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Recovery of Polyhydroxyalkanoates from Cooked Mussel Processing Wastewater at High Salinity and Acidic Conditions

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Abstract: Polyhydroxyalkanoates (PHA) are biodegradable polymers that can be intracellularly produced by microorganisms valorizing organic-rich wastes. In the present study, a PHA production system was fed with mussel cooker wastewater after acidogenic fermentation. Besides low pH (4.0 ± 0.3) and high salt (21.7 ± 2.9 g NaCl/L) concentrations, this wastewater also contained nitrogen concentrations (0.8 ± 0.1 g N/L), which were previously reported to be a challenge to the PHA accumulating bacteria enrichment. Bacteria with a PHA storage capacity were selected in an enrichment sequencing batch reactor (SBR) after 60 days of operation. The enriched mixed microbial culture (MMC) was mainly formed by microorganisms from phylum *Bacteroidetes*, and genera *Azoarcus*, *Comamonas* and *Thauera* from phylum *Proteobacteria*. The MMC was able to accumulate up to 25 wt% of PHA that was mainly limited by the wastewater nitrogen content, which promoted biomass growth instead of PHA accumulation. Indeed, when the presence of nutrient was limited, PHA stored in the accumulation reactor increased to up to 40.9 wt%. This work demonstrated the feasibility of the enrichment of a MMC with a PHA storage ability valorizing the fish-canning industrial wastewater at low pH, which is generally difficult to treat in wastewater treatment plants.

Keywords: fish-canning industry; industrial wastewater valorization; low pH; mixed microbial culture; PHA; value-added products

1. Introduction

Fish and seafood processing industries consume vast volumes of water and, consequently, generate large amounts of wastewater with high organic matter, nutrients and salt concentrations. Depending on the processed fish product (tuna, mussels, etc.) and seasonal variations, the generated waste streams show different compositions [1,2]. In fact, effluents with distinct qualities and flows may be produced even in the same facility, depending on the processing steps, challenging its adequate treatment [3,4]. The high loaded wastewaters generated in these facilities often contain chemical oxygen demand (COD) concentrations up to 42 g COD/L characterized by its high complexity with not only easily-biodegradable carbohydrates but also relevant protein fractions (15–20% of wet weight), and lipids (0.1–44 g/L) [2,5].

Different biological treatment technologies have been developed to treat these effluents, focusing on the achievement of more efficiency, less energy consumption and fewer surface requirements for implantation [5]. Méndez et al. [6] achieved, in an anaerobic digester, a COD removal efficiency of 80% treating effluents generated in a factory processing different fish products with high salinity (up to 15 g Cl^-/L). Picos–Benítez et al. [7] reported that the biogas yield decreases by 64% when the

salt concentration increases from 0 to 20 g NaCl/L while treating wastewater from the evisceration process in the fish processing industry. At fish and seafood canning facilities, the wastewater treatment plant (WWTP) configuration highly varies depending on the wastewater characteristics and the local discharge limits. The most common technologies are a dissolved air floatation (DAF) pretreatment to remove grease and oil followed by a biological treatment that might use anaerobic (in large industries) or aerobic systems to remove organic matter and, eventually, nitrogen. Indeed, there are fish and seafood canning facilities that treat their effluents by only applying physical-chemical treatment steps to remove grease and solids and then, the wastewater is discharged to the municipal WWTP to be polished. In the last few decades, in the frame of the circular economy concept, other added-value products were proposed as an alternative to biogas to obtain more sustainable materials. For example, fish and seafood industry wastes (as organic-rich streams) can be valorized to produce volatile fatty acids (VFA) suitable for use as a chemical platform to obtain other compounds such as biopolymers [8,9].

In search of new materials, polyhydroxyalkanoates (PHA) have appeared as an alternative to fossil fuel-based plastics due to their similar properties [8,10]. PHA constitute a group of biodegradable polymers that can be produced by microorganisms as carbon and energy reserves [11]. PHA production by pure microbial cultures are the most widely employed strategies at industrial scale due to the high accumulation capacities of single strains. However, these strategies are limited by the specific substrates and sterile conditions required, which increase the operational costs and, therefore, the final product price. Thus, mixed microbial cultures (MMC) have appeared as a promising alternative to pure cultures due to the expected reduction of PHA production costs, which is associated with the low price of the substrates (wastes) and the fact that sterile conditions are not required. The typical PHA production system using MMC comprises three stages [12]: (1) acidogenic fermentation of the raw feedstock to produce a VFA-rich effluent; (2) selection of PHA-accumulating microorganisms in an enrichment reactor; and, finally, (3) accumulation assays to maximize the PHA production.

Wastewater streams produced in the food processing industry containing high organic content appear as suitable substrates for PHA production, such as those from sugar factories [13], the brewery industry [14] and cheese whey from cheese production [15]. Therefore, PHA production using MMC represents an opportunity to recover organic carbon from wastewater. However, PHA production from complex waste streams must take into consideration certain characteristics of the wastewater, which might decrease the storage capacity of the system [8,16]. In the case of the cooked mussel processing wastewater, the presence of high NaCl concentrations has been traditionally considered as being inhibitory for aerobic [17] and anaerobic biological processes [18]. Passanha et al. [19] studied the effect of salts on a pure culture of *Cupriavidus necator* and obtained the highest PHA production with the addition of 9 g NaCl/L, which yielded 30% higher PHA than the control without salt. The effect of saline conditions over non-adapted MMC was evaluated by Palmeiro–Sánchez et al. [20] and observed that the salt provoked a decrease of the PHA production rate, with a half inhibitory concentration (IC_{50}) value close to 6 g NaCl/L. pH value is another crucial operational parameter since microbial activities are critically dependent on the pH homeostasis because most proteins and enzymes have different optimal pH range values to operate. For example, bacteria can grow at external pH ranges from 5.5 to 9.0, but they generally maintain their cytoplasmic pH in a narrow range of 7.5–7.7 [21]. Therefore, most of the microorganisms have strategies to maintain a significantly more alkaline cytoplasmic pH relative to the outside pH value.

