Microbial Culture. *J. Hazard. Mater.* **2016**, *306*, 332–339. [CrossRef]
17. Lagoa-Costa, B.; Kennes, C.; Veiga, M.C. Cheese Whey Fermentation into Volatile Fatty Acids in an Anaerobic Sequencing Batch Reactor. *Bioresour. Technol.* **2020**, *308*, 123226. [CrossRef]
18. Lagoa-Costa, B.; Kennes, C.; Veiga, M.C. Influence of Feedstock Mix Ratio on Microbial Dynamics during Acidogenic Fermentation for Polyhydroxyalkanoates Production. *J. Environ. Manag.* **2022**, *303*, 114132. [CrossRef]
19. Portela-Grandío, A.; Lagoa-Costa, B.; Kennes, C.; Veiga, M.C. Polyhydroxyalkanoates Production from Syngas Fermentation Effluents: Effect of Nitrogen Availability. *J. Environ. Chem. Eng.* **2021**, *9*, 106662. [CrossRef]
20. Lagoa-Costa, B.; Abubackar, H.N.; Fernández-Romasanta, M.; Kennes, C.; Veiga, M.C. Integrated Bioconversion of Syngas into Bioethanol and Biopolymers. *Bioresour. Technol.* **2017**, *239*, 244–249. [CrossRef]
21. Fra-Vázquez, A.; Santorio, S.; Palmeiro-Sánchez, T.; Val del Río, Á.; Mosquera-Corral, A. PHA Accumulation of a Mixed Microbial Culture Co-Exists with Ammonia Partial Nitritation. *Chem. Eng. J.* **2019**, *360*, 1255–1261. [CrossRef]

22. Tamang, P.; Banerjee, R.; Köster, S.; Nogueira, R. Comparative Study of Polyhydroxyalkanoates Production from Acidified and Anaerobically Treated Brewery Wastewater Using Enriched Mixed Microbial Culture. *J. Environ. Sci.* **2019**, *78*, 137–146. [CrossRef] [PubMed]
23. Silva, F.; Campanari, S.; Matteo, S.; Valentino, F.; Majone, M.; Villano, M. Impact of Nitrogen Feeding Regulation on Polyhydroxyalkanoates Production by Mixed Microbial Cultures. *New Biotechnol.* **2017**, *37*, 90–98. [CrossRef] [PubMed]
24. Johnson, K.; Kleerebezem, R.; van Loosdrecht, M.C.M. Influence of the C/N Ratio on the Performance of Polyhydroxybutyrate (PHB) Producing Sequencing Batch Reactors at Short SRTs. *Water Res.* **2010**, *44*, 2141–2152. [CrossRef] [PubMed]
25. Dionisi, D.; Majone, M.; Vallini, G.; Di Gregorio, S.; Beccari, M. Effect of the Applied Organic Load Rate on Biodegradable Polymer Production by Mixed Microbial Cultures in a Sequencing Batch Reactor. *Biotechnol. Bioeng.* **2006**, *93*, 76–88. [CrossRef]
26. Oliveira, C.; Silva, M.; Silva, C.E.; Carvalho, G.; Reis, M.A.M. Assessment of Protein-Rich Cheese Whey Waste Stream as a Nutrients Source for Low-Cost Mixed Microbial PHA Production. *Appl. Sci.* **2018**, *8*, 1817. [CrossRef]
27. Colombo, B.; Sciarria, T.P.; Reis, M.; Scaglia, B.; Adani, F. Polyhydroxyalkanoates (PHAs) Production from Fermented Cheese Whey by Using a Mixed Microbial Culture. *Bioresour. Technol.* **2016**, *218*, 692–699. [CrossRef]
28. Colombo, B.; Calvo, M.V.; Sciarria, T.P.; Scaglia, B.; Kizito, S.S.; D'Imporzano, G.; Adani, F. Biohydrogen and Polyhydroxyalkanoates (PHA) as Products of a Two-Steps Bioprocess from Deproteinized Dairy Wastes. *Waste Manag.* **2019**, *95*, 22–31. [CrossRef]
29. Albuquerque, M.G.E.; Torres, C.A.V.; Reis, M.A.M. Polyhydroxyalkanoate (PHA) Production by a Mixed Microbial Culture Using Sugar Molasses: Effect of the Influent Substrate Concentration on Culture Selection. *Water Res.* **2010**, *44*, 3419–3433. [CrossRef]
30. Simona, C.; Laura, L.; Francesco, V.; Marianna, V.; Cristina, M.G.; Barbara, T.; Mauro, M.; Simona, R. Effect of the Organic Loading Rate on the PHA-Storing Microbiome in Sequencing Batch Reactors Operated with Uncoupled Carbon and Nitrogen Feeding. *Sci. Total Environ.* **2022**, *825*, 153995. [CrossRef]
31. Carvalho, G.; Oehmen, A.; Albuquerque, M.G.E.; Reis, M.A.M. The Relationship between Mixed Microbial Culture Composition and PHA Production Performance from Fermented Molasses. *New Biotechnol.* **2014**, *31*, 257–263. [CrossRef]
32. Marang, L.; Jiang, Y.; van Loosdrecht, M.C.M.; Kleerebezem, R. Butyrate as Preferred Substrate for Polyhydroxybutyrate Production. *Bioresour. Technol.* **2013**, *142*, 232–239. [CrossRef]
