Novel bioprocess approach for valorisation of ammonia-rich resources towards the production of PHA biopolymers

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Abstract

Mixed microbial cultures (MMC) enriched in feast/famine regime can accumulate large amounts of polyhydroxyalkanoates (PHA) in the absence of ammonium. However, if waste streams are to be used as a substrate, nutrient limitation may not always be achievable. This study aimed to investigate the influence of ammonium availability in the PHA accumulation stage of the process, using fermented fish waste (fFW) as feedstock. Fish waste was anaerobically fermented at different operational conditions. The concentrations of volatile fatty acids (VFAs), soluble chemical oxygen demand (sCOD), and NH_4^+ increased with organic loading rate (OLR). pH within the range of 6.0-7.0 appeared to enhance fish waste acidification, reaching the highest yields of VFAs/sCOD (67-69%). The highest VFAs concentration (498±51 Cmmol L^{-1}) was achieved at pH 6.0 and OLR 20 gFW L^{-1} d^{-1} with butyrate and acetate accounting for 56% of total VFAs and a C:N:P ratio of 100:47:2. Donnan dialysis was performed to remove NH_4^+ from fFW, reaching a C:N:P ratio of 100:0.4:1. A halotolerant MMC was selected in a feast/famine sequential batch reactor (SBR) firstly acclimatised with synthetic VFA mixture and then shifted to fFW. In a typical SBR cycle, biomass reached a maximum PHA content of 12.3 and 13.7% (gPHA gVS^{-1}) with synthetic medium and fFW, respectively. In the PHA accumulation stage, under conditions of ammonium limitation, biomass reached higher PHA storage rates (0.31 and 0.21 Cmol-PHA Cmol-X^{-1} h^{-1}) and maximum PHA contents (49.5 and 38.3% gPHA gVS^{-1}), with both synthetic medium and fFW. Nutrient limitation appears to be the best strategy for maximal PHA production.

Keywords: *Acidogenic Fermentation; Polyhydroxyalkanoates; Mixed Microbial Cultures; Ammonium; Fish Waste; Donnan dialysis.*

Introduction

Considering the serious environmental concerns related to the production of petroleum-based plastics, the last two decades have been marked by a considerable interest about the use and development of bioplastics. Among all bioplastics, polyhydroxyalkanoates (PHAs) have been gaining special attention as a potential substitute to conventional plastics, when considering their biodegradable and physicochemical properties and the far more sustainable application. PHAs are synthesised as intracellular carbon and energy storage in various microorganisms from different renewable resources [1].

PHA production by mixed microbial cultures have been recognised as a promising alternative for lowering PHA production costs [2; 3]. For PHA production using mixed microbial cultures (MMC), a three-stage process is proposed, comprising: (1) Acidogenic fermentation, where the raw complex organic substrate is fermented to obtain a stream rich in VFAs, the precursors for PHA biosynthesis; (2) Culture selection of PHA-storing microorganisms by applying a high selective pressure through the use of alternative F/F regime; and finally (3) PHA accumulation, where the selected microorganisms are fed with the VFA-rich stream produced in the anaerobic fermentation aiming at accumulate PHA up to the culture's maximum capacity [4].

The performance and efficiency of the overall PHA production largely depends on the nature of the waste used as feedstock, in particular, its nutrients content (mainly nitrogen and phosphorous). Many waste streams, technically suitable for PHA production, are often N and P deficient (e.g. cheese whey permeate, paper mill and olive oil mill wastewaters or sugar-cane molasses). While, other feedstocks with potential sources of nitrogen (e.g. food waste, cheese whey, fish waste, chicken manure or sewage sludge), making nutrient supplementation unnecessary to sustain biomass growth in the selection stage [5].

Fish waste has attracted attention as an inexpensive alternative substrate for biopolymer production. It contains large amounts of proteins, resulting in the release of high levels of ammonia (NH_4^+) and fatty acids (long chained fatty acids (LCFA) and volatile fatty acids (VFAs)) when digested [6].

However, a bottleneck in the PHA production process when using nitrogenous feedstocks is that nitrogen availability may limit PHA yields in the final accumulation stage. If the biomass is continuously exposed to both nutrients and carbon substrates, a growth response will progressively increase (with gains in cell and PHA volumetric productivity), whereas the storage response will decrease. Therefore, the maximum PHA content reached under these conditions is expected to be lower than the cell's maximum storage capacity [7].

A promising solution can pass by ammonium removal from the fermented stream when assessing PHA production. Moreover, these high concentrations of ammonium, when removed and recovered from digestate, represents a potential application for diverse fields (e.g nitrogen fertilisers) [8] or even to implement the strategy of uncoupling nitrogen from the carbon source in the PHA selection stage [9].

A simple and cost-effective process that allows to achieve ammonia removal is Donnan dialysis (DD), a membrane

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separation process that uses only an ion-exchange membrane without applying an external potential difference across the membrane. The driving force of the process is the electrochemical potential gradient of an electrolyte that causes the transport, selectively removing ions from the feed solution, and enrich them in the receiving solution. For the electroneutrality of the solution to be retained, stechiometrically equal amount of counter-ions should flow from the feed to the receiving solution. The process of ion exchange between solutions lasts until the Donnan equilibrium is reached [10]. Besides such a driving force, DD may not be enough to quickly achieve an efficient separation, with some disadvantages as long exchange time, making this process not well to scale up. DD processes have already been reported when treating ions in the industrial processing effluent (e.g. nitrate, fluoride, phosphate, arsenate or valuable species such as aluminium or gold) [11]. These ions are often in a relatively small quantity whereas ammonium, with a drastically different concentration in various complex streams, is rarely studied in Donnan dialysis [12].

A novel combination of the Donnan dialysis process with PHA production is presented in this work. Selective ammonium removal and recovery from VFA-rich streams using Donnan dialysis process is presented as a possible solution to the critical aspect of the enrichment cultures which might prove to be sensitive to the presence of nutrients in the final accumulation stage. Because there is the possibility of removing ammonia from nitrogen-rich ferments using saline solutions is important to assess the effects that salt has on culture selection and subsequent PHA accumulation. Therefore, the aims and scope of this thesis include: Operation of an acidogenic fermentation reactor of fish waste using anaerobic granular sludge; Evaluate the performance of ammonia removal from a synthetic VFA-mixture solution using Donnan dialysis process; Ammonia removal of fermented fish waste using Donnan dialysis process; Acclimatisation of a halotolerant PHA-storing mixed microbial culture and finally the assessment of PHA production using fermented fish waste with different ammonia content.