The present study evaluates the potential of pre-fermented VFA-rich wastewater, coming from the cookers of a mussel-processing factory [9], to be used as a substrate to produce PHA. An enrichment reactor was operated with the ability to use high salt concentrations and a low pH. Main PHA-accumulating microorganisms selected in the MMC were identified. Furthermore, several fed-batch assays were carried out to test the maximum storage capacity in PHA of the microbial community selected.

2. Materials and Methods

2.1. PHA Production System

The PHA production system comprising two stages was operated. In the first stage, PHA-accumulating microorganisms were selected in an enrichment sequencing batch reactor (SBR) operated under the feast-famine regime. Then, the maximum PHA accumulation capacity of the enriched MMC was promoted in fed-batch assays.

2.1.1. Enrichment Reactor

A double jacket tubular SBR with a working volume of 2 L was operated under non-sterile and fully aerobic conditions to select a MMC enriched in PHA-accumulating bacteria. Since many microbial populations naturally produce PHA, activated sludge from a municipal WWTP was used as inoculum with an initial volatile suspended solids (VSS) concentration of 2.4 g VSS/L. The complete medium mixture was achieved by using the supply of air (6 L/min), which was introduced through a ceramic air diffuser located at the bottom of the reactor. The temperature was controlled at 30 °C by using a thermostatic bath (Techne Inc., Burlington, NJ, USA). The dissolved oxygen (DO) concentration was measured with an oxygen pocket meter provided with a membrane sensor (Hach-Lange, Loveland, CO, USA). The DO concentration and the pH value in the reactor media were not controlled. The SBR cycles lasted for 12 h and were divided into 3 phases: feeding (15 min), aerobic reaction (690 min) and withdrawal (15 min). Cycles were controlled by a programmable logic controller (PLC, Siemens S7-224 CPU). Continuous aeration (no settling stage) resulted in equal sludge retention times (SRT) and hydraulic retention times (HRT) of 1 day.

Cooked mussel processing wastewater was fermented at 35 °C to produce a VFA-rich stream (details provided in Fra-Vázquez, Pedrouso, Val del Rio and Mosquera-Corral [9]). Then, after solids separation by centrifugation, wastewater was fed to the system. The mixture of VFA in this wastewater was composed of acetic acid (HAc), propionic acid (HPr), butyric acid (HBu) and valeric acid (HVa). The wastewater (described in Table 1) was five times diluted with tap water to achieve an optimal organic loading rate of 2.5 g COD/(L·d) to feed the PHA production system. Fluctuations in the composition of the raw wastewater fed to the previous acidified system resulted in variable acidified effluents [9]. Hence, the feeding of the enrichment reactor also showed these fluctuations. Moreover, 10 mg/L of allylthiourea (ATU) were added to the feeding to prevent nitrification.

Table 1. Average composition of the acidified mussel cookers wastewater.

Parameter	Value
pH	3.99 ± 0.30
sCOD (g/L)	13.01 ± 3.25
VFA (g COD_{VFA}/L)	4.06 ± 1.27
% HAc (as COD)	42.06 ± 7.05
% HPr (as COD)	16.43 ± 9.67
% HBu (as COD)	36.73 ± 11.69
% HVa (as COD)	6.83 ± 3.74
Carbohydrates (g/L)	0.04 ± 0.02
Proteins (g/L)	2.34 ± 0.67
Total Nitrogen (g N/L)	0.82 ± 0.14
Ammonium (g NH_4^+-N/L)	0.21 ± 0.06
NaCl (g/L)	21.69 ± 2.92

2.1.2. Accumulation Assays

Biomass samples were collected from the enrichment SBR at the end of the operational cycle to perform the accumulation assays in a 2-L fed-batch reactor (FB-R). The accumulatio experiments were also carried out at 30 °C, controlled by using a thermostatic bath (Techne Inc., Burlington, NJ, USA). The DO

concentration was monitored but neither the DO concentration nor the pH value were controlled. Two types of carbon sources were tested: the acidified cooked mussel processing wastewater without dilution (Table 1) and a mixture of VFA. The latter was prepared to mimic the composition of the acidified wastewater in terms of VFA (43:7:42:8, as HAc:HPr:HBu:HVa in Cmmol fraction percentages), to serve as a basis to evaluate the effects of the complex matrix of the industrial wastewater on the accumulation experiment. Both substrates were manually added in pulses every time that an increase in the DO concentration was observed, which coincided with the complete depletion of the carbon source added in the previous pulse.

2.2. Identification of Microbial Populations

Fresh biomass samples were collected from the enrichment SBR. The fluorescence in situ hybridisation (FISH) technique was carried to identify the microbial populations. Biomass samples were fixed with paraformaldehyde 4% (*wt/vol*) solution according to the procedure described by Amann et al. [22]. Hybridization was performed at 46 °C for 90 min, adjusting the percentages of formamide to each probe. Bacterial cells were hybridized with several FISH probes.

General FISH probes used were: EUB338mix, for all *Bacteria*; a mix of CFB562 and CF319ab for phylum *Bacteroidetes*; and ALF1b, BET42a and GAM42a probes for classes *Alpha-*, *Beta-* and *Gamma-*, respectively, from phylum *Proteobacteria*. More specific probes were: PAE997, PAR1244, Cte, Zra23a, MZ1 and Azo644 for genera *Pseudomonas*, *Paracoccus*, *Comamonas*, *Zoogloea*, *Thauera* and *Azoarcus*, respectively. All probes were 5′ labeled by using fluorochromes FITC (Fluorescein-5-isocyanate) or Cy3 (Carbocyanine 3). DAPI (4, 6-diamidino-2-phenylindole) was used as a universal dye for the detection of all DNA in the samples. Fluorescence signals were captured using an acquisition system (Coolsnap, Roper Scientific Photometrics) coupled with an epifluorescence microscope (Axioskop 2, Zeiss, Obercochen, Germany). The semi-quantitative counting of the bacterial populations, based on the biovolume fraction, was performed with DAIME software [23].

