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Pilot-scale valorisation of salmon peptone into polyhydroxyalkanoates by mixed microbial cultures under conditions of high ammonia concentration

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ABSTRACT

The aquaculture industry produces vast amounts of organic side streams that are often ensilaged for later use in low value applications. However, the potential of converting fish silage, particularly the proteinaceous fraction, into more economically valuable products such as biodegradable polyhydroxyalkanoates (PHA), has not been explored. This study investigates the valorisation of salmon peptone, the proteinaceous fraction obtained from salmon silage, into PHA by mixed microbial cultures (MMC) under conditions without nutrient limitation, where the high concentration of ammonia (up to 2.3 ± 0.2 gN/L) which the culture was subjected to has not been previously reported. Acidogenic fermentation of salmon peptone (SP) was attained in a continuous reactor, resulting in the production of a fermentate rich in acetate and butyrate (16.4 ± 1.6 $gCOD_{FP}$/L). The enriched MMC with PHA-accumulating organisms was selected in a sequential batch reactor (SBR) under nutrient-rich conditions (100 gCOD: 27.6 gN: 0.48 gP at OLR 7.1 $gCOD_{FP}$/(L.d)). The dominant microorganisms in the enriched culture were from the genus *Brachymonas*, which are described as capable of simultaneous cell growth and PHA accumulation, storing up to 53.0 ± 3.4% wt. of P(3HB-co-3HV) with a 3HV content of 34% wt.. Despite the high ammonia concentration and constant nutrient availability, a good overall PHA yield (189 gPHA/kgSP) and global volumetric PHA productivity (3.10 ± 0.11 gPHA/(L.h)) were achieved. The results demonstrate the technological feasibility of producing PHA from salmon peptone under conditions that are typically considered unfavorable for PHA accumulation, which represents a significant novelty of this work.

Abbreviations: °Bx, Degrees Brix; 3HB, 3-Hydrobutyrate; 3HV, 3-Hydroxyvalerate; Acet, Acetate; But, Butyrate; C/N, Carbon/nitrogen; C/P, Carbon/phosphorus; C2SS, Category 2 salmon silage; CAGR, Compound annual growth rate; Cap, Caproate; COD, Chemical oxygen demand; DA, Degree of acidification; DO, Dissolved oxygen; EtOH, Ethanol; F/M, Food to microorganism; F/f, Feast/famine; FBR, Fed batch reactor; FP, Fermentation products; GC, gas chromatography; HPLC, High performance liquid chromatography; HRT, Hydraulic retention time; iBut, Isobutyrate; iVal, Isovalerate; MMC, Mixed microbial cultures; MW, Molecular weight; N, Nitrogen in the form of ammonia; OLR, Organic loading rate; OFMSW, Organic fraction of municipal solid waste; P(3HB-co-3HV), Poly(3-hydroxybutyrate-co-3-hydroxyvalerate); P, Phosphorous in the form of phosphorus oxide; PHA, Polyhydroxyalkanoates; PHA_0, PHA content at the beginning of the experiment; PHA_{max}, maximum PHA content; PLC, Programmable logic controller; Pro, Propionate; q_{PHA}, Specific PHA production rate; $-q_S$, Specific substrate consumption rate; $-q_{PHA}$, Specific PHA consumption rate; q_X^{Feast}, Specific growth rate on Feast period; q_X^{Famine}, Specific growth rate on famine period; rpm, Revolutions per minute; rRNA, Ribosomal ribonucleic acid; RRM, Raw rest materials; S, Substrate; SCOD, Soluble chemical oxygen demand; SBR, Sequential batch reactor; SP, Salmon peptone; SSW, Sewage sludge waste; SRT, Solids retention time; TC, Total carbon; TCOD, Total chemical oxygen demand; TN, Total nitrogen; TOC, Total organic carbon; TSS, Total suspended solids; UASB, Upflow anaerobic sludge blanket; Val, Valerate; VFA, Volatile fatty acids; VSS, Volatile suspended solids; V/v, Volume/volume; WWTP, Wastewater treatment plant; X, Active biomass; X_0^{Acc}, X at the beginning of the accumulation; $X_0^{F/f}$, X at the beginning of the F/f cycle; X_{max}^{Acc}, Maximum active biomass in an accumulation batch; $X_{max}^{F/f}$, Maximum active biomass in a F/f cycle; XRP, Active biomass productivity; $Y_{PHA/S}$, PHA storage yield on substrate; $Y_{X/PHA}$, Growth yields on PHA; $Y_{X/S}$, Growth yield on carbon substrate; Wt., weight.

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1. Introduction

The aquaculture industry is growing globally with a compound annual growth rate (CAGR) of 3.9% towards a market size estimated to reach € 310 billion by 2027 [1]. Although aquaculture is a sector with high nutritional and commercial value, it is also associated with a significant production of organic side streams that require adequate treatment and disposal. Norway's aquaculture industry produces 1.3 million tons of salmon annually, generating more than 400 thousand tons of raw rest materials (RRM) [2]. RRM from fish are often ensilaged and used for low value applications, such as animal feed or fertilizers. Fish silage (including category 2 material, i.e., fish that died while in the net pens) is typically produced by grinding up the RRM and mixing it with an acid, most often formic acid, to prevent bacterial spoilage. The endogenous enzymes present in the RRM subsequently digest solids, resulting in a liquid that is rich in proteinaceous and lipidic material. The proteinaceous fraction from fish silage consists mostly of peptones (i.e. a complex mixture of amino acids and short peptides resulting from the partial hydrolysis of proteins) and has recently been investigated for higher value applications, such as culture media for microorganism growth [3]. However, better utilization of fish peptone into products of even higher economic potential, such as polyhydroxyalkanoates (PHA), has not yet been explored.

PHAs are biobased polymers, fully biodegradable, and with excellent biocompatibility and physicochemical characteristics, allowing its use in a wide range of applications (e.g. packaging, food industry, cosmetics, medical implants) [4]. Based on these properties [5,6], these biopolymers are an interesting alternative to traditional fossil fuel-based plastics (recalcitrant material) which additionally offer significant societal and environmental benefits. The PHA industry is growing globally, with a CAGR of 15.3% and the PHA market size is estimated to reach € 167 million by 2027 [7]. However, production cost of PHA is still estimated to be four times higher (€ 4–5/kg) than conventional plastics manufacturing, thereby limiting PHA's wider commercialization and industrialization [4].

PHA production by open mixed microbial cultures (MMC) reduce production costs as it enables the use of inexpensive by-products, or waste streams, as feedstock and does not require aseptic conditions, thus reducing the process energy demand [4]. PHA production by MMC generally comprises three distinct stages: (i) *acidogenic fermentation*, where the organic matter is converted into volatile fatty acids (VFA) which serve as the PHA precursors; (ii) *culture selection*, where the culture is enriched in PHA-accumulating organisms by applying at least one selective pressure (e.g. Feast/famine (F/f) regime); and finally (iii) *PHA production*, where PHA accumulation batches are carried out using the culture selected from stage (ii) and fed with the fermented stream produced from stage (i). The PHA production stage is normally carried out in the absence of nutrients, as their presence might compromise PHA storage capacity of the culture when a fraction of the available carbon is unwantedly channeled towards cell growth. However, culture selection is the key step of the whole PHA production process. This stage is carried out in the presence of nutrients in order to sustain growth of the enriched culture with PHA-accumulating organisms and, subsequently, achieve higher global volumetric PHA productivity [5].

PHA storage occurs when cells are unable to grow at the same rate at which they take up carbon substrate. Therefore, restricting cell growth is the basis of culture selection strategies. Growth restriction can be imposed by means of internal limitation (i.e. decrease in the concentration of growth factors, such as a lack of anabolic enzymes, after subjecting the culture to prolonged carbon limitation), or external limitation (e.g. lack of nitrogen or phosphorous) [5].

MMC selections based on an internal pressure strategy are achieved generally by applying alternated periods of carbon excess and limitation. This is commonly referred to as a F/f regime. Typically, longer famine periods (i.e. where F/f ratio < 0.33 h/h) provides a sufficiently strong selective pressure for PHA-accumulating organisms [8]. However, the amount by which the F/f ratio can be lowered is constrained by operational factors such as the amount and rate of carbon feeding imposed on the bioreactor [8–11]. Most studies that have investigated PHA production by means of MMC used feedstocks with limited availability of nutrients (e.g. sugarcane molasses [12], cheese whey [13], candy bar factory wastewater [14], paper mill wastewater [15], potato-starch wastewater [16] and fruit waste [17]). When using these nutrient-limited feedstocks, the culture selection process can further be improved by controlling the nutrients availability in the F/f cycle (external pressure). For example, in some cases, it is possible to fully uncouple carbon-nutrient (e.g. C/N or C/P) feeding, i.e. exogenous nitrogen is introduced exclusively during the famine period of the F/f cycle. By doing so, only bacteria that accumulated carbon, in the form of PHA, during the preceding feast period has reserve carbon available for cell growth. The result is a selection pressure that strongly favors the propagation of PHA-accumulating organisms over non-accumulating species. Previous studies [17–20] demonstrated that an uncoupled carbon-nutrient strategy tends to benefit culture enrichment and thereby enhancing the potential of reaching higher PHA productivities.