Materials and methods

Acidogenic Fermentation

Fish waste (FW) acidogenic fermentation was carried out in a continuous stirred tank reactor (CSTR-5S, Bioprocess Control, Sweden) with a working volume of 5 L. The CSTR was inoculated with non-acclimatised granular sludge from an anaerobic digester of a brewery industry and fed with FW provided by an aquaculture industry plant.

The bioreactor was operated in continuous mode under anaerobic conditions for a period of 144 days, stirred at 250 rpm and the temperature maintained at 30 °C by means of hot water recirculation through the reactor double jacket, using a bath system (CW-05G, Lab.Companion). pH was controlled and kept relatively constant, through automatic dosing of 2M NaOH and 2M HCl.

The hydraulic retention time (HRT) was controlled by overflow in order to be kept at around 2 days. No nutrient supplementation was added to the feeding solution.

Table 1: Operational conditions used in the acidogenic reactor over the 144 days of operation.

Condition	Operation days	OLR ($gFW\ L^{-1}\ d^{-1}$)	pH
I	21	5.0	7.0
II	33	5.0	4.5
III	16	5.0	6.0
IV	31	12.5	6.0
V	43	20.0	6.0

A settler (1.6 L of working volume) was connected to the CSTR, promoting solids retention. The effluent from the acidification reactor was directed by gravity to the settling tank and the supernatant was collected. Sediments were recirculated back to the CSTR after separation in the settler in the first three operating conditions (I, II and III). Biomass recirculation was accomplished on a daily basis at 5 L d^{-1}, so the sediment volume was kept constant in the settler's bottom, i.e. 0.2-0.4 L. In the final two operating conditions (IV and V), the solids retained in the settler were removed at a flow rate of 500 mL d^{-1}.

About 80 L of fermented fish waste (fFW) were collected, during 30 days of CSTR stable operation of the last studied condition. The effluent from the CSTR was later clarified using an ultrafiltration set-up composed of a peristaltic pump and an ultrafiltration hollow fiber membrane module (5×10^5 MW cut-off, UFP-500-E-4X2MA, GE Healthcare). The clarified effluent was collected under sterile conditions and kept at -20 °C prior to its use as a feedstock for the membrane system for ammonium removal and later for the PHA accumulation stage.

Donnan Dialysis

Firstly, the ammonium recovery from a synthetic VFA mixture with a fixed concentration of NH_4^+ (10 NmM) was assessed. Different concentrations of NaCl in the receiver and varying volume ratio of the streams were examined. These tests were followed by experiments with synthetic medium mimicking a real ammonium-rich feedstock (300 NmM) produced in the acidogenic reactor.

Set-up The schematic drawing of Donnan dialysis apparatus operated in a batch mode is shown in Figure 1. These assays were performed in an enclosed steel module with two identical rectangular chambers separated by a single piece of CEM with an effective membrane surface area of 39 cm^2. The feed and receiving solution were placed in two beakers, respectively, which sit on a magnetic stirrer at 250 rpm. The solution in each beaker was respectively introduced into the chambers of the Donnan dialysis stack and circulated by two peristaltic pumps (LEAD FLUID YZ15), respectively. Counter-current flow mode was used and the flow rate for each solution was maintained identical as 15 L h^{-1}. All experiments were performed at room temperature. Experiments with both synthetic and real fermented fish waste solutions were carried out under this semi-batch mode.

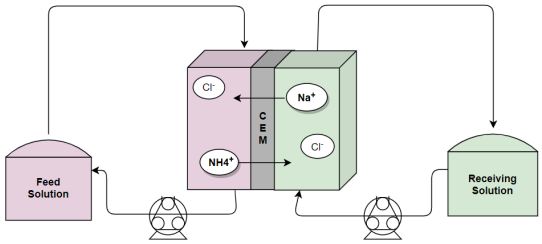

Figure 1: Donnan dialysis experiment apparatus.

Chemicals and Membrane In this study, Na^+ and NH_4^+ represent driving ion and target ion, respectively. The feed solution consisted of a solution of NH_4Cl with a fixed concentration of 10 Nmmol L^{-1} and a VFA-rich solution with 20 Cmmol L^{-1} in acetic, propionic, butyric and valeric acids in a equimolar carbon content. While the extracting solution consisted of a solution of NaCl at a varying concentration of 10, 20, 30, 60, 180 and 300 g L^{-1}. In this study, a CEM (RalexrCMH-PES 12-239) was used [13].

MMC Enrichment in PHA-accumulating Organisms

The PHA-accumulating culture selection using fermented fish waste as feedstock was accomplished in a SBR with a working volume of 2 L, operating under feast and famine regime. The reactor was inoculated with 1 L of sediments collected from a saline area of Rio Tejo (Samouco's salt pans, Portugal).

The SBR was operated with an aeration rate of 1-1.5 L min^{-1} controlled by a flow meter. Stirring was kept at 300 rpm. The reactor was kept at room temperature and the pH of the reactor liquid was maintained at 8.0, by automatic addition of NaOH (0.5 M) and HCl (0.5 M).

The SBR cycle length was 8 hour, consisting of four discrete periods: influent filling (5 min); aeration (440 min), a settling phase (40 min) and withdrawal of the exhausted effluent (8 min). In the first experiment, 100 mL of synthetic VFA mixture (acetic, propionic, butyric and valeric acids) and 900 mL of a mineral nutrient solution were fed, per cycle, to the SBR. While for the second run, 84 mL of fFW and 916 mL a mineral nutrient solution were fed to the SBR. Following the settling phase, the exhausted supernatant was withdrawn in order to keep an HRT of 16 hours. And a SRT of 3 days was kept by imposing a purge of mixed liquor (222 mL) at the end of each aeration phase (end of famine).