2.3. Analytical Methods

Total Suspended Solids (TSS) and VSS were analyzed according to *Standard Methods for the Examination of Water and Wastewater* [24]. Liquid samples were filtered through a cellulose-ester filter of 0.45 µm of pore size (Advantec, Japan) for the quantification of total organic carbon (TOC), total nitrogen (TN), ammonium (NH_4^+), soluble COD (sCOD) [24], ions (e.g., Na^+ and Cl^-), proteins, carbohydrates and VFA concentrations. TOC and TN concentrations were determined by catalytic combustion in a TOC-L CSN analyzer (Shimadzu, Kyoto, Japan). Ammonium was determined following the methodology described by Bower and Holm–Hansen [25]. Ion chromatography (861 Advanced Compact IC system, Methrom, Herisau, Switzerland) was used to determine the concentration of Na^+ and Cl^-, among other ions, to calculate the salt concentration. VFA concentration was determined by gas chromatography (GC) (Hewlett Packard 5890 A, Palo Alto, CA, USA). Protein content was measured according to Lowry et al. [26] using bovine serum albumin (BSA, Sigma) as the standard. Carbohydrate concentration was measured following the methodology described by Loewus [27] and quantified as glucose (Sigma, St. Louis, MO, USA) equivalents.

For the quantification of the PHA content inside cells, fresh biomass samples were collected, and formaldehyde was added to stop the microbial activity. Then, samples were immediately centrifuged, frozen and freeze-dried to obtain a solid phase. The method proposed by Smolders et al. [28] was applied. The PHA sample content was analyzed by GC (6850 Series II, Agilent Tehnologies) equipped with the HP-INNOWAX detection column (Agilent, Santa Clara, CA, USA). PHA quantification was done using a commercial PHA standard (Sigma) containing 88% of hydroxybutyrate (HB) and 12% of hydroxyvalerate (HV). HB and HV are distinguished by the different retention times in the obtained chromatograph.

2.4. Calculations

Detailed calculations can be found in the Supplementary Material. The amount of PHA accumulated inside the cells was determined, on dry weight basis, as the percentage (wt%) of the measured volatile solids (VS). The HB:HV ratio was calculated as Cmmol. The active biomass (X) was obtained by subtracting the mass of the stored compounds from the VSS mass. The elemental composition of the active biomass was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ [29].

The specific VFA consumption rates (q_{VFA}, $Cmmol_{VFA}/(Cmmol_X \cdot h)$) and PHA production rates (q_{PHA}, $Cmmol_{PHA}/(Cmmol_X \cdot h)$) were determined from the maximum slopes of the curves representing the obtained experimental data, divided by the active biomass. Yields (Y) of biopolymers (PHA or separate HB and HV) on substrates ($Cmmol_{PHA}/Cmmol_{VFA}$) were obtained by dividing the corresponding production rate ($Cmmol_{PHA}/h$) by the VFA consumption rate ($Cmmol_{VFA}/h$). A similar procedure is applied for the calculation of the active biomass yield ($Cmmol_X/Cmmol_{VFA}$). The HB:HV ratio was calculated as the amount of each homopolymer divided by the total amount of PHA.

The concentration of proteins and carbohydrates as COD was calculated using the following factors: 1.5 g $COD_{protein}$/g protein and 1.1 g $COD_{carbohydrate}$/g carbohydrate [30]. The nitrogen content in proteins was assumed to be 15% of the weight.

3. Results and Discussion

3.1. Selection of PHA-Accumulating Microorganisms

3.1.1. Enrichment of the Mixed Microbial Culture

The enrichment SBR was operated for 180 days to obtain a MMC with PHA-accumulating capacity treating cooked mussel processing wastewater. This substrate showed a complex composition, which mainly consisted of proteins, carbohydrates and salt. The C/N of the feeding during the operation presented an average value of 6.5 ± 0.9.

The performance of the enrichment SBR was monitored by the feast phase length (Figure 1), which was measured by the change in the DO concentration profile. The feast phase corresponds to the period with low DO concentrations and the famine phase to the opposite situation. The shorter the feast length, the more enriched the system was. In the present study, whereas the DO concentration profile randomly varied during the first cycles, after one week of operation a clear feast-famine profile was observed, with feast length values of approximately 6 h (approximately 50% of the cycle length). Then, from day 60 of operation onwards, the feast phase length remained on average at a value of 2.9 ± 0.4 h, which was of 22% of the cycle length on day 180. Dionisi et al. [31] found that the selection of microorganisms with storage response takes place when the feast phase length is lower than approximately 20% of the overall length of the cycle. The increase of the degree of enrichment of the PHA-accumulating culture coincided with the rise of its storage capacity. The average PHA accumulation was of 4.5 ± 2.2 wt% during the first weeks and increased up to 12.8 ± 0.8 wt% from day 60 of operation onwards (Figure 1).

The obtained results indicated that the enrichment of the system was accomplished after two months of operation. However, the complex composition of the wastewater used as a substrate could prevent the further decrease of the feast length and the achievement of higher PHA accumulation values. Furthermore, the presence of different carbon sources (not only VFA but also proteins were present) probably allowed both accumulating and non-accumulating bacterial groups to coexist in the mixed culture.

The main bacterial populations present in the enrichment SBR were identified by the FISH technique. The activated sludge used as inoculum was characterized by the presence of a wide range of microbial populations but in a low relative abundance. However, once the MMC was enriched, the diversity decreased and the dominance of a few groups increased. The PHA-accumulating mixed culture was composed of two phyla: *Bacteroidetes* and *Proteobacteria*. Members of this latter were identified mainly as class *Betaproteobacteria* and in a low abundance as class *Gammaproteobacteria*. Microorganisms from

genera *Azoarcus* and *Thauera* were observed as the most abundant ones (Figure 2a,b), followed by genera *Comamonas*, all of them from class *Betaproteobacteria* (Figure 2c). The presence of these microbial populations is ubiquitous in PHA-producing systems from wastewater and residual streams. Carvalho et al. [32], for example, observed that the PHA-producing community fed with fermented molasses was dominated by a combination of genera *Azoarcus*, *Thauera* and *Paraccocus*. Microorganisms affiliated to phylum *Bacteroidetes* were also identified (Figure 2d), which have been demonstrated to be able to store PHA using mixed cultures fed with different substrates [33].