However, when using feedstocks that are not nutrient-poor, a complete carbon-nutrient uncoupling is not always possible. In these cases external selection pressures, based on nutrients availability/limitation, can still be implemented but only by controlling the degree of carbon and nutrient limitation and this is often done by supplementing the bioreactor with exogeneous carbon or nutrients. The adjusted carbon-nutrient ratio will not only affect the amount of carbon channeled toward PHA storage versus growth during the feast period, but also affect the growth yield during the famine phase. It was found that for the culture selection stage, imposing a carbon limitation strategy (i.e., nutrients are in excess during the feast period) does indeed lead to a culture with improved carbon uptake rates and maximum PHA storage capacity compared to a nitrogen limitation strategy (i.e., nitrogen is depleted before carbon depletion) [21]. However, the carbon-nutrient ratio to choose in the subsequent accumulation stage has been less clear for cultures selected by carbon limitation as selective pressure. Indeed, in some studies, higher productivities were achieved in the accumulation stage after changes made to nutrient availability (e.g. nitrogen starvation [22], nitrogen limitation [23] or even a combination of nitrogen and phosporous-limitation [24]). Specifically, higher PHA accumulation was observed under ammonia starvation (89% wt. of PHA with C/N $\sim \infty$) than under ammonia limitation (79% wt. of PHA for 91.7 gCOD/gN) or excess of ammonia (69% wt. of PHA for 18.3 gCOD/gN) using a culture selected by carbon limitation conditions [22]. However, twice the PHA concentration and higher PHA storage capacity (20% higher PHA content) were observed with partial ammonia content compared to the same conditions without ammonia, with respect to a culture selected by carbon limitation conditions [23]. Furthermore, an optimal range of combined nitrogen ($67 <$ gCOD/gN < 500) and phosphorus ($333 <$ gCOD/gP < 2000) limitation was suggested to offer an improvement in productivity compared to those in nutrient starvation [24]. These discrepancies emphasize that other factors (e.g., non-VFA carbon sources, operational conditions) can also affect the culture's community profile, the degree of culture enrichment in PHA-accumulating organisms, and ultimately the effectiveness of PHA accumulation.

Finally, selecting an efficient MMC using nutrient-rich feedstocks is also possible, but it comes with a special set of challenges. Generally, when high levels of nutrients are present throughout the F/f cycle, it is not possible to control the carbon-nutrient ratio, or nutrients availability as external selections pressures thereby making the whole process much more difficult to control. Therefore, when using nutrient-rich feedstocks, culture selection is mainly driven by internal selection pressure (carbon limitation). Subsequently, other factors such as the the feedstock's composition (e.g. the amount of non-VFA carbon sources) can have a stronger influence in the culture selection than would normally be the case.

Despite these challenges, it was previously shown that protein-rich

resources (e.g. the organic fractions of municipal solid waste and sewage sludge [23,25–27]) can indeed be valorized using this approach. Characteristically, during the acidogenic fermentation of these protein-rich feedstocks, fermented streams high in nutrient content, especially nitrogen (in the form of NH_3/NH_4), are produced. Although this constant presence of nutrients makes culture selection, and subsequent PHA accumulation, more challenging, the economical upside is that nitrogen and phosphorous supplementation is avoided.

In the present study, the valorisation of peptone obtained from salmon silage into PHA by MMC was investigated. Since the salmon peptone used in this study falls within the nutrient-rich feedstock category, the main challenges were (i) to obtain a good acidogenic fermentation of SP and to produce a VFA-rich stream with low non-fermented solids content to minimize unwanted cell growth (non-PHA accumulating organisms) during the culture selection stage, (ii) to enrich an efficient PHA-accumulating MMC in conditions without limiting nutrients, where ammonia has a high concentration and (iii) to attain good PHA productivity under conditions without restriction of cell growth. This work was carried out at pilot-scale by MMC using a three-stage process, where three separate reactors were operated for acidogenic fermentation, culture selection and PHA accumulation, respectively.

2. Materials and methods

2.1. Feedstock

The SP was produced from category 2 salmon silage (C2SS) at Biotep, a test processing facility operated by Nofima (Tromsø, Norway). Approximately 3800 kg of C2SS was provided by Brødrene Karlsen AS for this purpose. First, the C2SS was added to two hydrolysis reactors and heated to 90 °C for 15 min. The heating was performed by steam-caps surrounding the reactors. The C2SS was then transferred to a tricanter (Flottweg SE, Germany) where an oil, an aqueous and sediment phases were formed. The oil and sediment phases were not analysed in this study and were therefore discarded. However, potential valorisation routes for these fractions may be explored in future research. The aqueous phase evaporated to 1200 kg resulting in a solution with an approximated dissolved solid content of 47 °Bx. The concentrated aqueous phase was dried in a spray drier (Gea, Germany).

A summary of the dried SP's physicochemical properties is presented in Supplementary Material (Table S1). The SP feedstock was prepared by dissolving the dried SP in tap water to a final concentration of 49 $gTCOD_{SP}$/L and then stored in a 100 L buffer tank at 4 °C.

2.2. General experimental setup

A schematic representation of the general experimental setup is provided in the Fig. 1. The SP feedstock was fed to a 100 L upflow anaerobic sludge blanket (UASB) reactor (stage I). The fermented stream obtained from the UASB was decanted using a 45 L decanter installed downstream of the UASB. The clarified fermented stream was stored at 4 °C in a 300 L buffer tank and fed to a 200 L sequential batch reactor (SBR) operated for culture selection (stage II) and to a 200 L fed batch reactor (FBR) operated for PHA production (stage III).

2.2.1. Stage I: Acidogenic fermentation

The UASB reactor was inoculated with 30 L of anaerobic granular sludge harvested from a brewery waste treatment facility (Porto, Portugal). The bioreactor was operated with a working volume of 90 L and under anaerobic conditions. The hydraulic retention time (HRT), temperature and pH, which was controlled through automatic dosing of 5 M NaOH solution, were set at 1 day, 30 °C and 4.5 or 7, respectively. The superficial velocity inside the reactor was 3 m/h by means of an external recirculation flow controlled at 136.5 L/h. The OLR was increased stepwise from 9.8 to 24.5 $gTCOD_{SP}$/(L.d) by reducing the dilution factor (i.e. reducing the amount of tap water fed). The decanter unit was operated at room temperature and a HRT of 12 h.

2.2.2. Stage II: Culture selection

The SBR was inoculated with aerobic activated sludge from the Mutela wastewater treatment plant (WWTP) (Almada, Portugal). The SBR was operated with a working volume of 100 L under aerobic conditions (airflow of 5.9 m^3/h). The HRT and solids retention time (SRT) were set to 1 day and 3 days, respectively.

Each F/f cycle period lasted 12 h (720 min) and consisted of 6 steps: (i) the addition of allylthiourea, as nitrification inhibitor, and micro-nutrients (as described in Huang et al. [28]) was done over a period of 1 min; (ii) influent filling over 11 min; (iii) aeration and agitation over 630 min; (iv) biomass purge over 6 min (16.7 L); (v) settling over 60 min; and (vi) effluent withdrawal over 12 min (50 L). The SBR was controlled at 21 °C, stirred at 1500 rpm using a two-blade impeller with

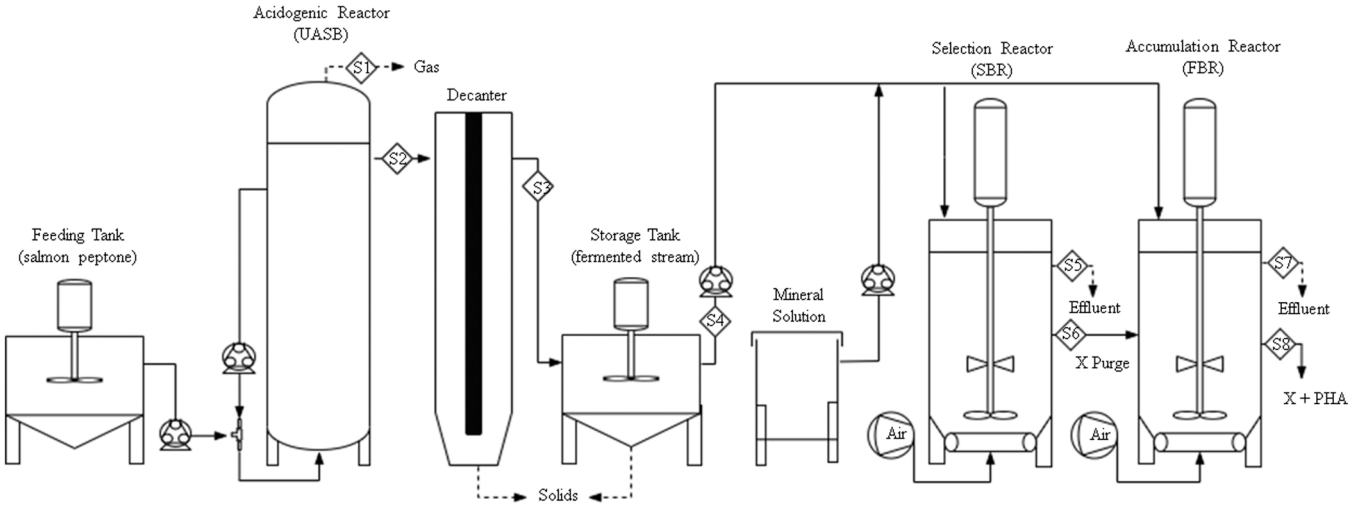

Fig. 1. The general experimental setup of the pilot PHA production process using salmon peptone side-stream. Diamonds labelled with S1 to S8 identify the sampling points of the gas and effluent stream of the acidogenic reactor (UASB), clarified fermented streams obtained downstream the decanter and storage tank, selection reactor (SBR) and accumulation reactor (FBR), respectively. Solids were removed from the effluent of UASB resulting in a clarified fermented stream. Effluent was discarded from the SBR and FBR. Purges (enriched with PHA-accumulating organisms) from the SBR were collected and utilized for FBR inoculation. Active biomass with high PHA content was collected at the end of the accumulation batches (X + PHA).

a pitched blade turbine and six-blade Rushton turbine and at pH between 7.5 and 8.3 controlled through automatic dosing of 2.5 M HCl or 2.5 M NaOH. Dissolved oxygen (DO) concentration and pH were monitored in real-time using a Programmable Logic Controller (PLC). DO levels ranged from approximately 2.5 to 8.3 mg_{O2}/L, indicating that there was no significant oxygen limitation during operation. The pH was consistently within the range of 8.0–8.3.