Both feedstocks were supplied with NaCl (30 g L^{-1}) and pH was adjusted to 8. Concerning synthetic VFA mixture, an ammonium solution was supplemented to the SBR in a C:N (mol) ratio of 100:47 to mimic CSTR's effluent. Coupled with the carbon and nitrogen source, mineral nutrient solution was added to the reactor accordingly to [14], with the following composition (mg L^{-1}) : $FeCl_3.6H_2O$: 1.5, H_3BO_3: 0.15, $CoCl_2.6H_2O$: 0.15, $MnCl_2.4H_2O$: 0.12, $ZnSO_4.7H_2O$: 0.12, $Na_2MoO_4.2H_2O$: 0.06, $CuSO_4.5H_2O$: 0.03, KI: 0.03, EDTA.2Na.$2H_2O$: 50, $CaCl_2.2H_2O$: 300, ATU: 10, $MgSO_4.7H_2O$: 50, $CaCl_2$: 50 and KH_2PO_4: 84.7. Potassium phosphate was supplied to the mineral solution, with a C:N:P ratio of 100:47:1.

After 38 days of operation using synthetic VFA mixture, the fFW was fed to the SBR as the final condition. A mineral medium supply with the same composition as used for the synthetic VFA mixture condition was provided to the bioreactor.

MMC PHA Accumulation Performance

Biopolymer accumulation assays were performed in an aerobic fed-batch reactor (BIOSTAT® Aplus), with a working volume of 1 L. Air flow rate was controlled at 1-1.5 L min^{-1} (vvm) by a mass flow controller and mixing was provided by mechanical stirring (300 rpms). For PHA accumulation tests, a biomass purge (1.5 L) was collected from the SBR at the end of the famine phase (i.e. 8h from cycle started). Before each accumulation experiment, the mixed-liquor biomass that was harvested from the SBR was resuspended in salt water (NaCl 30g L^{-1}) after decanting the supernatant, in a medium without nitrogen. In this manner, the potential for influence of any uncontrolled variation in nutrient carrier over from the enrichment SBR mixed liquor was mitigated. For each accumulation experiment, two reactors were operated in parallel with the same initial SBR biomass grab sample split into two (750 mL).

The accumulation assays were carried out at room temperature. pH was monitored (but left uncontrolled and ranging between 8 and 9). Accumulation experiments were performed by feeding the VFA-rich stream pulse-wise, controlled by DO. When DO increased, a new pulse of carbon source was fed. This procedure was repeated until no DO response was observed.

In the first set of two parallel accumulation tests (A1 and B1), one accumulation reactor was fed with a synthetic VFA mixture mimicking the VFA composition of the fermented fish waste, supplemented with NH_4Cl with a C:N:P ratio of 100:47:1 (mol) and the other one with the same VFA mixture without a nitrogen source 100:0:1. A second set of two parallel accumulation tests (A2 and B2) was conducted with a dialysed clarified fermented fish waste supplemented with NH_4Cl, with a C:N:P (mol) ratio of 100:47:1 and dialysed clarified fermented fish waste, with a C:N:P (mol) ratio of 100:0.4:1. Micronutrients were added to all prepared feedstocks (A1, B1, A2 and B2), in order to ensure the absence of other possible forms of growth limitation with the same composition used in the culture selection stage.

Analytical Methods

Monitoring the SBR was carried out after observing reactor stability, in terms of constant F/F and VSS concentration (three cycles were monitored in consecutive days) in order to assess biomass growth, substrates consumption and the PHA production. In the PHA accumulation fed-batch tests, samples were collected periodically in order to assess the substrates consumption and the PHA production during all the pulses.

TSS, TS, VSS and VS were determined according to the standard methods [15].

Ammonia and phosphate concentrations were determined in the filtered samples (0.2 μm) using a segmented continuous flow analyser (Skalar SNA^{++}).

Organic acids and ethanol were determined in filtered samples (0.2 μm) by high performance liquid chromatography (HPLC) using a VWR Hitachi Chromaster equipped with a RI detector 5450, Diode Array Detector 5230, Aminex HPX-87H 300x7.8MM column and Biorad 125-0129 30x4.6mm pre-column. Sulphuric acid (H_2SO_4 0.005 N) solution was used as eluent at a flow rate of 0.6 mL min^{-1} and 60°C of operating temperature. Concentrations were calculated through a standard calibration curve in the range of 25-1000 mg L^{-1} for each organic acid.

The total organic carbon (TOC) content was analysed in TOC-VCSH (Shimadzu) analyser by Total carbon (TC) analysis with a combustion catalytic oxidation at a temperature of 680 °C and high purity air as a carrier gas at a flow rate of 150 mL min^{-1}, and by Inorganic Carbon (IC) analysis that consists in a pre-acidification of the solution with hydrochloric acid (2M). The TOC values are obtained by subtracting the IC from TC (TOC = TC – IC).

Gas chromatography (GC) was performed for PHA quantification and characterisation using a protocol adapted from [16]. Briefly, 2 to 4 mg of lyophilised biomass were incubated with 1 mL of acidic methanol and chloroform (1 mL). The chloroform solution comprised heptadecanoate (HD) at around 1 g L^{-1}, which acted as an internal standard. The mixture was digested for 3.5 hours at 100 °C. The organic phase (methylated monomers dissolved in chlorophorm) was extracted and injected (2 μL) into a gas chromatograph (Trace 1300, Thermo Scientific), equipped with a Restek column (60 m, 0.53 mm internal diameter, 1 μm film thickness, Stabilwax). Helium was used as carrier gas at a flow rate of 1 mL min^{-1} and a constant pressure of 14.50 psi. Each run lasted 32 minutes and a volume of 2 μL of sample was injected in the equipment. The temperature started at 40 °C and rose at 20 °C min^{-1} up to 100 °C (3 min), followed by a period during which temperature rose up to 155 °C at 3 °C min^{-1} (18 min and 20 s), encompassing the elution times of the hydroxyalkanoate monomers. Lastly, temperature was raised up to 220 °C, again at 20 °C min^{-1} (3 min and 15 s), and kept for 7 more minutes for cleaning. Calibration standards were made using an Aldrich copolymer of P(3HB-co-3HV) containing 14% (HV) and 86% (HB) (%(w/w)) with concentrations between 0 and 6.3 g L^{-1}.

Intracellular PHA granules were identified using Nile Blue staining according to [17] Samples were taken and Nile Blue solution was added. After a period of incubation at 55 ºC for 20 minutes the sample was observed with epifluorescence microscope Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software).