The complex composition of the substrate contributed to select microbial populations that adapted to unfavorable operational parameters. Salt concentrations present in the acidified cooked mussel processing wastewater may select certain microorganisms that, despite being non-halophilic with optimal growth in less than 11 g NaCl/L, are able to tolerate high NaCl concentrations and are defined as halotolerant [34]. For example, bacteria belonging to phylum *Bacteroidetes* and *Proteobacteria* were found to be the dominant groups in an acetate-enriched MMC of a PHA production system using estuarine sediments as inoculum [35]. Certain members from class *Gammaproteobacteria* such as genera *Pseudomonas* and *Halomonas*, have been identified as halotolerant but also PHA-storing bacteria [34]. Therefore, the selection of members from phylum *Bacteroidetes* and class *Gammaproteobacteria* in the mixed culture operated with acidified cooked mussel processing wastewater correlated with the operational conditions in terms of high salt concentrations in the enrichment SBR.

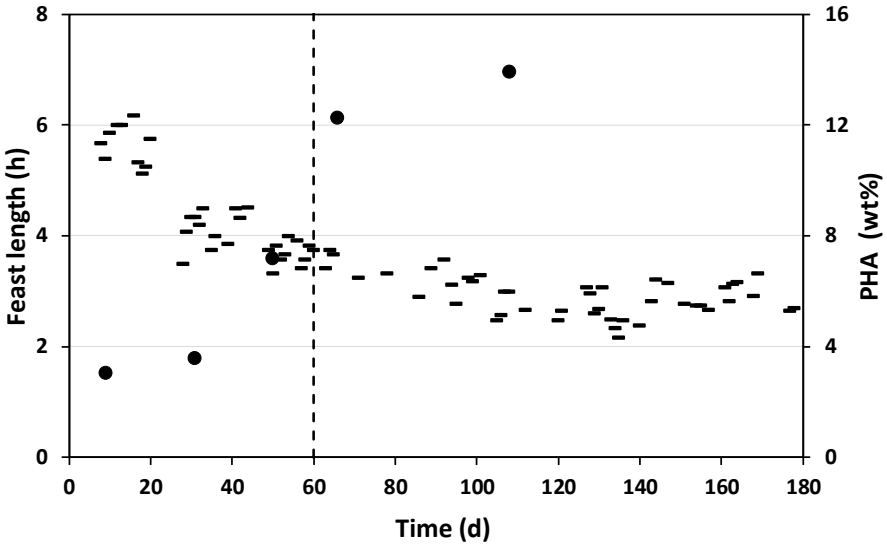
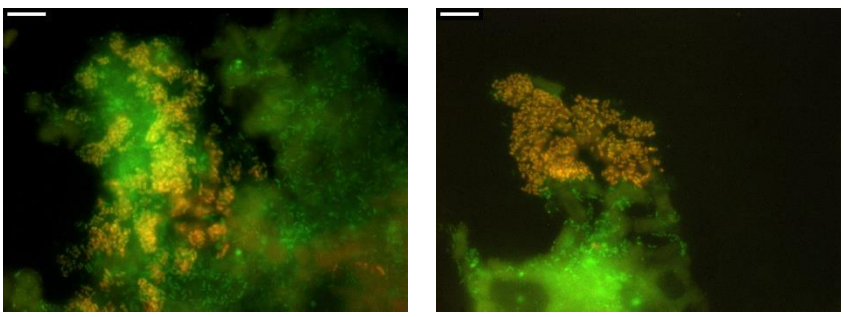
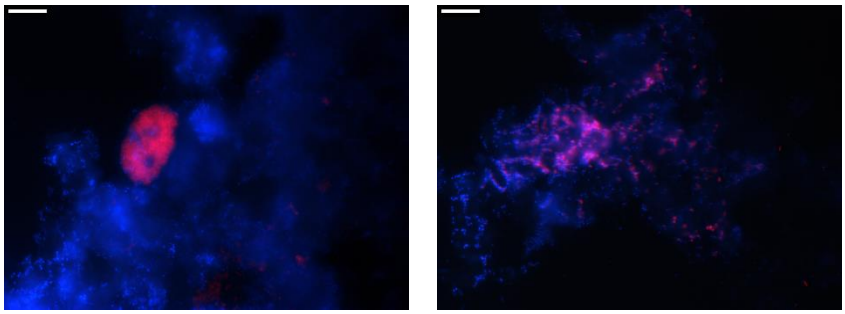


Figure 1. Evolution of the feast length during the operation of the enrichment sequencing batch reactor (SBR) (-) and maximum Polyhydroxyalkanoates (PHA) percentage accumulated at the end of the feast phase (•). The discontinuous vertical line indicates the beginning of the enrichment of the mixed culture.



(**a**) (**b**)

Figure 2. *Cont.*



(**c**) (**d**)

Figure 2. FISH images of the mixed microbial culture (MMC) enriched with acidified cooked mussel processing wastewater. (**a**) *Azoarcus* (Cy3-red) and *Bacteria* (FITC-green). (**b**) *Thauera* (Cy3-red) and *Bacteria* (FITC-green). (**c**) *Comamonas* (Cy3-red) and all DNA (DAPI-blue). (**d**) *Bacteroidetes* (Cy3-red) and all DNA (DAPI-blue). In A and B, the orange color indicates both Cy3 and FITC-labeled probes hybridized; In (**c**,**d**), the pink color indicates both Cy3 and DAPI-labeled probes that are hybridized. The bar represents 10 μm.

3.1.2. Characterization of Enrichment Cycles

The mixed culture performance was characterized by monitoring several enrichment cycles throughout the operation of the SBR. Data from a representative operational cycle on day 66 once the steady-state conditions were achieved showed the correlation between the end of the feast phase, after approximately 2.8 h of the cycle, and the complete depletion of the VFA and the maximum PHA production (as the sum of HB and HV) of 12.3 wt% (Figure 3a). The composition of the produced PHA was 90:10 as the HB and HV ratio. Even after the complete depletion of the VFA, the soluble COD was still present in the liquid media and was mainly associated with the incomplete degradation of the proteins (Figure 3b). This enrichment cycle was carried out at a concentration of approximately 5 g/L of NaCl.