The OLR was increased stepwise from 1.78 to 7.1 $gCOD_{FP}$/(L.d) by reducing the dilution factor, whenever the F/f ratio and VSS were steady for at least three SRT cycles (9 days).

2.2.3. Stage III: PHA production

During the final stage of the famine phase of a F/f cycle, the SBR underwent automatic purging with a pump. The purged material, enriched with PHA-accumulating organisms, was then stored in an agitated vessel before being utilized for PHA accumulation. For each PHA accumulation batch in the FBR, roughly three purges were employed as inoculum. The FBR operated with a working volume ranging from approximately 50 L at the beginning of the accumulation batch to 150 L at the end. The clarified fermented stream produced in stage I was fed to the FBR through two feeding modes. Specifically, 84% v/v of the total organic carbon fed in an accumulation batch was given in pulses, while the remaining part of the total organic carbon (16% v/v) was given continuously to maintain exogenous carbon always available throughout accumulation. The bioreactor was operated under aerobic conditions (airflow of 5.9 m^3/h) and controlled as in the SBR (pH 7.5–8.0), stirring (1500 rpm) and temperature (~21 ºC). The DO concentration response was monitored online. The biological activity was halted by quenching the system to a pH of 2–3 with sulfuric acid whenever the selected culture reached its maximum PHA capacity. The maximum PHA capacity was determined by interpreting the online DO profile. Typically, after a carbon feeding pulse is given to the culture, the DO value reaches its minimum and starts to rise with the consumption of VFAs, achieving the maximum DO value when the VFAs are depleted. However, when the culture reaches its maximum PHA capacity, the DO value no longer drops as significantly as before after a pulse is given, indicating that the culture has exhausted its capacity for PHA production. At this point, the accumulation batch is stopped by quenching the pH to halt the biological activity and prevent PHA consumption.

2.3. Analytical methods

Total suspended solids (TSS) and volatile suspended solids (VSS) were quantified following standard methods [29]. Total chemical oxygen demand (TCOD), soluble COD (SCOD) and total nitrogen (TN) were determined by a colorimetric method using Hach Lange kits. The total carbon (TC) analyses were performed by combustion catalytic oxidation using TOC-VCSH equipment. Total organic carbon (TOC, expressed as substrate (S)) was determined by the subtraction of the inorganic carbon (IC) from TC (TOC = TC – IC). The fermentation products (FP) concentration was determined by high performance liquid chromatography (HPLC). Nitrogen in the form of ammonia (expressed as N or N-NH_4) and phosphorus in the form of phosphorous oxide (expressed as P or P-PO_4) were determined by a colorimetric method implemented in a continuous flow analyser. Chloride (Cl^-) concentration was determined using liquid chromatography. Sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), and calcium (Ca^{2+}) concentrations were determined using an inductively coupled plasma-atomic emission spectrometry. PHAs were quantified by gas chromatography (GC) with a flame ionization detector. The gas composition (CH_4, CO_2, O_2, N_2, and H_2) was determined by GC equipped with a thermal conductivity detector. Intracellular PHA granules were identified using Nile Blue staining and observed with epifluorescence microscope (Axio Imager D2, Carl Zeiss) at 1000X. Samples from the UASB reactor and SBR were taken for microbial community analysis by means of 16 S rRNA gene amplicon sequencing.

The exact experimental conditions of the analytical methods described above are provided in the Supplementary Material.

2.4. Calculations

The degree of acidification (DA) was calculated dividing FP concentration in the reactor by the TCOD of the influent [30]. The degree of ammonification (i.e., the conversion of all nitrogen species to the form of ammonia) during the acidogenic fermentation of salmon peptone was calculated as the ratio between the content of free ammonia produced during acidogenic fermentation of SP (i.e., the content of free ammonia in the fermented stream minus the free ammonia originally present in the influent) and the content of ammonia present in organic nitrogen compounds in the feedstock (i.e., the total nitrogen content in the influent minus the free ammonia content in the influent). The F/f ratio was calculated as the ratio between the period lengths of the feast phase (when exogenous carbon is available, such as VFAs) and the famine phase (after exogenous carbon is depleted) of the SBR cycle. The length of the feast period was determined by monitoring VFA consumption in the SBR. The PHA content (% wt., g_{PHA}/g_{VSS}) in the biomass was calculated by dividing the PHA concentration (g_{PHA}/L) by the concentration of VSS (g_{VSS}/L), considering VSS to be constituted of active biomass (X) and PHA. For determining cell growth the generic chemical formula for MMC under carbon-limited conditions ($CH_{1.8}O_{0.5}N_{0.2}S_{0.002}P_{0.02}$) [31], with a molecular weight (MW) of 25.30 g/Cmol and $gCOD/g_X$ ratio of 1.36, was used. The maximum active biomass ($X^{F/f}_{max}$ or X^{Acc}_{max}, g_X/L) was determined as function of the generic formula for MMC and ΔP consumed during a F/f cycle or accumulation batch. The amount of stored PHA (ΔPHA) was determined as the maximum PHA content (PHA_{max}, g_{PHA}/L) minus the PHA content at the beginning (PHA_0, g_{PHA}/L) of the experiment. Stoichiometric and kinetic performance parameters were determined for the SBR and PHA accumulation assays, in pseudo-steady state conditions, with the reactors operated at the highest OLR. The specific rate of substrate consumption (-q_S, $Cmol_S/(Cmol_X.h)$), PHA storage (q_{PHA}, $Cmol_{PHA}/(Cmol_X.h)$), PHA consumption (-q_{PHA}, $Cmol_{PHA}/(Cmol_X.h)$) and cellular growth (q_X, $Cmol_X/(Cmol_X.h)$) were determined from the linear regression of the specific concentrations of S, PHA, and X plotted over time. Storage yield ($Y_{PHA/S}$, $Cmol_{PHA}/Cmol_S$) was calculated as the ratio between q_{PHA} and the -q_S. Growth yields on carbon substrate ($Y_{X/S}$, $Cmol_X/Cmol_S$) and on stored PHA ($Y_{X/PHA}$, $Cmol_X/Cmol_{PHA}$) were calculated as the ratio between the specific growth rate during the Feast (q_X^{Feast}) and during the famine (q_X^{famine}) phases and the -q_S and -q_{PHA}, respectively. The active biomass productivity (XPR, g_X/(L.h)) was calculated as the ratio between the produced active biomass ($X^{F/f}_{max}$ minus $X^{F/f}_0$ or X^{Acc}_{max} minus X^{Acc}_0) per the corresponding time of F/f cycle or accumulation. Volumetric PHA productivity (g_{PHA}/(L.h)) for the accumulation stage was calculated as the ratio between the produced PHA (ΔPHA) at maximum value per the corresponding time of accumulation. Specific PHA productivity ($g_{PHA}/(g_X.h)$) was calculated as the ratio between the volumetric PHA productivity for the accumulation per X at the beginning of the accumulation (X^{Acc}_0). Global volumetric PHA productivity (g_{PHA}/(L.d)), taking in consideration the second and third stages of the PHA production process, was calculated as the ratio of the PHA_{max} achieved per X^{Acc}_0 in the accumulation assay multiplied by XPR determined for the SBR.

Standards errors associated with the determined parameters were estimated using standard errors propagation formulae.