Calculations

Acidogenic Fermentation The degree of acidification (DA) was the main parameter used to evaluate the acidogenic potential of the organic waste stream and it is considered to be the fraction of the organic matter converted into fermentation products (FP). It was determined using the following equation:

$$DA(\%) = \frac{[FP]}{[COD_{\text{total in}}]} \times 100 \quad (1)$$

where [FP] corresponds to the sum of the concentration of VFAs and ethanol in the reactor in gCOD L^{-1} and [$COD_{\text{total in}}$] corresponds to the total COD concentration in the feed in gCOD L^{-1}.

Organic acids profile (in %(Cmol-HA Cmol-$VFAs^{-1}$)) was obtained by fraction of each hydroxyl acid produced in relation to total VFAs concentration.

Culture Selection and PHA Accumulation Stages The performance of both SBR and accumulation reactors was characterised by calculation of relevant ic parameters. Feast/famine ratio (F/F, h h^{-1}) was calculated by dividing the time needed to consume all VFAs by the remaining time of the cycle. The PHA content in the biomass was determined in terms of percentage of VSS on a mass basis, considering VSS to be constituted by active biomass (X) and PHA. In this study, it was considered that VS was equal to VSS. Both ratios VS/TS and VSS/TSS were constant during the monitored period.

Active biomass was estimated by subtracting PHA from VSS, assumed to be represented by the molecular formula $CH_{1.8}O_{0.5}N_{0.2}S_{0.02}P_{0.02}$ [18], and based on phosphorous uptake curve, where 40 mg of active biomass were obtained from 1 mg of P. The PHA produced in each cycle (ΔPHA, % gPHA gVS^{-1}) was determined as the maximum PHA content (% gPHA gVS^{-1}) during the cycle, generally achieved at the end of the feast phase, minus the PHA content at the beginning of the feast phase. Specific VFAs consumption rates (-qVFAs, Cmol-VFAs Cmol-X^{-1} L^{-1}), specific PHA storage (q_{PHA}, Cmol-PHA Cmol-X^{-1} h^{-1}) and consumption (-q_{PHA}, Cmol-PHA Cmol-X^{-1} h^{-1} were obtained by adjusting linear functions to the experimental data for each variable concentration plotted over time, and calculating the first derivative at time zero. Storage and growth yields on substrate consumed ($Y_{PHA/S}$, Cmol-PHA Cmol-$VFAs^{-1}$ and $Y_{X/S}$, Cmol-X Cmol-$VFAs^{-1}$), respectively) were calculated by dividing q_{PHA} by -q_{VFAs} and q_X by -q_{VFAs}, respectively. In the accumulation assays, the specific rates and yields were calculated as described before, for each pulse. In order to compared the different accumulation experiments, the average values of each parameter on the first two pulses was considered.

Results and Discussion

Acidogenic Fermentation

FW was used as substrate in this study and it was mostly composed by peptides. The characterisation of the substrate used in this work is presented in Table 2.

Suspended Solids and Biomass Settling Properties The incorporation of a settling unit was necessary for the effective removal of solids from the CSTR effluent, so that solids did not proceed to the selector and accumulator reactors, diluting the eventual PHA content of the biomass. It should be noted that the dilution of PHA content and increasing downstream processing costs.

As a first approach the retained solids in the settler were recycled to the CSTR to promote higher substrate solubil-

Table 2: Characterisation of the fish waste treated in the acidogenic fermentation reactor.

Parameter	Value
pH	4.5
VSS/TSS (%)	88.5±0.04
Total COD ($gCOD_T$ gFW^{-1})	0.98±0.02
Soluble COD ($gCOD_S$ gFW^{-1})	0.84±0.03
Chloride ($mgCl^-$ gFW^{-1})	33.26
Total nitrogen (mgTNb gFW^{-1})	95.72
NH_4^+-N (mgN L^{-1})	7.4
PO_4^{3-}-P (mgP L^{-1})	11.8

isation and VFA production (conditions I, II and III). By means of a TSS balance at the settler different percentages of solids removal were achieved for each condition: I (not measured); II (95%); III (89%), IV (75%) and V (70%). As the OLR increased, a depletion in the settler capacity to remove solids at initial defined flow rate was observed. Thus, the new approach was followed by stopping solids recirculation into the CSTR (conditions IV and V). COD analyses to solids samples indicated that around 60% were solids and about 90% of it were organic matter. Hence, another approach would be to allow the collected solids to be reused for new biological treatments (e.g. biogas production, composting), thus promoting a higher efficiency of the treatment process.

As mentioned above, an ultrafiltration module was used to obtain a filtered VFA-rich stream for Donnan dialysis experiments and PHA accumulation assays. The characterisation of the fermented effluent after filtration indicated a volumetric recovery of filtrate of 75%, with TSS removal of 55% in the filtrate. Soluble COD, VFAs, ammonia and phosphorous concentrations were not affected by the filtration at all.

Fermentation Profile and Acidification Degree For an organic load of 5 gFW L^{-1} d^{-1}, at different pH values (i.e. condition I, II and III), total VFAs concentration remained approximately constant. However, changes in organic acids profile could be noticed. In the first condition at pH 7, acetic and propionic acids were the main FP, accounting with 65% (Cmol basis) of total VFAs concentration. Moreover, it was observed a low concentration of iso-valerate, butyrate and valerate of 25±4%, 8±7% and 2.5±0.4% (Cmol basis), respectively. This was probably due to their easy biodegradation to form acetic acid, which was subsequently utilised by methanogens, confirmed by their high activity under these conditions. Through the second phase, at pH 4.5, acetic acid remained the primary product with 36±6% (Cmol basis) of the total FP. However, a significant increase in butyric acid proportion was observed, reaching 24±1% (Cmol basis) of total FP. In the third condition, at pH 6, acetic and butyric acids remained the major products, but with the appearance of hexanoic acid, reaching 3±2% of total VFAs concentration. These results are in agreement with those observed by Jankowska *et al.* (2017) [19], where under acidic conditions, FP composition consists mostly on acetic and butyric acids, and acetic and propionic acids under neutral conditions. Jiang *et al.* (2013) [20] have also reported that butyric acid from food waste was dominant under treatment with pH 6.0-7.0, whereas acetic acid production was promoted under treatment with pH 5.0. Other scientific reports have shown different results suggesting that the production on specific VFAs and distribution of VFAs depend not only on pH but on the type of substrates as well. Indeed, biodegradation of peptides present in the FW could also make a slight contribution to butyrate, acetate and propionate composition. Moreover, the use of anaerobic granular sludge can also explain the spectrum of FP obtained, by resulting in a complex combination of metabolic pathways [21].