The observed yields of PHA over VFA during the feast phase on day 66 of operation were 0.21 $Cmmol_{HB}/Cmmol_{VFA}$ and 0.03 $Cmmol_{HV}/Cmmol_{VFA}$. The observed values were in the range of those obtained by other authors working with PHA production systems from complex substrates. Oliveira et al. [15] obtained a maximum PHA accumulation of 17% during the enrichment of a MMC fed with fermented cheese whey, with a production yield of 0.18 $Cmol_{PHA}/Cmol_{substrate}$. Tamis et al. [36] observed a PHA yield value of 0.37 g COD_{PHA}/g $COD_{substrate}$ using fermented wastewater from a candy bar factory, whereas Korkakaki et al. [37] employed raw leachate from the process of hydrolysis of the organic fraction of municipal solid waste and estimated a yield of 0.3 g COD_{PHA}/g COD_{VFA}.

Approximately 60% of the TN was consumed during the enrichment cycle, which was initially composed by ammonium (40%) and proteins (60%) (Figure 3b). Since ATU was added to inhibit the nitrifying activity, nitrogen was only consumed during the enrichment cycle for biomass growth. During the feast phase, the increase of the active biomass and the decrease of the TN concentration indicated that VFA were used as a carbon source both for growth and PHA storage (Figure 3). Then, during the famine phase, the ammonium concentration increased due to the degradation of proteins. Part of that ammonium, generated by the protein hydrolysis, was consumed for the microrganisms growth, as shown by the TN consumption during this phase. Approximately 40% of the TN remained in the effluent, which corresponded to 30 mg N/L of protein. Biomass concentration at the end of the cycle doubled the initial value.

Finally, the evolution of the feast/famine regime also correlated with the pH profile (Figure 3a). As no pH control was used in the enrichment SBR, the pH sharply dropped from 7.9 to 4.6 at the beginning of the enrichment cycle, after the addition of the acidified wastewater as a substrate. The reactor operated under acidic conditions during the feast phase, but the pH value increased along with the VFA consumption up to 8. Then, the pH remained in this value during the famine phase until the end of the cycle. These results indicated the successful enrichment, in PHA-accumulating bacteria, of a MMC operated under acidifying

conditions during the feast phase. Few authors have previously reported on studies of PHA enrichment systems that operated at different pH values in the neutral or basic range: 7 and 8 [38], 7.5, 8.5 and 9.5 [39], between 8 and 9 [40]. Moreover, Montiel–Jarillo, et al. [41] studied the MMC enrichment at a controlled pH of 7.5 or with uncontrolled pH that was established at 9 using pure acetate as carbon source. These authors found slightly higher PHA accumulation when pH was not controlled. There is no previous information on the enrichment of a PHA-storing mixed culture developed under acidic conditions, as in the case of the present study, performed without pH control.

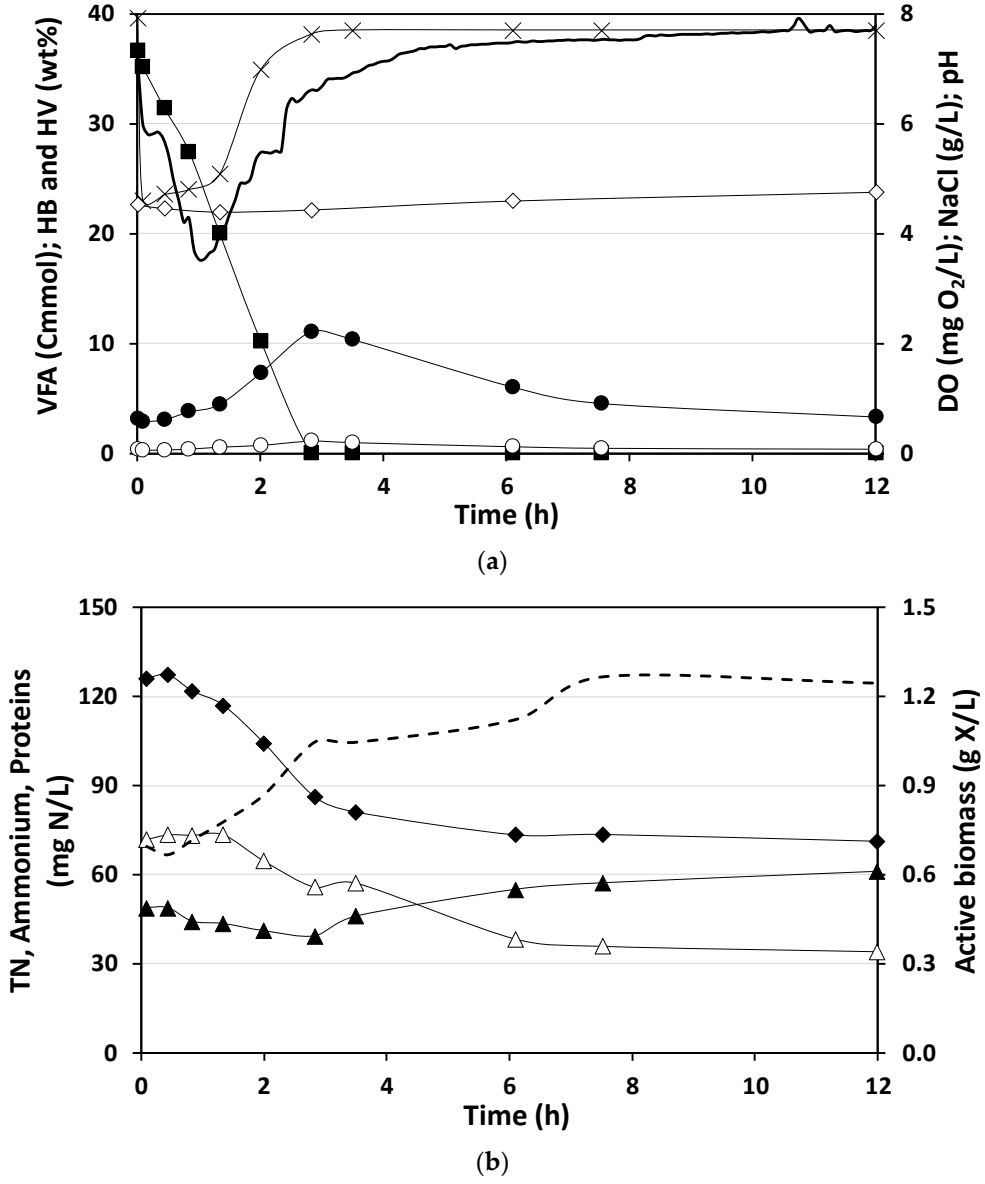


Figure 3. Characterization of the operational cycle of the enrichment SBR measured on day 66. (**a**) VFA (■), DO (-) and NaCl (◇) concentrations; HB (●) and HV (○) percentages; and pH value (×). (**b**) TN (♦), ammonium (▲), proteins (△) and active biomass (- - -) concentrations.