3. Results and discussion

3.1. Stage I: Acidogenic fermentation of salmon peptone

The acidogenic fermentation of SP was conducted in a UASB reactor with a working volume of 90 L and under anaerobic conditions. The reactor was inoculated with granular sludge aiming at minimizing the long period associated with the granulation processes and taking advantage of the high SRT of this type of biomass in continuous

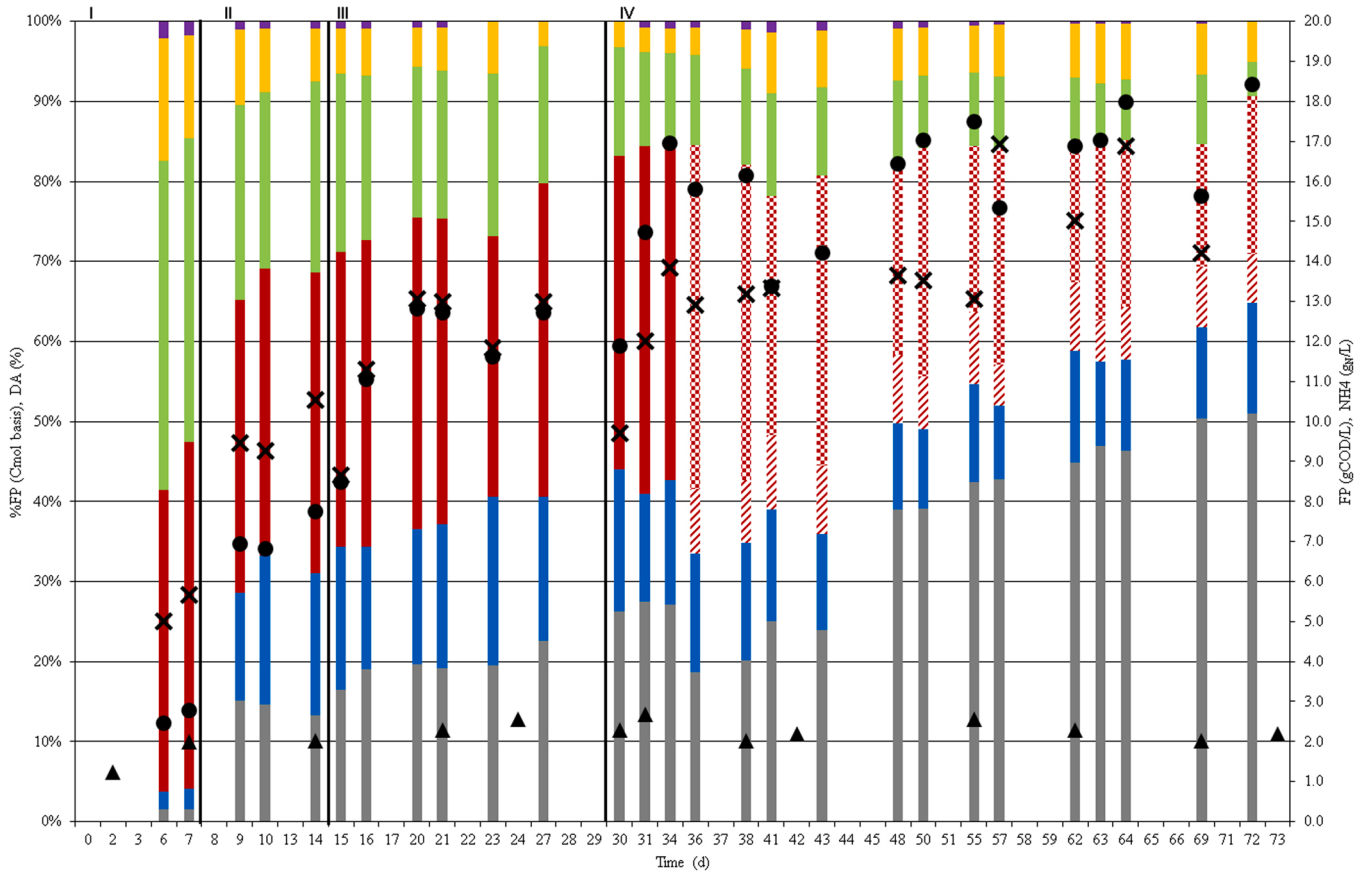

Fig. 2. Profile of the acidogenic fermentation of salmon peptone over 73 days of operation. OLR was increased stepwise, periods I to IV correspond to OLR at 9.8, 14.7, 19.6 and 24.5 $gTCOD_{SP}/(L.d)$), respectively. Degree of acidification (DA, %, X), concentration of fermentation products (FP, gCOD/L, •), abundance of fermentation products (%FP, Cmol-basis, acetate (■), propionate (■), isobutyrate+ethanol+butyrate (■), isobutyrate+ethanol (■), butyrate (■), isovalerate (■), valerate (■), caproate (■)), concentration of ammonia in the effluent (N-NH4, gN/L, ▲).

operation. This stage aimed at achieving high DA and producing a fermented stream with high concentration of VFAs and low non-fermented solids content. This would minimize the loss of fermentable carbon through the effluent and the growth of non-PHA accumulating organisms on peptone during the second stage of the process. In addition, reactor volumes and energy requirements in the PHA production process would also be kept to a minimum.

Initially, the acidogenic fermentation of SP was carried out at pH 4.5 with the premise of limiting carbon losses to methane, as most methanogens are inhibited at acidic pH [32]. Although the methanogenic activity was almost suppressed after 34 days of operation, reaching a CH_4 content of 7% (based on Cmol) as measured in the gas outflow, the DA was low (29 ± 5%) and there was a large accumulation of solids inside the reactor. Furthermore, the deamination of amino acids led to ammonia release and pH increase, requiring large amounts of HCl addition to control the reactor at acidic pH.

Therefore, the UASB reactor operation was interrupted and a new operation started with a fresh inoculum and the bioreactor controlled at pH 7. In this approach, our main focus was to favour peptone hydrolysis and VFA production that is faster at pH 7 than at acidic pH [33], excepting carbon losses through increased methanogenic activity as a trade-off [32].

This new experiment was carried out for 73 days (Fig. 2). The UASB was initially operated with an OLR of 9.8 $gTCOD_{SP}/(L.d)$ and then incrementally increased to 24.5 $gTCOD_{SP}/(L.d)$ (Fig. 2), aiming at the gradual acclimatisation of the microbial culture to the SP and increasing the volumetric VFA productivity.

The acidogenic fermentation of SP responded best to pH 7. As can be seen in Fig. 2, an extensive release of ammonia from amino acid degradation was accompanied by an increase in DA throughout the operation. An overall conversion of ~94% of the nitrogen species present in salmon peptone (95.7 mgTN/gSP) into ammonia were obtained at the highest OLR applied. This resulted in the production of an ammonia-rich fermented stream comprising a concentration up to 2.26 ± 0.23 gN/L at the highest applied OLR (Table S2). Despite of the ammonia concentration was above the inhibitory threshold reported for microbial population involved in WWTP [34], it was obtained a DA of 72.9 ± 7.8% in the present study. This value is comparable to those reported in previous studies carried out with feedstocks that are nutrient-poor (low protein content) [14,15,17] and higher than those reported for proteins-rich resources [23,25,26] (Table S2). This could be explained by the low content of soluble compounds present in those feedstocks (<48% gSCOD/gTS) [23,25] compared with SP (~84% gSCOD/gTS).

Methanogenic activity was not suppressed at pH 7, but the molar content (%mol) of CH_4 in the gas outflow was halved with an increase in OLR, decreasing from 64.1 ± 0.4% at OLR 9.4 $gTCOD_{SP}/(L.d)$ to 30.5 ± 5.3% at OLR 24.5 $gTCOD_{SP}/(L.d)$. This could be explained by the partial inhibition of methanogenic activity due to the presence of high concentrations of ammonia (mainly NH_4 form at pH 7) [35] and/or high concentrations of propionate [36].

In addition to CH_4, significant amounts of CO_2, N_2 and H_2 were also present in the gas outflow, comprising a concentration of 57.3%, 11.7% and 0.5% in the highest OLR applied, respectively. Furthermore, to

make the process more energy favourable, the methane produced could then be evaluated for recovery and energy production.

High gas production was observed for the highest OLRs applied. This disturbed the blank sludge contributing to the deterioration of the granular sludge and the release of particulate debris (e.g. deteriorated biomass and non-fermented peptone) into the effluent stream, which reached a concentration of 5.3 ± 1.7 gVSS/L and 10.3 ± 5.0 gTSS/L at the highest OLR applied. Therefore, a two-stage decanter unit was installed at the outlet of the UASB to remove the solids from the fermented stream and to minimize the negative impact it would have in the downstream stages. The first unit (45 L settler) removed 77% wt. of solids, while the second unit (300 L effluent storage tank) removed an additional 10% wt. of solids. The decanting process resulted in a clarified fermented stream with a solids content of only 1.6 ± 0.5 g_{TSS}/L.

A fermented stream with a concentration of 16.4 ± 1.6 $gCOD_{FP}$/L and a FP content of 87 ± 11% ($gCOD_{FP}$/gSCOD) was produced at the highest OLR applied. The VFA profile changed with the increase of OLR, with acetate levels increasing from 2% to 44.7% at the highest OLR applied, reaching a maximum concentration of 6 gCOD/L, while most other VFAs decrease its content (Fig. 2). The increase in acetate content may be related to the inhibition of acetoclastic methanogens, likely due to the increase of ammonia and propionate, which were detected in the acidogenic reactor (see below).

During the pseudo-steady state period at the highest OLR, the fraction of 3HV precursors was estimated to be ~35% (based on Cmol), considering that 3HV monomers can be produced by a single molecule of valerate or by a combination of acetate and propionate in a ratio of 1:1 molecules.