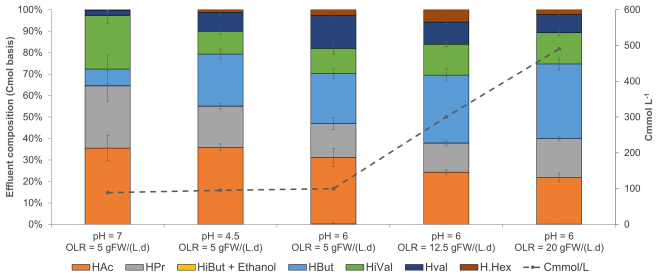

Figure 2: Average effluent composition in terms of FP profiles obtained for each condition tested in the acidogenic reactor (conditions presented in chronological order). Error bars represent one standard deviation.

In this study, FP profiles were similar for different OLRs at the same pH. For higher OLRs (12.5 and 20 gFW L^{-1} d^{-1}) the dominant FP were butyric and acetic acids in the ranges of 32-35% and 24-21%, respectively, and the sum of produced butyric and acetic acids was around 56% of the total VFA. In addition, with the increase of OLR from 5 to 12.5 gFW L^{-1} d^{-1}, the content of butyric acid increased up to 32% as well as for caproic acid (6%). Despite of these differences, a similarity between different OLRs was observed suggesting that OLR did not have relevant effect on the final acids distribution (at least in the explored range). These findings imply that pH may significantly control the fermentation profile of the effluent, bringing advantages in controlling PHA composition.

The maximum VFAs concentration was reached at the highest OLR (20 gFW L^{-1} d^{-1}, pH 6.0), as expected, since the quantity of organic matter subjected to fermentation was higher. Other studies have also reported maximum VFAs concentration at pH 6.0 derived from the optimal activities of hydrolytic enzymes at this pH.

The degree of acidification, defined as the capacity of the system to convert acids from a substrate degradation, did not suffer significant changes with organic load, pH and absence/presence of recirculation. A global DA of 36% was obtained for all operating conditions. Recirculation of solids, worked very well despite resulting in relatively low ratios of VFAs to soluble COD in the fermentation broth (data not shown). Solids recirculation may have resulted in a worse mass transfer due to solids accumulation in the mixed liquor of the CSTR. Regarding pH changes, an increase in acidification degree was expected with the rise in pH 4.5 to 6.0 (condition III to IV). However, this was not observed, probably due to the effects of recirculation.

The positive effect of higher pH on VFA production was further evident from the VFAs/sCOD ratio, which represents the proportion of solubilised material transformed to VFAs. Higher ratios were obtained for condition I (pH 7) with 69% and V (pH 6) with 67% (II: 40%, III: 22% and IV:56%). These findings confirm the optimum pH range for solubilisation and acidification of this substrate.

Donnan Dialysis

Three series of runs (A, B and C) were carried out enabling study of the ics of ammonium Donnan dialysis. In series A, the effect of varying the initial concentration of NaCl in the range 10 to 30 g L^{-1} and permeability of the selected membrane for the VFAs were investigated at a constant initial feed concentration of 10 Nmmol L^{-1} and 20 Cmmol L^{-1}. In Series B, the effect of varying volume ratio (Receive:Feed) was investigated. And finally, Series C consisted of studying the effect of varying the initial NaCl concentrations over the range of 60 to 300 g L^{-1}, using an initial ammonium concentration of 300 NmM.

Table 3 gathers the average values of maximum removal of NH_4^+ (%) and average removal rates when reaching a plateau for each studied NaCl concentration for series A and B.

Concerning Series A, it was observed over time that ammonium removal from the feed phase increased almost linearly for the first 10 hours, while a plateau was reached at around 40 hours of experiment running (data not shown). Results suggest that the flux of ammonium increases with higher NaCl concentration. The reason for this described phenomenon is the higher concentration gradient of counter-ions (Na^+) that results in high counter-ion flux to the feeding solution causing an increase in the flux of removed ion [22].

Moving on to series B, it was observed a higher transfer flux and shorter time necessary to reach equilibrium when compared to the tests using the same a volume ratio of 1:1 (Table 3). This may be an indication that the concentration gradient changes with the volume ratio, as well as the time required to achieve the equilibrium concentration of the exchanged ion (NH_4^+). Theoretically, under such conditions (when the volume of the receive is few times larger than the volume of the feed), the concentration gradient of sodium ions increases, and at the same time the driving force of the Donnan dialysis becomes stronger. The amount of ammonium ions transferred to the receiver is respectively large because of significant dilution in the receiving solution. Simultaneously, there is transport of stechiometrically equal amount of Na^+ ions to the feed, where these ions are concentrated. In such circumstances, the concentration gradient of NH_4^+ is higher and the equilibrium concentration of NH_4^+ ions in the receive is lower, accelerating the process. As the result of this phenomenon, the efficiency of ion removal is higher in the system with greater volume of the receiving solution. These findings were also reported but in contrary approach, where Wisniewski *et al.* (2005) [23] concluded that the increase in volume of the feeding solution resulted in lower efficiency of ion removal.

Organic acids were not found in receiver compartment (data not shown), indicating a total impermeability of the membrane.

The attained NH_4^+ recovery rate, higher than 90% (Series A and B), suggests the potential application of this process to reduce the N-content of a stream with NH_4^+ concentrations up to 10 NmM (e.g. domestic wastewater, pig manure digestate and urine-containing wastewater), at low cost and salt consumptions [24; 25; 26].

Given the high ammonium content of the fermented fish waste, different expenses of driving ion as sodium were expected when compared to tests with ammonium concentrations up to 10 NmM (series A and B). Therefore, to investigate the feasibility of applying Donnan dialysis to recover the NH_4^+ from ammonia-rich streams, the feed phase was enriched with NH_4Cl at 300 NmM to simulate acidogenic reactor's effluent (fFW) (Series C - Test 8, 9 and 10).