3.2. PHA Accumulation Capacity of the Enriched MMC

3.2.1. PHA Accumulation with Acidified Cooked Mussel Processing Wastewater

PHA fed-batch accumulation assays were conducted with biomass harvested from the enrichment SBR at the end of operational cycles. At this point, the biomass concentration was approximately 1 g VSS/L. The acidified mussel cookers processing wastewater was fed, as carbon source, and added

in pulses to prevent substrate inhibition. Considering the link between the DO concentration and the external carbon source consumption, substrate pulses were added each time the DO concentration increased. The DO concentration increased indicated that the previously added VFA were completely depleted.

The maximum PHA content obtained in accumulation assays (day 120) had a 24.95 wt%, which corresponded to a volumetric productivity of 278.3 mg PHA/(L·h) (Figure 4). The presence of nitrogen as ammonium and proteins in the acidified wastewater had a negative effect on the maximum PHA content and promoted the biomass growth instead of the PHA storage during the batch accumulation assays. Carbon balance calculations showed that only approximately 20% of carbon contributed to PHA accumulation ($Y_{PHA/TOC}$ = 0.24), and that the 50% was used for biomass growth ($Y_{X/TOC}$ = 0.52). Indeed, Oliveira, et al. [42] already reported that it is impossible to obtain a culture enriched in PHA-accumulating bacteria capable of using proteins as the sole nitrogen source requiring the presence of ammonium to growth.

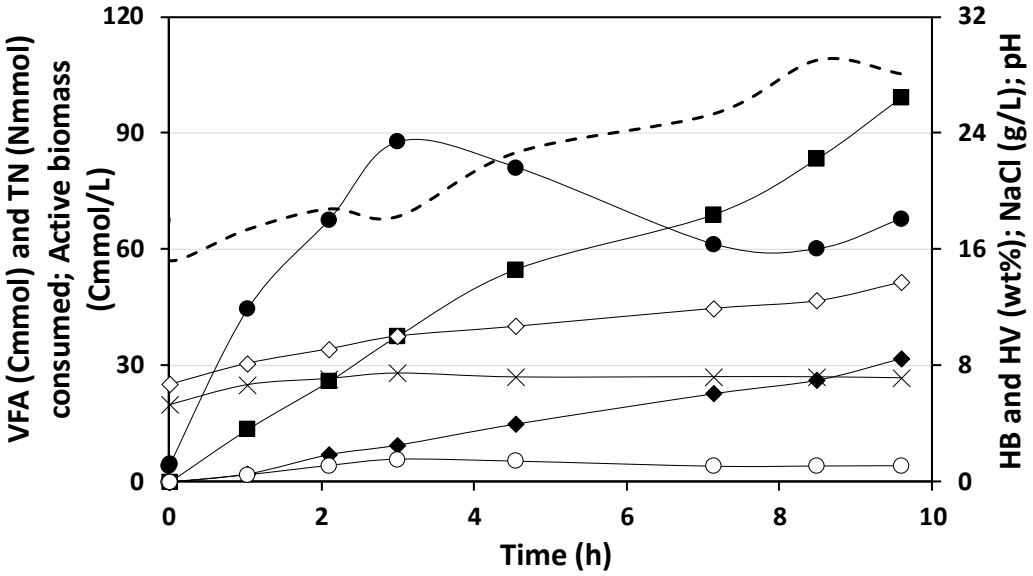


Figure 4. Characterization of an accumulation assay on day 120 of operation using the acidified mussel cookers wastewater as a substrate. Cumulative amount of consumed VFA (■) and TN (♦); active biomass (- - -) and NaCl (◇) concentrations; HB (●) and HV (○) accumulated percentages; and pH value (×). The time of pulse addition is the same as the data points since pulses were supplied just after the sample collection.

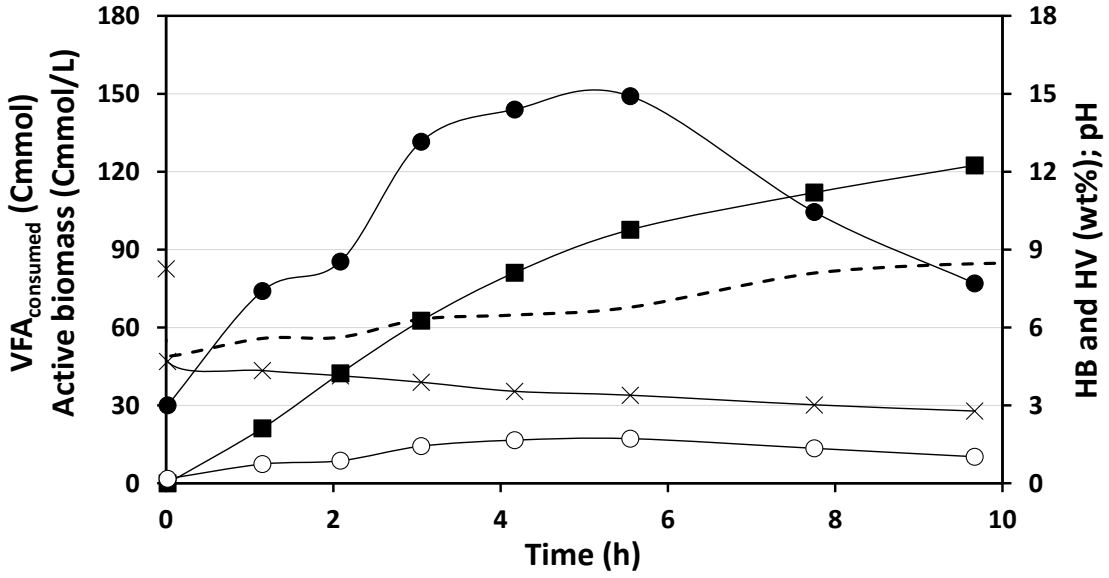
The biomass growth, associated with the addition of nitrogen in each pulse, was clearly observed in the active biomass concentration profile during the fed-batch accumulation experiment. The solids concentration at the end of the experiment almost doubled the initial value (Figure 4). Previous studies have demonstrated that the presence of nutrient and carbon sources in the accumulation assays promote cell growth instead of the intracellular accumulation of PHA [43]. However, the biomass production rate was lower during the first 3 h of the experiment, when 25% of the total biomass was produced, which correlated with the 25% of the nitrogen consumed during the same period. Once the maximum accumulation was achieved, after 3 h of the experiment, the percentage of PHA decreased due to cell growth, which leads to a decrease of PHA-accumulation percentage by the formation of new biomass. The HB:HV average ratio in all the accumulation fed-batch experiments was of 83:17. Small variations in the composition of the biopolymer were due to changes in the mixture of the VFA present in the acidified effluent. The highest HB production (HB:HV ratio of 95:5) corresponded to the lowest concentrations of valeric and propionic acids (precursors of HV synthesis) in the substrate.