The composition of the fermented stream was not only influenced by the operation conditions applied and type of the feedstock, but also by the microbial community present in the reactor. In the present study, the microbial community had to consist of organisms with the ability to secrete extracellular peptidases for hydrolysing SP into amino acids. The formed amino acids can then be further degraded, via different pathways, to various end products such as VFAs, ammonia, carbon dioxide and hydrogen. Typically, amino acids are degraded under anaerobic conditions through (i) the degradation of pairs of amino acids (Stickland reaction), (ii) the reductive deamination of single amino acids, and (iii) the oxidative deamination of individual amino acids [37,38].

The microbial community selected at the end of the operation was dominated by amino acid-degrading bacteria and methanogenic archaea (Supplementary Fig. S1). The amino acid-degrading bacteria selected were undetected by 16 S rRNA gene sequencing in the starting inoculum, suggesting a significant microbial community shift during operation due to the feedstock type (i.e. peptone). The enriched bacterial culture include species belonging to *Peptostreptococcus* (59%), *Peptoniphilus* (8.8%) and *Anaerosalibacter* (5.1%) genera from Firmicutes and *Aminobacterium* (10.7%) genus from Synergistetes. Most of these species are described in the literature as negative for the Stickland reaction which is the most common pathway of amino acid degradation (90%) [37,39]. Therefore, amino acid degradation must have occurred via alternative pathways such as reductive or oxidative deamination. Reductive deamination is favourable and consumes hydrogen, while oxidative deamination is a thermodynamically unfavourable process (produce H_2) and requires an extremely low hydrogen partial pressure. The latter therefore requires the presence of a hydrogen scavenging partner such as hydrogenotrophic methanogens [37,38].

Methanogenic archaea were found to be present both in the inoculum and at the end of operation. These species belong to the hydrogenotrophic genus *Methanobacterium* (73.5%) and to the aceticlastic genus *Methanosaeta* (16.5%) which use H_2/CO_2 and acetate as sole carbon and energy source, respectively [40].

The selected mixed culture appears to have a syntrophic oxidation of H_2 because hydrogenotrophic methanogens use the H_2 produced by amino acid-degrading bacteria and thus maintaining low partial pressure of H_2 inside the reactor (~0.5%mol in the biogas), while amino acid-degrading bacteria need low partial pressure of H_2 for the unfavourable reductive deamination of single amino acids to occur [37].

Overall, the acidogenic fermentation of SP was largely achieved, as shown by the extensive acidogenic fermentation (DA ~73%) and the clarified, VFA-rich (~87%, $gCOD_{FP}$/gSCOD) and NH_4-rich (2.3 ± 0.2 g_N/L) stream produced.

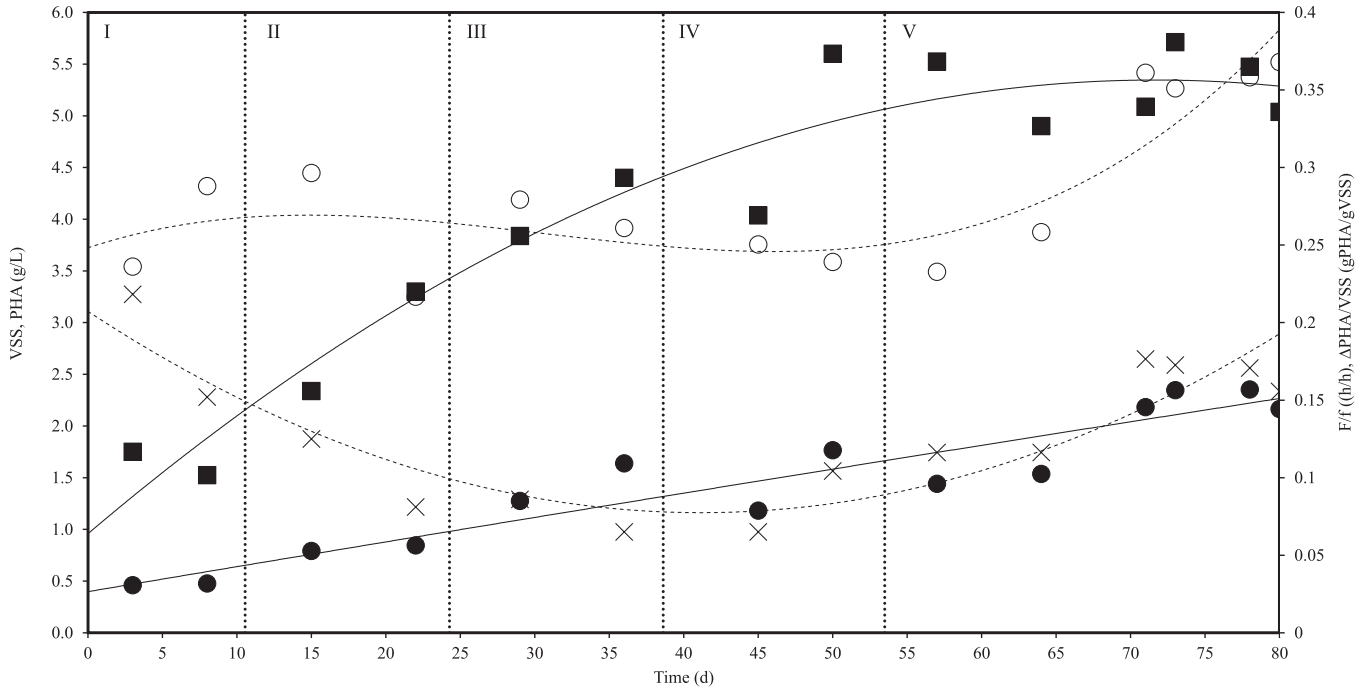

Fig. 3. Profile of the culture selection over 80 days of operation. OLR was increased stepwise, periods I to V correspond to OLR at 1.78, 2.66, 4.44, 6.21 and 7.10 $gCOD_{FP}$/(L.d), respectively. F/f (X), VSS_0 (■), PHA_{Feast} (•), $\Delta PHA/VSS_0$ (o). Solid and dotted trend lines are adjusted using a 3rd order polynomial.

3.2. Stage II: Culture Selection

The selection of a PHA-accumulating MMC was conducted in a SBR with a working volume of 100 L under aerobic conditions and using the nutrient-rich fermented stream produced as source of carbon and nutrients (Table S3).

In order to select an efficient PHA-accumulating culture, a conventional F/f regime was applied as selective pressure throughout the operational period (80 days). The F/f ratio was continuously monitored, since it is a useful indicator for following the culture enrichment. Generally, a F/f of 0.33 h/h, or less, is considered to boost the selection of an efficient PHA-accumulating MMC [8]. Throughout the operation, an average F/f value of 0.12 ± 0.04 h/h was achieved, except for the first 8 days (Fig. 3). The SRT was kept short (3 days) to enhance the selection of a PHA-accumulating MMC with a high growth rate and, therefore, increasing the volumetric productivity of the process. The cell concentration in the SBR was increased by incrementally increasing the OLR up to 7.1 $gCOD_{FP}$/(L.d) whilst the selective pressure for PHA-accumulating organisms was maintained (Fig. 3).

An OLR increase to 6.21 $gCOD_{FP}$/(L.d) more than tripled biomass growth, reaching maximum concentration of 5.0 ± 0.1 gX/L in period IV and maintained for the highest OLR applied (Fig. 3). Overall, a biomass productivity (XPR) of 1.7 ± 0.33 g_X/(L.d) was determined for the SBR at the highest OLR applied (Table S4). Notably, the increase in OLR from period IV to V did not result in a significant increase in biomass concentration. This led to a higher food to microorganism (F/M) ratio imposed on the culture (0.68 $gCOD/g_{VSS}$) and, consequently, to a higher F/f ratio (Fig. 3). To prevent the loss of selective pressure for PHA-accumulating organisms, we decided not to further increase the OLR. Additionally, at the highest OLR, the culture exhibited an increase in specific PHA accumulation ($\Delta PHA/VSS_0$), which rose from 26.2 ± 2.6% wt. to 36.0 ± 0.6% wt. (Fig. 3). This indicates that the culture achieved a greater capacity for storing PHA at the highest OLR tested. With respect to PHA composition, an average 3HB: 3HV ratio of 64 ± 3: 36 ± 3% (based on Cmol) was obtained from fourteen monitored F/f cycles at different OLRs (1.78–7.1 $gCOD_{FP}$/(L.d)). This corresponded well with the theoretical monomeric content estimated from the composition of VFA (65% HB: 35% HV).

The selected PHA-accumulating MMC was characterized considering six monitored cycles at the highest OLR applied (7.1 $gCOD_{FP}$/(L.d)) (Table 1). A representative F/f cycle is shown in Fig. 4. The VFA and TOC consumption profiles were similar (Fig. 4), whereas the specific substrate consumption rate ($-q_S$) based on TOC was 1.29 times higher than that obtained based on VFA (Table 1). This result suggests that the unidentified organic carbon compounds (~0.92 $Cmol_{FP}/Cmol_S$) are readily taken up by cells. A complete VFA consumption after 1.8 h (Table 1 and Fig. 4), with a greater preference for consumption of acetate (0.194 ± 0.03 $Cmol_{Ac}/(Cmol_X.h)$) and butyrate (0.185 ± 0.03 $Cmol_{But}/(Cmol_X.h)$) was observed. The $-q_S$ for acetate and butyrate were three times higher than for the other VFAs (0.061 ± 0.015, 0.038 ± 0.018, 0.035 ± 0.011, 0.021 ± 0.010 $Cmol_{VFA}/Cmol_X.h$ for isobutyrate+ethanol/propionate/valerate/isovalerate respectively and negligible for caproate). The preference for butyrate may be explained from an energetic point of view, as less ATP is required for taking 1 Cmol of long-chain VFAs (e.g. butyrate and valerate) than of short-chain VFAs (e.g. acetate and propionate) [17,41].