Results obtained from these tests are in line with those obtained in the series A, as expected. Figure 3 shows that higher sodium concentration in the receiver container can offer stronger electrochemical potential differences, as explained above.

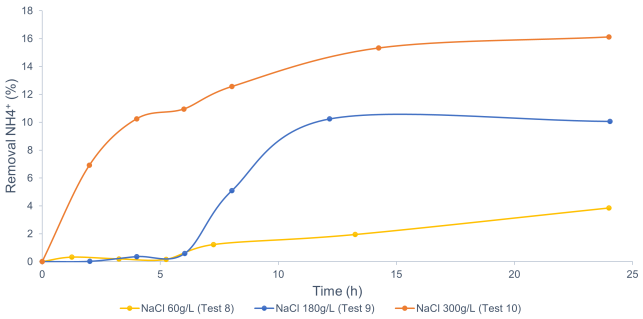

Figure 3: NH_4^+ removal (%) over time at different initial concentrations of NaCl in the receive phase. Feeding phase solution of $[NH_4Cl]$ = 300 NmM, receiving phase solution of [NaCl] = 60 (Test 8), 180 (Test 9) and 300 (Test 10) g L^{-1}, represented by yellow, blue and orange lines, respectively.

The highest NH_4^+ removal of 16%, was achieved in 24 hours with the highest NaCl concentration studied (300 g L^{-1}). According to these results, a total removal of NH_4^+ would be expected in about 6 days with exchanges of the receive phase every 24 hours. These results suggest that, although higher concentrations of NaCl enhance the removal rate of ammonium, there are still some limitations to overcome in laboratory scale studies, in particular, long exchange time.

Clarified fFW was latter subjected to an ammonium removal process. However, since the optimal parameters using NaCl as receiver phase were not determined, it was necessary to follow a previously tested approach using a solution of HCl 0.25M, which ensured the NH_4^+ removal. The process of removing ammonia from 2 L of fFW was achieved (99.3%) in 20 days with 5 exchanges of the receiving solution (10 L of HCl 0.25M).

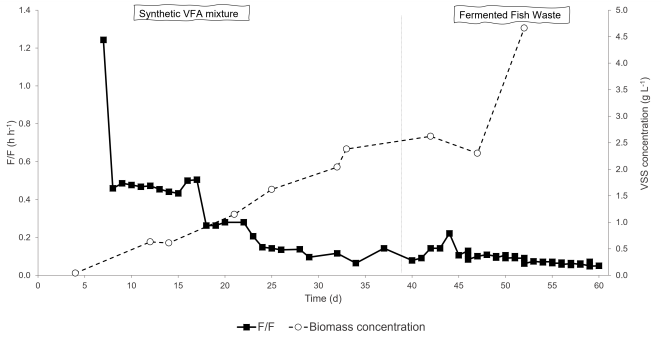

Figure 4: Evolution of F/F ratio and biomass concentration (gVSS L^{-1}), along the SBR operational period, operated operated with both feedstocks: Period I - synthethic VFAs mixture; and Period II - fermented fish waste.

MMC Enrichment

The F/F ratio was regularly measured and used to monitor biomass adaptation and process stability (Figure 4). It is known that low F/F values, <0.2, ensure physiological adaptation of the microorganisms, causing an internal growth limitation and favouring PHAs storage [27]. During the operation using a synthetic mixture, F/F value decreased from 1.2 to 0.1, stabilising at around day 32.

After inoculum, the VSS concentration increased substantially, then stabilised at an average value of around 2.5 gVSS L^{-1} on the 32nd day.

Regarding period I, it was observed a sudden decrease in the DO concentration in correspondence to the start of the influent filling, due to the increase of the metabolic activity of the biomass in the presence of the external carbon and other nutrients. From the moment of influent filling, cells had all nutrients available for growth since ammonium and mineral solutions were coupled to the carbon source. Along the feast period, butyric acid was consumed preferentially with an uptake rate of 0.29 Cmmol L^{-1} h^{-1}. While acetic, valeric and propionic acids were consumed at 0.076, 0.071 and 0.056 Cmmol L^{-1} h^{-1}, respectively.

The end of the feast period and the beginning of the famine period was easily identified by the sudden DO increase, when all VFAs were exhausted. Together with the end of the feast phase a maximum PHA content of 12.3% gPHA gVS^{-1} was obtained at the end the feast phase. Despite the presence of intracellular PHAs at the beginning of the cycle (3.3% gPHA gVS^{-1}), the MMC was able to store more PHA during the cycle. In this cycle, the ΔPHA was 8.7% (gPHA gVS^{-1}) and a $Y_{PHA/S}$ of 0.57 Cmol-PHA Cmol-VFAs^{-1} was obtained. The monomeric composition of the produced copolymer was uniform throughout the cycle with 75:25 (3HB:3HV, Cmol basis).

After 38 days of operation, the SBR feedstock was shifted to fermented fish waste, still operating at 60 Cmmol L^{-1} d^{-1}. The feast phase length destabilised, reaching values of F/F up to 0.2. However, after 19 days of operation using fFW, the value stabilised again at around 23 min, corresponding to an average F/F ratio of 0.05.

In the feast phase, DO rise partially divided into two stages, likely due to the complexity of the feedstock. It is worth mentioning that total organic carbon coincided with VFAs uptake during the feast phase of the SBR cycle (40 minutes) (depletion of VFAs observed by HPLC) with VFAs accounting for 67-69% of the soluble TOC. It should also be noted that peaks with longer retention times were observed in the HPLC analysis of fFW, however they were not identified or considered (data not shown). Moreover, from the HPLC analysis of the SBR cycle sampling, the consumption of these unidentified peaks (longer-chain VFAs) was detected along the feast phase. After VFAs exhaustion, it was observed a consumption of about more 11% TOC during 10 minutes. The initial rapid increase in the DO concentration was due to the consumption of the readily biodegradable COD, mainly consisting of VFAs; whereas the subsequent increase due to the remaining soluble TOC. Based on these findings, it is hypothesised that part the organic carbon consumed right after the known and unknown VFAs (end of the feast phase) could be associated to biodegradable non-VFAs, mainly amino acids, which could be used as carbon sources for non-PHA-storing bacteria to survive, thereby wakening the selective pressure for PHA-storing MMC enrichment [28]. On the whole, only 80% of the total organic carbon was consumed. After 80% of organic carbon consumption (50 minutes), soluble TOC remained approximately constant during the rest of the cycle, suggesting that not all non-VFAs fractions could be used by bacteria, despite its biodegradability [29]. Other studies have also reported the stepwise increase of the DO due to the complexity of the feedstock and, consequently, the existence of undesired populations leading to a reduction of the overall production yield [30; 31; 32].