Moreover, the addition of the substrate in different pulses generated the increase of salt concentration in the reactor throughout the time of the experiment (Figure 4). The initial salt concentration corresponded to that present in the effluent of the enrichment SBR, which was approximately 5 g NaCl/L (the wastewater fed was 5 times diluted). However, this value doubled up to 13.74 g NaCl/L after subsequent addition of substrate pulses (added as acidified wastewater without dilution). Although the biomass continued growing according to the nitrogen consumption and solids measurements, the PHA accumulation decreased after 4 h when the salt concentration was above 10 g NaCl/L. Palmeiro–Sánchez et al. [20] evaluated the effects of NaCl over PHA accumulation in a non-adapted mixed culture. They observed a decrease in the PHA accumulation from 34.6 to 17.4 wt% when the salt concentration increased from 7 to 13 g NaCl/L, respectively. They also observed the degradation of the accumulated polymer, probably as a reaction of the microorganisms to overcome the stress produced by the high salt concentration.

3.2.2. Maximum Accumulation Capacity Evaluated with Mimicked VFA Mixture

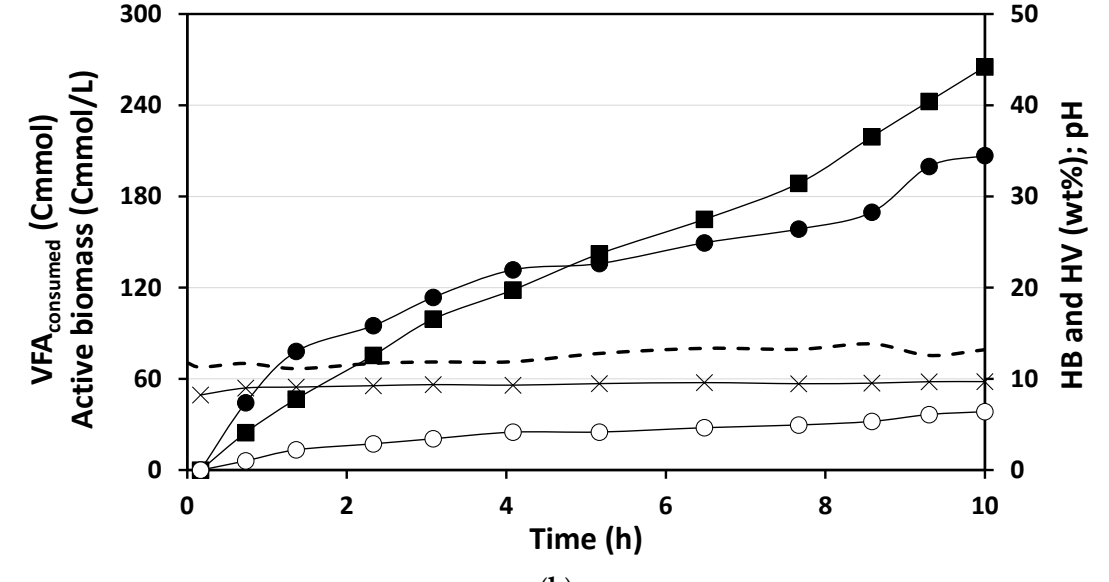
As previously discussed, the presence of nitrogen in the acidified effluent used as substrate limited the maximum PHA accumulated by the enriched MMC and promoted the biomass growth. For this reason, to determine the maximum accumulating capacity of the culture, an experiment with a mimicked media was carried out. A mixture of VFA in the same proportion as in the composition of the acidified cooked mussel processing wastewater was used as a substrate in fed-batch assays and no nitrogen source was added.

When the industrial wastewater was replaced by the mimicked VFA mixture, maximum PHA storage of 16.6 wt% was achieved in 6 h, with a yield of 0.30 $Cmmol_{PHA}/Cmmol_{VFA}$. However, the pH of the liquid media after the addition of the VFA-pulse gradually decreased and finally reached a value of 3 after 8 h of the experiment (Figure 5a). Most bacteria can grow at pH values of 5.5–9.0, and maintain their cytoplasmatic pH in a narrow range of 7.5–7.7 [21]. Although the MMC was enriched at acidic conditions, these results indicated that the PHA production was inhibited when the pH dropped below 4, which was the average pH value during the feast phase. The active biomass remained stable at the beginning of the experiment but later, it increased from 60 to 90 Cmmol/L. This growth correlates with the decrease of PHA accumulated (after 6 h of experiment) inside the cells. Residual ammonium coming with the seeding sludge collected at the end of the enrichment cycle (70 mg N/L) was used for growing. At the end of the cycle, ammonium concentration was 6 mg N/L, while protein concentration variation was not observed.



(**a**)

Figure 5. *Cont.*



(**b**)

Figure 5. Performance of accumulation assays using a mixture of VFA as a substrate. (**a**) Without pH adjusted. (**b**) With the pH adjusted to approximately 7.0. VFA consumed (■), pH value (×), active biomass (- - -), HB (●) and HV (○) accumulated percentages. The time of pulse addition is the same as for the data points, since pulses were supplied just after the sample collection.