During the final period of operation (period V), a specific PHA production rate (q_{PHA}) of 0.24 ± 0.02 $Cmol_{PHA}/(Cmol_X.h)$ (0.26 ± 0.02 $gCOD_{PHA}/(gCOD_X.h)$) was observed (Table 1). This value is in agreement with the q_{PHA} obtained by Oliveira et al. [18], where the culture was selected with controlled nitrogen availability during the feast phase (q_{PHA}= 0.25 ± 0.01 $Cmol_{PHA}/(Cmol_X.h)$), but lower than the value (0.40 ± 0.03 $Cmol_{PHA}/(Cmol_X.h)$) obtained when the culture was selected in conditions of uncoupled availability of C/N.

Despite the excess of nutrients during the feast phase (100 gCOD: 27.6 gN: 0.48 gP), PHA storage dominated carbon uptake ($Y_{PHA/S}$ = 0.72 ± 0.04 $gCOD_{PHA}/gCOD_S$), indicating that the strong selective pressure (i.e. a long famine phase) applied resulted in the selection of an efficient PHA-accumulating MMC (i.e. with high capacity of channelling the majority of the exogeneous carbon towards PHA storage instead of cell growth). The PHA storage yield is among of the highest values previously reported in studies at pilot scale (Table S4).

With respect to specific growth rate (q_X), the q_X^{Feast} value (VFA used as substrate) was approximately 3.5 times higher than the specific growth rate based on PHA (q_X^{famine}), which accounts for 37% of the total cellular growth of a F/f cycle (Table 1). A growth yield of 0.12 ± 0.04 $gCOD_X/gCOD_S$ for the feast phase ($Y_{X/S}$) and 0.21 ± 0.09 $gCOD_X/gCOD_{PHA}$ for the famine phase ($Y_{X/PHA}$) were obtained (Table S4). A global yield on S ($Y_{PHA/S} + Y_{X/S}$) of 0.84 ± 0.04 $gCOD_{(PHA+X)}/gCOD_S$ was determined, suggesting that the remaining carbon was likely used for cell respiration and maintenance.

PHA-accumulating organisms can be divided into two groups based on the conditions required for PHA synthesis. The first group requires the limitation of an essential nutrient such as nitrogen or phosphorous for the synthesis of PHA from an excess carbon source (e.g. *Cupriavidus necator*), whereas the second group do not require nutrient limitation for PHA synthesis [42]. *Brachymonas* sp. P12 belong to the second group, as it was found to accumulate PHA in a growth-associated pattern, whereby cell growth and PHA accumulation occurred simultaneously but were enhanced by nitrogen limitation [43].

In this study, microorganisms belonging to *Brachymonas* genus were found with high abundance (69.9%) at the selected culture, whereas microorganisms belonging to Microbacteriaceae and Alcaligenes families were also enriched with lower abundance (Supplementary Fig. S2). Organisms from both these two families have previously been shown capable of PHA accumulation [44,45].

Overall, an efficient and stable PHA-accumulating culture was successfully enriched under nutrient-rich and high ammonia conditions, which, to the best of our knowledge, is the first report of culture selection at such a high ammonia concentration (0.962 ± 0.040 g_N/L at the highest OLR applied).

3.3. Stage III: PHA production

The accumulation of PHA under nutrient-rich and high ammonia

Table 1
Operating conditions applied and performance parameters determined for the culture selection stage considering five monitored cycles carried out at OLR 7.1 $gCOD_{FP}$/(L.d).

Parameter		Average ± Standard Deviation
OLR	gCOD/(L.d)	7.1
	$Cmmol_S$/(L.d)	225 ± 32
	$Cmmol_{FP}$/(L.d)	206 ± 13
FP profile	Acet/ Pro/ EtOH+iBut/ But/ iVal/ Val/ Cap, % Cmol basis	44.7 / 11.5 / 7.0 / 21.8 / 8.1 / 6.4 / 0.4
Feast/ famine	h/h, S basis	0.17 ± 0.02
	h/h, FP basis	0.15 ± 0.02
$X_0^{F/f}$	g_X/L	4.5 ± 0.1
	$Cmol_X$/L	176 ± 5
PHBV @$X_0^{F/f}$	% wt., g_{PHA}/g_{VSS}	5 ± 1
ΔPHBV	% wt., g_{PHA}/g_{VSS}	24 ± 3
HB:HV ratio	% wt., $g_{Monomer}/g_{PHA}$	66 ± 3: 34 ± 3
	%.,$Cmol_{Monomer}/Cmol_{PHA}$	64 ± 3: 36 ± 3
q_X^{Feast}	$gCOD_X/(gCOD_X.h)$	0.045 ± 0.017
q_{PHA}	$gCOD_{PHA}/(gCOD_X.h)$	0.26 ± 0.02
$-q_S$[a]	$gCOD_S/(gCOD_X.h)$	0.36 ± 0.03
	$Cmol_S/(Cmol_X.h)$	0.34 ± 0.03
	$Cmol_{FP}/(Cmol_X.h)$	0.44 ± 0.08
q_X^{famine}	$gCOD_X/(gCOD_X.h)$	0.013 ± 0.005
$-q_{PHA}$	$gCOD_{PHA}/(gCOD_X.h)$	0.06 ± 0.02

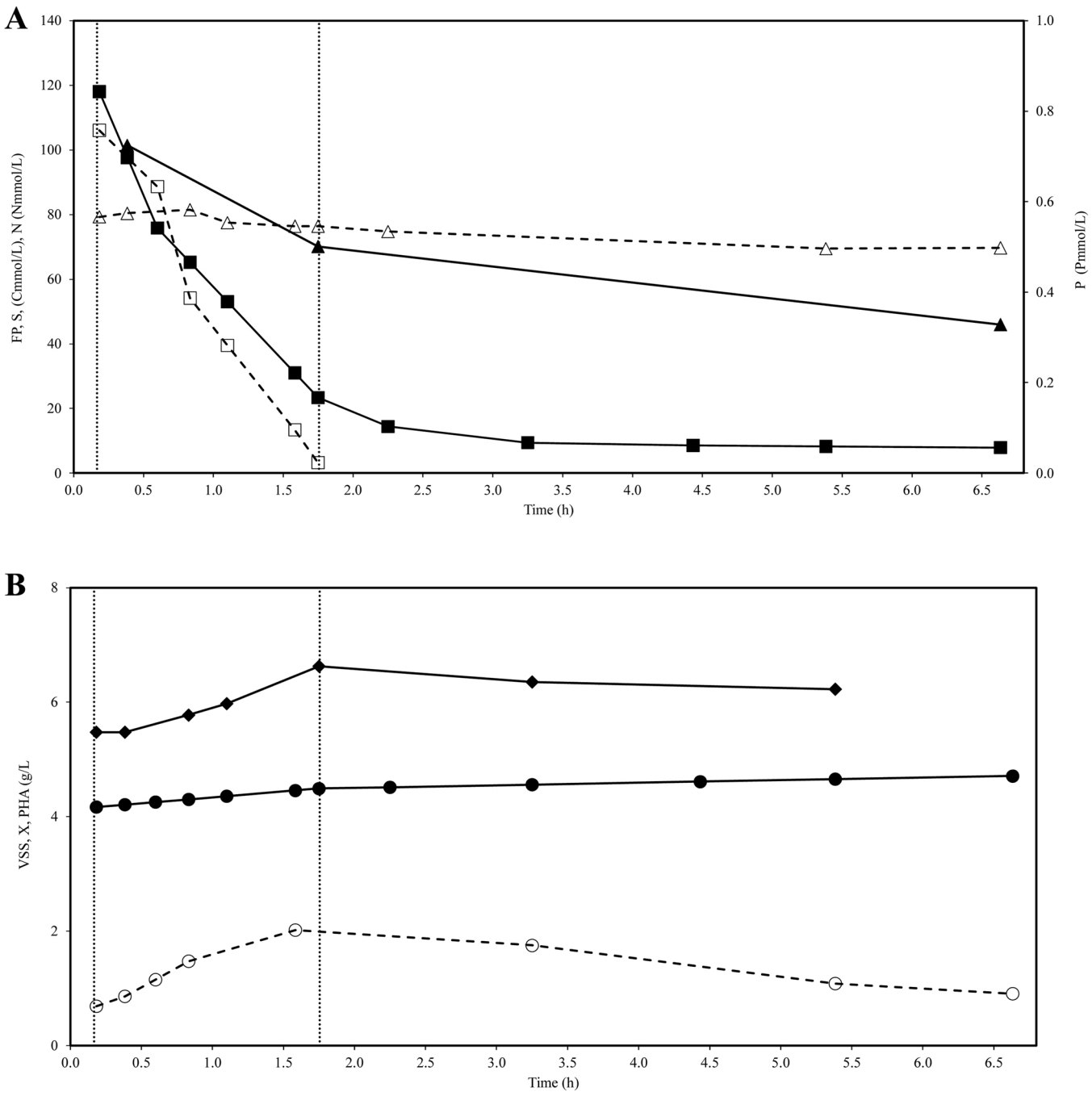

Fig. 4. Profile of one monitored SBR cycle (6.5 h out of 12 h) conducted at OLR 7.1 $gCOD_{FP}$/(L.d). The left vertical dot lines limit the end of the feeding period (10 min). The right vertical solid line limit both feast (I) and famine (II) phases. Feast phase was obtained based on the TOC analysis. Panel A: S (■), FP (□), P-PO_4 (▲), N-NH_4 (Δ). Panel B: VSS (◆), X (●), PHA (o).

conditions was the last challenge of this study. PHA accumulation was carried out in twenty-one production batches over 49 days of operation, using the PHA-accumulating MMC selected at OLRs between 4.44 and 7.1 $gCOD_{FP}$/(L.d) as inoculum.