Concerning VFAs specific uptake preference during the feast phase, it was observed uptake rates of 0.2, 0.09, 0.03, 0.025, 0.020 and 0.003 Cmmol L^{-1} h^{-1} for butyric, propionic, acetic, valeric, iso-valeric and hexanoic acids, respectively.

Table 3: Average values regarding Series A and B: Maximum NH_4^+ removal (%), removal rates and removal rates per area until reaching a plateau.

Series	NaCl (g L^{-1})	Volume ratio [Feed:Receive]	NH_4^+ Removal (%)	Removal rate (Removal (%) h^{-1})	Removal rate per area (Removal (%) h^{-1} m^{-2})
A	**10**	1:1	83	3.4	871.8
	20		87	3.9	1000.0
	30		87	4.5	1153.8
B	**30**	1:2	91	5.6	1435.9

Removal rates were determined for the first 10 hours of the experiment (until reaching a plateau).

Table 4: Average performance of the enriched MMC in the selection reactor (main parameters monitored in the SBR runs), operated at 60 Cmmol L^{-1} d^{-1}, using two different influent substrates (synthetic VFA mixture (period I) and fermented fish waste (period II)).

	Synthetic VFA mixture	**fFW**
F/F ratio	0.1	0.05
PHA_{max} (% gPHA gVS^{-1})	12.3±0.25	13.7
Δ PHA (% gPHA gVS^{-1})	8.2±0.46	8.1
$Y_{PHA/S}$ (Cmol-PHA Cmol-$VFAs^{-1}$)	0.57±0.020	0.56
$Y_{X/S}$ (Cmol-PHA Cmol-$VFAs^{-1}$)	0.15±0.0050	0.22
q_{PHA} (Cmol-PHA Cmol-X^{-1} h^{-1})	0.28±0.010	0.23
$-q_{VFAs}$ (Cmol-VFAs Cmol-X^{-1} h^{-1})	0.50±0.035	0.41
HB:HV ratio (Cmol basis)	75:25	80:20
VS/TS (%)	77.4±0.00	71.2

Average values were determined using data of three days (the data from one day was an outlier and was neglected); For fermented fish waste: data from two days (data from one day was an outlier and was neglected).

In this case, overall growth occurred at higher extent when compared with the previous period using synthetic mixture, reaching a VSS concentration of 4 gVSS L^{-1} in the most stable phase of the reactor. This fact indicates that, besides phosphate, another source of phosphorous could be being used for growth, a hypothesis mentioned above.

By the end of the feast phase, the selected culture was able to store PHA, accumulating 13.7% (gPHA gVS^{-1}), during the cycle, with a storage yield of 0.56 Cmol-PHA Cmol-$VFAs^{-1}$ and a specific storage rate of 0.23 Cmol-PHA Cmol-X^{-1} h^{-1} (Table 4).

Interestingly, it appears that ic and stoichiometric parameters were not significantly affected by using real fermented fish waste instead of the synthetic mixture mimicking fFW. No significant variations were reported in terms of maximum PHA content and PHA yield on substrate between the synthetic and fermented feedstock. Slightly differences in growth yields and PHA composition are related to the presence of unknown residual organic fraction besides VFAs. Lower specific substrate uptake rates when using fFW were expected due to the complexity of the substrate, containing longer-chains VFAs and other potentially available carbon sources to metabolise. The difference of F/F ratio among the two periods of SBR operation was attributed to the biomass concentration, as a consequence of the slightly different influent concentration.

The selected PHA-storing MMC preference to consume butyric acid over acetic, propionic and valeric was exhibited in both periods. This preference was also noted in several other studies using different feedstock (e.g. Kourmentza *et al.* (2016) [33] and Marang *et al.* (2013) [34]), suggesting that butyrate may be a more appropriate feedstock for PHA production.

All, these results suggest that fermented fish waste matrix does not introduce significant variation on the enrichment culture, confirming that the reactor performance and microbial culture were not affected by the substrate shift, showing the robusteness of the microbial culture.

Staining with Nile blue confirmed that most of the population accumulated PHA under that conditions (data not shown). These observations coupled with the low feast and famine ratio observed suggest an efficient culture enrichment in PHA-producing microorganisms.

PHA Accumulation

PHA accumulation fed-batch experiments were performed to determine the influence of the ammonium presence over the maximum PHA storage capacity of the mixed culture in both periods: synthetic VFA mixture (period I) and fermented fish waste (period II). For this aim, two different conditions were applied in parallel tests: nitrogen-excess (A) and nitrogen-limitation (B) conditions.

From the results obtained, presented in Table 5, it can be found that biomass from different periods of SBR operation showed different PHA production capacities. This observation may be an indication that the enrichment with a more complex waste stream as fFW may have affected the physiologic response of the culture, by reducing the selective pressure in the enrichment stage. Similarly to that obtained in the selection stage, produced PHA with the synthetic mixture and fFW showed equal compositions in terms of HV content in both tests (with and without nitrogen source). This was expected due to its similarity in the main organic acids profile.

The maximum PHA content achieved in both experiments was similar to the results obtained in other studies using synthetic VFA mixtures, for example 48 wt% [35], 50 wt% [36] and 52 wt% [4]. As for fFW, there is no report in the literature for performance of PHA production using MMC. Nevertheless, high yields and storage rates were obtained under different nitrogen content, when comparing with previous studies using real complex waste streams.

Concerning PHA accumulation tests using fFW, it was observed that enriched cultures tended to saturate its PHA storage capacity earlier in the limitation of nutrients than with its presence. It makes sense once, in the presence of nutrients, new biomass could be continuously formed and thus, extending time to reach the storage saturation. In view of, it would have been interesting to extend these tests to investigate when it would be the proper time to stop PHA accumulation in order to get the maximum PHA productivity.