Since the PHA storage was improved by limiting the nitrogen source but inhibited by the acidic pH, another experiment was carried out by adding NaOH to the VFA mixture to buffer the influent mixture at pH 7 (Figure 5b). In this way, the acidification of the medium after the addition of the carbon pulses was avoided. The pH value only slightly increased due to the CO_2 stripping, and the pH value was maintained at an average value of 9.3 ± 0.2 during the whole experiment. The maximum accumulation of the total PHA increased up to 40.9 wt% after 10 h and it might potentially be higher, since a plateau on the VFA consumption rate was not observed. The PHA production yield was of 0.48 $Cmmol_{PHA}/Cmmol_{VFA}$, which was higher than in the experiment without the pH control and no

variations on active biomass concentration were observed. Moreover, the PHA productivity was of 0.2 g PHA/(L·h), three times higher than in the previous experiment. Therefore, the pH control in the substrate promoted the maintenance of the pH value inside the reactor, and eventually the increase of the PHA storage. The HB:HV ratio in both experiments was similar to that obtained in the fed-batch experiments with acidified cooked mussel processing cooker wastewater. Since the mixture of VFA was prepared in the same proportion as the acidified mussel cookers wastewater, the enriched MMC showed the same response in terms of the type of PHA produced.

3.3. Global PHA Production System

The performance of the complete PHA production system from cooked mussel processing wastewater, including acidification (data published in previous work [9]), enrichment and accumulation units, corresponded to a global yield of 0.09 kg COD_{PHA}/kg COD_{fed} (Figure 6). Approximately 10% of the soluble COD present in the raw wastewater from the mussel cookers factory is recovered as PHA. This value was calculated considering the values obtained during the operation of the enrichment SBR under steady-state conditions and during the accumulations assay with the acidified mussel cookers wastewater.

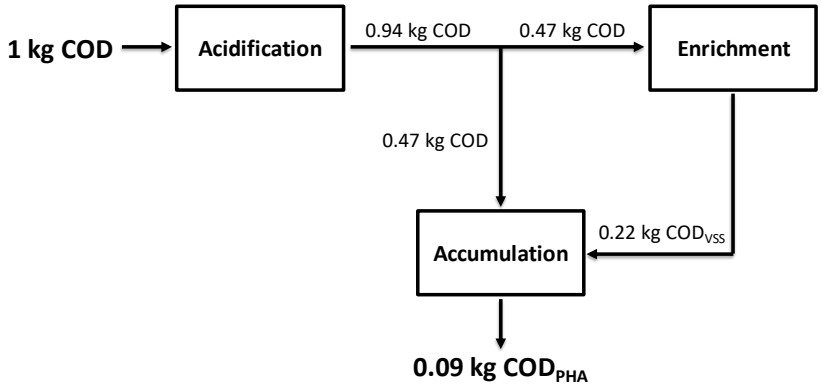


Figure 6. Overall efficiency of the PHA production process with the acidified mussel cookers wastewater, using the conversion of 1 kg of soluble COD from the influent wastewater into PHA as a basis.

This approximately 10% recovery of COD as PHA is much lower than the 34% reported by Tamis, et al. [44] fed with paper mill wastewater. Nevertheless, the wastewater used in the present study had higher complexity due to the presence of salt and proteins.

One important factor that should be noted is the absence of pH control in the whole PHA production system. Even though the enrichment SBR was operated under a low pH during the feast phase, due to the acidic nature of the acidified mussel cookers processing wastewater, the MMC has demonstrated to be enriched in PHA-accumulating microorganisms. This is important for further implementation at large scale since the reduction in the use of chemicals is economically advantageous. However, operational conditions need to be optimized to reduce the large pH decrease. A possible alternative is to use alternative alkalinity sources such as mussel shells that are also generated in the factory.

The effluent generated in the accumulation unit still contained a high nitrogen concentration due to the presence of ammonium and proteins that were not degraded. For the integration of the PHA production system into the industry, the produced effluent must cope with the discharge limits in accordance with the established legislation. Further studies must be developed to include a polishing treatment of the generated effluent for nitrogen removal. In this way, autotrophic nitrogen removal processes such as anammox based ones [45] are a preferred option, since no organic matter is needed and it can be fully converted in the PHA accumulation system.

Another factor that may be further optimized is the substrate conditioning to maximize the PHA accumulation. Since the wastewater from mussel cookers processing shows a high nitrogen

concentration, removing nitrogen to a fraction of the acidified wastewater previous to be fed to the accumulation reactor will be advantageous. Another option will be to mix the acidified stream with other effluent generated in the same facility, or even with the effluent of the acidification unit if the cited nitrogen removal process is implemented, in order to obtain an influent stream to the PHA-system with a higher carbon to nitrogen ratio for fostering the PHA production and increased economic attractiveness [16].

4. Conclusions

Acidified industrial wastewater from mussel cookers was demonstrated to be a suitable substrate for PHA production while its pollutant load is reduced. PHA-accumulating microorganisms were successfully selected in the enrichment SBR under high salinity and acidic conditions (pH 4 during the feast phase) in only 60 days. Microorganisms from phylum *Bacteroidetes*, and genera *Azoarcus*, *Comamonas* and *Thauera* from phylum *Proteobacteria* were identified in the enriched MMC. The mixed culture accumulated up to 25 wt% of PHA, with a HB:HV ratio of 83:17, at a salt concentration of 13.7 g NaCl/L. However, the wastewater nitrogen content promoted biomass growth and limited the PHA accumulation. Thus, the evaluation of nitrogen removal processes must be considered not only as polishing step for discharging the wastewater into the environment but before the acidification unit to maximize the PHA accumulation. The enriched culture showed a higher PHA accumulating capacity (up to 40.9 wt%) when nutrients were limited in the substrate of the accumulation assay, showing the relatively high PHA-accumulating microorganisms selection using this complex substrate. Moreover, when pH value in the accumulation reactor falls below 4, the PHA accumulation is hindered and accumulation products are consumed.

The final recovery of PHA in the global system (acidification, enrichment and accumulation units) was approximately 10% of the soluble COD contained in the raw cooked mussel processing wastewater.

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