In this respect, the production batches were characterized considering three accumulations carried out with sludge enriched with PHA-accumulating organisms (inoculum) purged from the SBR at the highest OLR applied (7.1 $gCOD_{FP}$/(L.d)). As the sludge was collected from purging the SBR during the final stage of the famine phase, the PHA content at the beginning of the accumulation was low (7.3 ± 1.9% wt., based on VSS).

To maintain a consistent supply of exogenous carbon and minimize PHA consumption for cell growth, we implemented a feeding strategy during PHA accumulation that involved a combination of standard feeding mode, pulse-wise feeding (84% v/v), and continuous feeding (16% v/v). The continuous feeding was essential to prevent any potential starvation periods between pulse-wise feedings, thereby ensuring a steady supply of exogenous carbon throughout the assay.

The culture was fed with undiluted fermentate containing an ammonia concentration of 2.3 ± 0.2 g_N/L. Therefore, the culture was subjected to 2.4-fold higher concentration of ammonia at the end of the production batch. These production batches were carried out at the highest ammonia concentration and nitrogen to carbon ratio reported so far in the literature (Table S5).

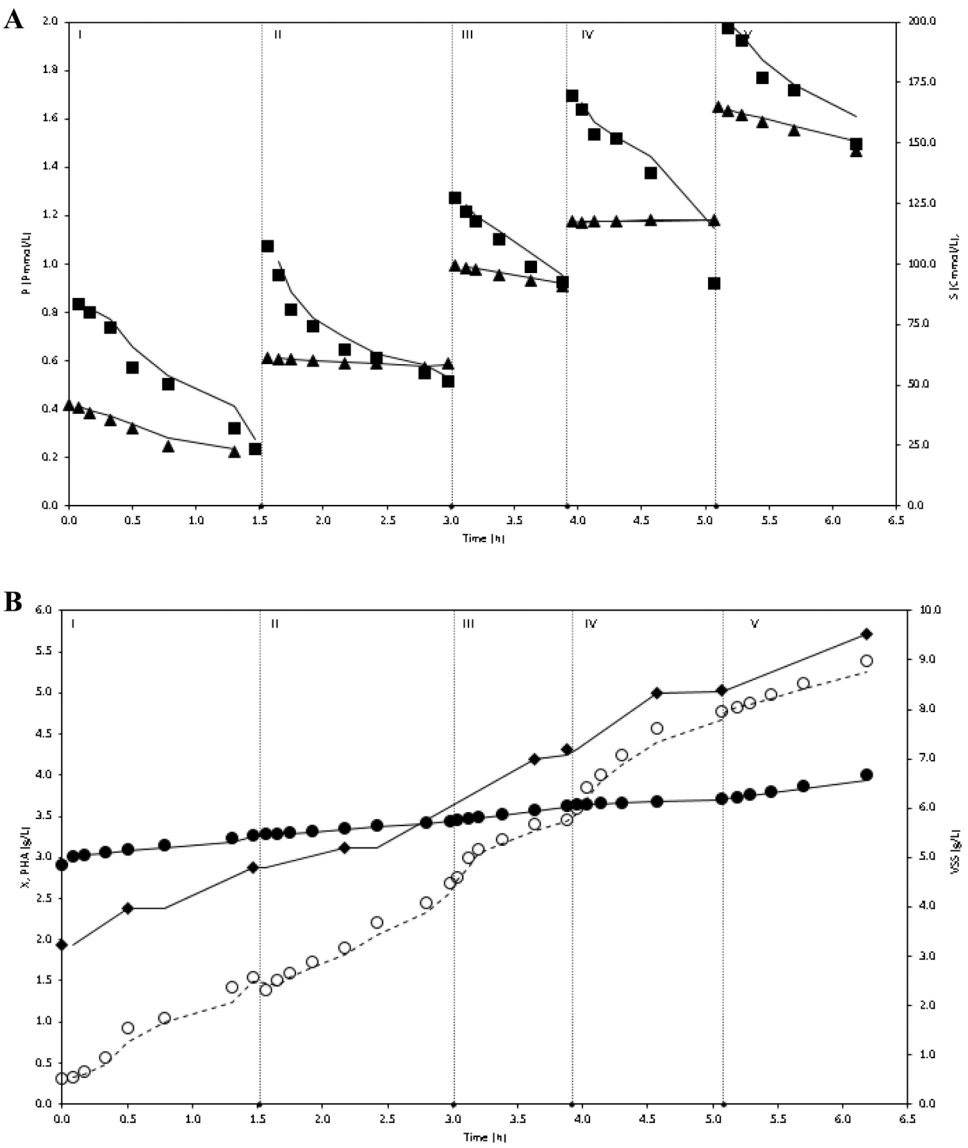

Fig. 5. Profile of a fed-batch accumulation conducted with the culture selected at OLR 7.1 $gCOD_{FP}/(L.d)$. Five pulses periods are numbered between I to V and limited by the vertical solid lines. Panel A: P (▲) and S (■); Panel B: VSS (◆), X (●), PHA (o). An ammonia concentration of 2.26 ± 0.23 g_N/L was achieved at the end of the accumulation assay. Solid and dotted trend lines are adjusted using a 2nd order moving-average. The C/N ratio (gCOD/gN) at the beginning of each pulse were 3.6, 2.6, 1.8, 1.4, and 1.2, which were calculated by using feed volumes, FBR volumes, and the initial ammonia concentration in the inoculum and feedstock.

A representative carbon consumption profile using the above-mentioned strategy is shown in Fig. 5. A higher specific substrate consumption rate ($-q_S$) was obtained for the PHA accumulation stage than for the F/f cycle (0.44 ± 0.05 $gCOD_S/(gCOD_X.h)$ vs 0.36 ± 0.03 $gCOD_S/(gCOD_X.h)$, Table 1 and Table 2), which might have been caused by the higher food to microorganism ratio applied.

In respect of PHA storage capacity, an average maximum PHA cell content of $53.0 \pm 3.4\%$ wt. (based on VSS) with a 3HV content of 34% wt. was reached after 5.3 ± 0.3 h (4 pulses). This value is comparable to those reported in previous studies carried out at much lower ammonia concentrations and nitrogen to carbon ratio (Table S5).

An average PHA storage yield ($Y_{PHA/S}$) of 0.62 ± 0.03 $gCOD_{PHA}/gCOD_S$ was achieved for the first four pulses (Table S5). The $Y_{PHA/S}$ obtained in this study was higher than those reported in previous studies where PHA-accumulations were performed in conditions without nutrient limitation (up to 0.59 $gCOD_{PHA}/gCOD_{VFA}$, [23,25,26]), and in the range of those studies carried out under conditions of ammonia starvation, except for the recent publication by Matos et al. (0.98 $gCOD_{PHA}/gSCOD$, [17]) (Table S5).

With regard to cellular growth, an average growth yield ($Y_{X/S}$) of 0.12 ± 0.02 $gCOD_X/gCOD_S$ and an average specific growth rate (q_X) of 0.053 ± 0.014 $gCOD_X/(gCOD_X.h)$ were obtained for the first four pulses (Table 2 and S4), which resulted in a XPR of 0.20 ± 0.04 $g_X/(L.h)$. Despite of the observed cellular growth, the PHA accumulation during the first four pulses was not compromised. These results are in accordance with previous studies where accumulations batches carried out in the presence of nutrients resulted in higher PHA productivity due to PHA-accumulating biomass growth than those without nutrients [23,27].

After the culture reached its maximum PHA content after four pulses ($PHA_{max} = 53.0 \pm 3.4\%$ wt.), the organisms have shifted their primary metabolism from PHA accumulation to cellular growth ($\Delta PHA_{Pulse5} = 1.2 \pm 0.8\%$ wt.). This shift led to a sharp decrease in the q_{PHA} (0.106 ± 0.075 $gCOD_{PHA}/(gCOD_X.h)$) and $Y_{PHA/S}$ (0.26 ± 0.10 $gCOD_{PHA}/gCOD_S$) and an increase in the q_X (0.100 ± 0.034 $gCOD_X/(gCOD_X.h)$) and $Y_{X/S}$ (0.33 ± 0.19 $gCOD_X/gCOD_S$) in the fifth pulse. Overall, the culture has reached its maximum PHA storing capacity after four pulses, thereby compromising the amount of carbon channeled toward PHA storage during the fifth pulse. This resulted in a global yield on S ($Y_{PHA/S} + Y_{X/S}$) of ~ 0.59 $gCOD_{(PHA+X)}/gCOD_S$ in the fifth pulse.