Despite different ammonium content, the enriched cultures demonstrated high PHA storage capacity with both feedstocks, producing a polymer-content in biomass higher than 37% (gPHA gVS^{-1}), reaching storage yields higher than 0.50 Cmol-PHA Cmol-FP^{-1}, and high specific storage rates (qPHA) (between 0.14 and 0.31 Cmol-PHA Cmol-X^{-1} h^{-1}) (Table 5).

Increase in PHA storage rates was observed under nitrogen limitation, using both feedstocks. This clearly indicates that the imposed limitation, the absence of growth, increased storage rates with respect to the internal one (F/F conditions). Moreover, specific PHA production rates were lower when ammonium was present, while growth rates were higher, leading to a dilution of the cellular PHA content by the formation of new biomass. Higher volumet-

ric PHA productivities were obtained under ammonium limitation conditions.

It is generally assumed that nutrient absence is the best strategy to enhance PHA accumulation. The results of this research confirmed these assumptions, showing that the presence of nitrogen had a negative effect on the PHA accumulation performance.

Clearly, many factors can affect the enriched culture response to nitrogen availability and can be therefore the reason for the different behaviour observed. Substrate's composition and operating conditions imposed to the reactor are likely the main factors to influence this response. In addition, future work could rely on understanding how these factors in combination with nutrient availability could affect biomass response and, consequently optimise nutrient level to maximise PHA production.

In summary, the ammonium limitation was shown to be an effective strategy for enhancing higher PHA productivities.

Conclusions and Perspectives

In the present work, a three-stage process was implemented for PHA production using MMC. In the first step, fish waste was submitted to acidogenic fermentation in order to produce a VFA-rich stream. The concentrations of VFAs, sCOD, and NH_4^+ increased as OLR increased. pH values within the range of 6.0-7.0 appeared to enhance the potential of fish waste to be used as a substrate for production of PHA. The incorporation of a settling unit resulted in an efficient effluent clarification. However, solids recirculation resulted in lower VFAs productivities, suggesting that it would be more advantageous to remove solids from the settling unit and take advantage of them through parallel biological treatment. In future work, it would be interesting to test even lower HRTs, higher OLRs and optimise pH range to maximise the amount of treated waste.

Concurrently, ammonium was efficiently removed from fermented fish waste through Donnan dialysis, however with a different driving ion (H^+) than initially planned (Na^+). Concerning synthetic experiments, more characterisation studies must be performed to evaluate: (1) the most suitable membrane when using saline solutions; (2) how many receiver content exchanges would be necessary for a faster process; (3) the influence of NH_4^+ transport in the presence of other cations; (4) effect of volumes ratio on the transport rate. Finally, to better understand the implications of these results, future studies should focus on the implementation of the process using a real complex waste stream.

This study demonstrated the feasibility of using NaCl concentrations mimicking seawater to acclimatise a halotolerant MMC able to store PHA in the continuous presence of ammonium. Finally, the ability of the selected MMC to accumulate PHA under different ammonium content was tested. The results obtained led to the conclusion that PHA production is best performed under conditions of nitrogen limitation using both feedstocks. Nitrogen limitation led to the highest PHA contents, which is important for an efficient downstream processing. Moreover, PHA production rates and yields were higher when compared to nitrogen excess conditions.

Even though the overall steps still require further optimisation, these findings pinpoint the great potential of using Donnan dialysis to remove and recover ammonium when treating nitrogenous complex waste streams aiming to enhance PHA production.

Acknowledgements

This document was written and made publicly available as an institutional academic requirement and as a part of the evaluation of the MSc thesis in Biological Engineering of the author at Instituto Superior Técnico. The work presented in this thesis was performed at Biochemical Engineering research group of Faculdade de Ciências e Tecnologias, Universidade Nova de Lisboa, during the period February-November 2020, under the supervision of Doctor Bruno Marreiros and Doctor Mónica Carvalheira. The thesis was co-supervised at Instituto Superior Técnico by Doctor Teresa Cesário.

Table 5: Overview of fed-batch PHA-accumulation assays with the MMC selected on both periods using two different feedstocks (synthetic and fermented fish waste). Overall F/M of 1.5, with 30 Cmmol L^{-1} (average) per pulse.

	Synthetic VFA mixture		Fermented Fish Waste	
	NH_4^+excess	NH_4^+ starvation	NH_4^+ excess	NH_4^+ limitation
Accumulation assay length (hours)	4.3	4.1	4.9	3.3
Maximum PHA (wt.% gPHA gTS^{-1})	29.0	36.1	23.6	26.6
Maximum PHA (% gPHA gVS^{-1})	40.2	49.5	33.8[(a)]	32.5[(b)]
Δ PHA (% gPHA gVS^{-1})	36.7	45.5	31.1	31.3
$Y_{PHA/S}$ (Cmol-PHA Cmol-$VFAs^{-1}$)	0.64±0.12	0.78±0.09	0.50±0.04	0.43±0.11
$Y_{X/S}$ (Cmol-X Cmol-$VFAs^{-1}$)	0.24±0.10	0.00±0.00	0.10±0.01	0.09±0.05
q_{PHA} (Cmol-PHA Cmol-X^{-1} h^{-1})	0.24±0.04	0.31±0.01	0.14±0.00	0.21±0.09
$-q_{VFAs}$ (Cmol-VFAs Cmol-$X^{-1}h^{-1}$)	0.38±0.01	0.40±0.06	0.24±0.03	0.47±0.09
HB:HV ratio (Cmol basis)	75:25	75:25	74:26	75:25
Productivity (Cmmol-PHA L^{-1} h^{-1})	16.8	18.5	11.5	16.1

PHA accumulation assays with fermented fish waste were performed by feeding a total of 5 and 6 pulses (for NH_4^+ excess and limitation experiments, respectively). For reasons of comparison with the results of the tests using synthetic mixture, the values for fFW (NH_4^+ excess and limitation) are presented up to the 4th pulse.
(a) Maximum PHA (gPHA gVS^{-1}) of 37.3% for a total of 5 pulses; (b) Maximum PHA (gPHA gVS^{-1}) of 38.3% for a total of 6 pulses.

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