33. Bengtsson, S.; Hallquist, J.; Werker, A.; Welander, T. Acidogenic Fermentation of Industrial Wastewaters: Effects of Chemostat Retention Time and PH on Volatile Fatty Acids Production. *Biochem. Eng. J.* **2008**, *40*, 492–499. [CrossRef]
34. Venkata Mohan, S.; Venkateswar Reddy, M. Optimization of Critical Factors to Enhance Polyhydroxyalkanoates (PHA) Synthesis by Mixed Culture Using Taguchi Design of Experimental Methodology. *Bioresour. Technol.* **2013**, *128*, 409–416. [CrossRef]
35. Duque, A.F.; Oliveira, C.S.S.; Carmo, I.T.D.; Gouveia, A.R.; Pardelha, F.; Ramos, A.M.; Reis, M.A.M. Response of a Three-Stage Process for PHA Production by Mixed Microbial Cultures to Feedstock Shift: Impact on Polymer Composition. *New Biotechnol.* **2014**, *31*, 276–288. [CrossRef]
36. Anjum, A.; Zuber, M.; Zia, K.M.; Noreen, A.; Anjum, M.N.; Tabasum, S. Microbial Production of Polyhydroxyalkanoates (PHAs) and Its Copolymers: A Review of Recent Advancements. *Int. J. Biol. Macromol.* **2016**, *89*, 161–174. [CrossRef]
37. Crognale, S.; Tonanzi, B.; Valentino, F.; Majone, M.; Rossetti, S. Microbiome Dynamics and PhaC Synthase Genes Selected in a Pilot Plant Producing Polyhydroxyalkanoate from the Organic Fraction of Urban Waste. *Sci. Total Environ.* **2019**, *689*, 765–773. [CrossRef]
38. Bhuwal, A.K.; Singh, G.; Aggarwal, N.K.; Goyal, V.; Yadav, A. Isolation and Screening of Polyhydroxyalkanoates Producing Bacteria from Pulp, Paper, and Cardboard Industry Wastes. *Int. J. Biomater.* **2013**, *2013*, 752821. [CrossRef]
39. Ginestet, P.; Audic, J.-M.; Urbain, V.; Block, J.-C. Estimation of Nitrifying Bacterial Activities by Measuring Oxygen Uptake in the Presence of the Metabolic Inhibitors Allylthiourea and Azide. *Appl. Environ. Microbiol.* **1998**, *64*, 2266–2268. [CrossRef]
40. Jiang, Y.; Marang, L.; Kleerebezem, R.; Muyzer, G.; van Loosdrecht, M.C.M. Polyhydroxybutyrate Production from Lactate Using a Mixed Microbial Culture. *Biotechnol. Bioeng.* **2011**, *108*, 2022–2035. [CrossRef]
41. Farghaly, A.; Enitan, A.M.; Kumari, S.; Bux, F.; Tawfik, A. Polyhydroxyalkanoates Production from Fermented Paperboard Mill Wastewater Using Acetate-Enriched Bacteria. *Clean Technol. Environ. Policy* **2017**, *19*, 935–947. [CrossRef]
42. Carvalho, G.; Pedras, I.; Karst, S.M.; Oliveira, C.S.S.; Duque, A.F.; Nielsen, P.H.; Reis, M.A.M. Functional Redundancy Ensures Performance Robustness in 3-Stage PHA-Producing Mixed Cultures under Variable Feed Operation. *New Biotechnol.* **2018**, *40*, 207–217. [CrossRef] [PubMed]
43. Wen, Q.; Ji, Y.; Hao, Y.; Huang, L.; Chen, Z.; Sposob, M. Effect of Sodium Chloride on Polyhydroxyalkanoate Production from Food Waste Fermentation Leachate under Different Organic Loading Rate. *Bioresour. Technol.* **2018**, *267*, 133–140. [CrossRef] [PubMed]
44. Lemos, P.C.; Levantesi, C.; Serafim, L.S.; Rossetti, S.; Reis, M.A.M.; Tandoi, V. Microbial Characterisation of Polyhydroxyalkanoates Storing Populations Selected under Different Operating Conditions Using a Cell-Sorting RT-PCR Approach. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 351–360. [CrossRef] [PubMed]
45. Wang, X.; Oehmen, A.; Freitas, E.B.; Carvalho, G.; Reis, M.A.M. The Link of Feast-Phase Dissolved Oxygen (DO) with Substrate Competition and Microbial Selection in PHA Production. *Water Res.* **2017**, *112*, 269–278. [CrossRef]
46. Johnson, K.; Jiang, Y.; Kleerebezem, R.; Muyzer, G.; Van Loosdrecht, M.C.M. Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity. *Biomacromolecules* **2009**, *10*, 670–676. [CrossRef]

47. Jiang, Y.; Marang, L.; Tamis, J.; van Loosdrecht, M.C.M.; Dijkman, H.; Kleerebezem, R. Waste to Resource: Converting Paper Mill Wastewater to Bioplastic. *Water Res.* **2012**, *46*, 5517–5530. [CrossRef]