A volumetric and specific PHA productivity of 0.76 ± 0.08 $g_{PHA}/(L.h)$ and 0.17 ± 0.03 $g_{PHA}/(g_X.h)$ were achieved for the PHA accumulation stage. The volumetric PHA productivity achieved in this study was more than double that obtained in previous studies where the culture

Table 2
Operating conditions applied and performance parameters determined for the PHA accumulation stage. It presents data from three monitored batches conducted with the culture selected at OLR 7.1 gCODFP/(L.d). Only the first four pulses were considered for the average values, as during the fifth pulse the exogenous carbon was mainly channelled for growth.

Parameter		Average ± Standard Deviation
FP profile	Acet/ Pro/ EtOH+iBut/ But/ iVal/ Val/ Cap, % Cmol basis	44.7/ 11.5/ 7.0/ 21.8/ 8.1/ 6.4/ 0.4
X_0^{Acc}	g_X/L	3.1 ± 0.2
	$Cmol_X$/L	122.6 ± 6.49
X_{max}^{Acc} [a]	g_X/L	3.9 ± 0.2
	$Cmol_X$/L	154.7 ± 9.3
PHBV @X_0^{Acc}	% wt., g_{PHA}/g_{VSS}	7.0 ± 2.9
PHBV @X_{max}^{Acc} [a]	% wt., g_{PHA}/g_{VSS}	53.0 ± 4.2
HB:HV ratio[a]	% wt., $g_{Monomer}/g_{PHA}$	68: 32
	%., $Cmol_{Monomer}/Cmol_{PHA}$	66: 34
q_X	$gCOD_X/(gCOD_X.h)$	0.05 ± 0.01
q_{PHA} [a]	$gCOD_{PHA}/(gCOD_X.h)$	0.27 ± 0.05
$-q_S$ [a]	$gCOD_S/(gCOD_X.h)$	0.44 ± 0.05
	$Cmol_S/(Cmol_X.h)$	0.41 ± 0.05

[a] considering three accumulation tests with four pulses.

selection and accumulation batch were performed in the presence of nutrients (Table S6). The global volumetric productivity (3.10 ± 0.11 gPHA/(L.d)), when taking into account both the biomass productivity and the biomass collected in the purges during the second stage, was found to be higher than the values reported in previous studies conducted under conditions of nutrient excess. However, it was still lower than the productivity of cultures selected with uncoupled C/N availability (Table S6).

Overall, our results showed a large PHA accumulation under nutrient-rich and high ammonia conditions with a positive synergetic effect between cell growth and PHA accumulation.

3.4. Overall PHA Production Process

The overall PHA yield was calculated for the long-term operation, taking into account the reactor operation at the highest applied OLRs. In this respect, a global COD mass balance was done considering as basis 1 kg (1.75 kgCOD) of P(3HB-co-3HV) with a 3HV content of 34% wt. and using the experimental parameters and assumptions obtained for each stage of the process (as detailed in Fig. S3). Based on this, it was determined that 5.30 kg of salmon peptone are required to produce 1 kg of P(3HB-co-3HV), resulting in an overall PHA yield of 0.34 $gCOD_{PHA}/gTCOD_{SP}$ and 0.4 $gCOD_{PHA}/gSCOD_{SP}$.

The global process parameters are good indicators to evaluate the economic/technical feasibility of a PHA production process. The global PHA yield and PHA productivity obtained in the present study are in the range of those studies previously carried out in conditions of nutrient limitation (see Table S6). Therefore, there is a potential to valorise salmon peptone into PHA, since there are no costs associated with nutrient supplementation.

In our point of view, good performance results are also achievable for conditions without nutrient limitation, but a firm control over the culture selection is of paramount importance. Below is a summary of key factors that led to a successful valorisation of salmon peptone into PHA:

(i) a feedstock with a high SCOD content (~0.9 SCOD/TCOD) was used. This was achievable since the feedstock was obtained from the proteinaceous fraction from fish silage, which consists of degraded proteins in the form of peptones or free amino acids.
(ii) a clarified VFA-rich stream (16.4 ± 1.6 $gCOD_{FP}$/L) was produced from the acidogenic fermentation of salmon peptone. This was possible due to the successful optimisation of the operating conditions of the UASB reactor, namely the pH, and the installation of a decanter unit to remove solids from the fermented stream;
(iii) The selection of an efficient PHA-accumulating MMC under conditions without nutrient limitation (100 gCOD: 27.6 gN: 0.48 gP at OLR 7.1 gCOD/L/d), where ammonia has a high concentration (0.962 ± 0.040 gN/L). This was achieved by using an F/f regime with short SRT (3 d) together with a VFA-rich carbon source (0.87 ± 0.11 $gCOD_{FP}$/gSCOD). This allowed a strong selective pressure for PHA-accumulating organisms as a long famine phase devoid of exogenous carbon source besides PHA was successfully kept thorough operation. Moreover, these operating conditions allowed to select a *Brachymonas*-dominated culture (69.9%) that includes organisms described to accumulate PHA in a growth-associated pattern;
(iv) Adequate maximum PHA capacity (53.0 ± 3.4% wt., based on VSS) and exceptional volumetric PHA productivity (0.76 ± 0.08 gPHA/(L.h)) were achieved under nutrient-rich and high ammonia (up to 2.3 ± 0.2 gN/L) conditions during accumulation. This resulted from the use of a mixed feeding strategy, which avoided periods of operation without VFA presence and allowed to reach good XPR (0.20 ± 0.04 g_X/(L.h)) without PHA consumption.

3.5. Generation and treatment of production side streams

The primary motivation for developing PHA production toward increased commercialization is to lower the environmental impact related to modern society's dependence on plastic items and packaging. It is therefore prudent to ensure that the side streams generated during the production process is not by itself harmful to nature.

Fortunately, SP protein-rich resources that was generated during the production process could successfully be treated for COD and solids content as a low content of COD (0.77 ± 0.35 g TCOD/L) and suspended solids (0.61 ± 0.08 g_{TSS}/L and 0.50 ± 0.08 g_{VSS}/L) were present in the main effluent of the process (100 $L_{effluent}$/d for the SBR). However, to ensure the safe disposal of effluent, an additional remediation step is necessary as residual amounts of phosphorus (9.7 ± 1.2 mgP/L) and a high concentration of ammonia (0.963 ± 0.040 gN/L) are still present. In addition, during the withdrawal of effluent that needs to be disposed of after the settling of PHA-enriched biomass at the end of the accumulation batch, an average of 75 ± 6 L of effluent per accumulation batch was obtained. Since not all the exogenous carbon fed was consumed to avoid PHA consumption, there was still a residual content of fermented products (3.4 ± 1.72 gCODFP/L) and nutrients (41.6 ± 3.69 mgP/L for phosphorus and 2.26 ± 0.23 gN/L for ammonia). Therefore, an additional remediation step is required to ensure the safe disposal of both effluents.

Regarding the oil and sediment phases, which were not the primary focus of this study and were thus excluded, future research could investigate their potential valorisation routes. Specifically, the oil fraction could be evaluated for the production of medium-chain-length PHA.

4. Conclusions

The bioconversion of SP into PHA using MMC has been proven to be feasible at pilot-scale. Acidogenic fermentation of SP produced a VFA-rich stream (16.4 ± 1.6 $gCOD_{FP}$/L) with high content in acetate and butyrate. An efficient PHA-accumulating culture dominated by *Brachymonas* was selected under conditions without nutrient limitation, where ammonia has a high concentration up to 0.962 ± 0.040 g_N/L, which was successfully used in twenty-one PHA accumulation batches carried out with an ammonia concentration up to 2.3 ± 0.2 g_N/L. This study was carried out at the highest ammonia concentration reported so far in the literature.

The operational strategy chosen for the production batches allowed

high biomass productivity (0.20 ± 0.04 g_X/(L.h)) to be achieved without compromising the PHA content of the cells ($53 \pm 3.4\%$ wt.). The positive synergetic effect between PHA accumulation and cell growth during the production batches led to good global parameters for the process, which includes a specific PHA productivity of 0.17 ± 0.03 $g_{PHA}/(g_X.h)$ and a global volumetric PHA productivity of 3.10 ± 0.11 g_{PHA}/(L.d). The high potential for the valorisation of SP was demonstrated by the global yield of process (1 kg of polymer per 5.3 kg of SP).

The operating conditions used for the selection of a culture enriched in microorganisms with the capacity to simultaneous accumulate PHA and growth, as well as the feeding strategy adopted in the accumulation stage were the key factors for the success of the conversion of a rich ammonia feedstock into PHA.

CRediT authorship contribution statement

Bruno C. Marreiros: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Mónica Carvalheira:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Cláudia Henriques:** Investigation, Data curation, Visualization. **Daniela Pequito:** Investigation. **Yen Nguyen:** Investigation. **Runar G. Solstad:** Investigation. **J. Johannes Eksteen:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Maria A.M. Reis:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2023.110100.

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