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Polyhydroxyalkanoates production from cheese whey under near-seawater salinity conditions

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ABSTRACT

Treating saline streams presents considerable challenges due to their adverse effects on conventional biological processes, thereby leading to increased expenses in managing those side streams. With this in consideration, this study explores into the potential for valorizing fermented cheese whey (CW), a by-product of the dairy industry, into polyhydroxyalkanoates (PHA) using mixed microbial cultures (MMC) under conditions of near-seawater salinity (30 g_{NaCl}/L). The selection of a PHA-accumulating MMC was successfully achieved using a sequential batch reactor operated under a feast and famine regime, with a hydraulic retention time of 14.5 h, a variable solids retention time of 3 and 4.5 days, and an organic loading rate (OLR) of 60 Cmmol/(L d). The selected culture demonstrated efficient PHA production rates and yields, maintaining robust performance even under high salinity conditions. During PHA accumulation, a maximum PHA content in biomass of 56.4 % wt. was achieved for a copolymer P(3HB-co-3HHx) with a 3HHx content of 7 %. Additionally, to asses the capacity of the culture to produce polymers with different compositions, valeric acid was supplemented to the real fermented feedstock which resulted in the production of terpolymers P(3HB-co-3HV-co-3HHx) with varied monomeric content and a higher maximum PHA content of 62 % wt. Additionally, this study highlights the potential utilization of seawater as alternative to freshwater for PHA production, thereby enhancing circular economy principles and promoting environmental sustainability.

1. Introduction

Sustainable industrial practices such as circular economy are of paramount importance today to reduce the environmental impact of human activity. Resource recovery from waste streams is strongly encouraged to reduce raw material extraction and processing as well as to limit the impact of waste disposal [1]. The agri-food sector is a relevant source of organic side streams that can be positively affected by resource recovery and circularity principles and applications [2].

Saline side streams generated by agri-food industries require adequate treatment for safe disposal. However, the high saline concentration inhibits conventional biological processes, making their treatment more expensive [3,4]. Among others, fishery and dairy industries are a relevant source of saline side streams [4,5], having a worldwide market volume of € 570 billion with a compound annual growth rate (CAGR) of 6.23 % (2023–2027) [6].

Worldwide, cheese production has reached 22 million tonnes [7], making its by-product, cheese whey (CW), a substantial resource in need of sustainable management [8]. The characteristics of cheese whey (CW) vary depending on the milk source, cheese-making processes, and other factors, leading to three main categories: acidic, sweet, and salty. Of these, salty whey has the most limited utility in the industry due to its high salinity [9].

Developing biological processes capable of valorizing and treating saline side streams and CW would favor environmental and economic sustainability in the agri-food industry. Despite this potential, the utilization of salty CW for Polyhydroxyalkanoates (PHA) production remains unexplored.

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PHA are polymers produced by microorganisms primarily as intracellular carbon and energy reserves. Additionally, recent studies have highlighted their role as stress protectants, particularly under conditions of extreme temperatures, oxidative and osmotic stress, and UV radiation exposure [10,11]. PHA are biobased, fully biodegradable, and exhibit excellent biocompatibility along with a wide range of physicochemical characteristics similar to those found in petroleum-based plastics, making it a viable substitute [12]. The properties of PHAs heavily rely on the monomer composition of the polymers. Depending on the side chain length, PHAs are categorized into three groups: short-chain-length (scl-PHAs), consisting of monomers with 3 −5 carbon units; medium-chain-length PHAs (mcl-PHAs), composed of monomers with 6–14 carbon units; and long-chain-length (lcl-PHAs), comprised of more than 14 carbon units [13]. The versatility of PHA allows its application across various sectors, including agriculture [14], animal feed [15], biomedical [16], fabrics and coatings [17].

The production of these biopolymers is of growing interest, as the worldwide market value is projected to increase from € 85 million in 2023 to € 178 million in 2028, at a CAGR of 15.9 % [18]. Nevertheless, the production process of PHAs still incurs high costs, rendering them more expensive than conventional plastics. This limitation hampers the broader commercialization and industrialization of PHAs [12].

PHA production by open mixed microbial cultures (MMC) reduces production costs as it enables the utilization of inexpensive feedstocks such as industrial by-products or waste streams, as mixed cultures are more amenable to dealing with complex matrices. Furthermore, they do not require aseptic conditions, thus reducing process energy costs. In this context, saline conditions may be applied when utilizing salty side streams as a substrate or as a consequence of using seawater instead of freshwater as process water. Reducing freshwater consumption is critical to enhancing the overall economic and environmental sustainability of the process.

PHA production by MMC is usually carried out through three distinct stages: (i) Acidogenic fermentation, where organic matter is converted into volatile fatty acids (VFAs) serving as precursors for PHA production. (ii) Culture selection, where the mixed culture is enriched with PHA-accumulating organisms by applying at least one selective pressure, such as feast and famine (F/f) regime. (iii) PHA production, during which PHA accumulation is performed to reach the maximum capacity of the culture selected in stage (ii), which is fed with the fermented stream produced in stage (i).

The selection stage is critical since the culture's stability is essential for long-term operation [19]. Typically, the primary selection driver is the F/f regime, which involves short periods of providing the exogenous carbon source to the culture followed by extended periods of carbon unavailability. This strategy enriches the culture with PHA-accumulating organisms capable of surviving the starvation period by utilizing PHA as a carbon and energy reserve [20]. Furthermore, uncoupling the feeding of the carbon source from the nitrogen source also promotes a more efficient selection for PHA-accumulating organisms, thereby acting as additional selection pressure [20,21].

Current knowledge about PHA production using mixed cultures under saline conditions remains limited, with only few studies exploring PHA production from saline organic resources. These studies investigated a variety of saline side streams, including pre-acidified cooked mussel processing wastewater [22,23], fermented tuna processing wastewater [24], fish-canning wastewater [25], waste fermentation leachate [26], industrial waste fish oil streams [27], and saline mixtures of VFAs [24,26,28,29], aiming to assess the impact of different salinity and substrates composition in the PHA production performance and polymer composition.

In these studies, it is noteworthy that culture enrichment was conducted under different operational conditions and transient salinities, resulting in PHA-accumulating cultures composed of diverse microbial communities. This diversity led to varying results: Palmeiro-Sánchez *et al.* observed a sharp drop in PHA storage when a mixed culture adapted to 3.35 g_{NaCl}/L was tested under higher salt concentrations (51–53 % wt. without NaCl vs. 8–9 % wt. at ~20 g_{NaCl}/L), with a half maximal inhibitory concentration (IC50) value near 7 g_{NaCl}/L. [24,29]. Conversely, Wen *et al.* found higher PHA storage (50.5 % wt.) with 5 g_{NaCl}/L compared to 10.0 g_{NaCl}/L, 15.0 g_{NaCl}/L, or no salt [26]. Carvalho *et al.* demonstrated that a halotolerant culture could accumulate PHA up to 84.1 % wt. under 30 g_{NaCl}/L [28].

In all these studies, copolymers of P(3HB-co-3HV) were produced with varying monomeric ratios. The 3HB:3HV ratio was observed to be influenced by factors such as substrate composition, NaCl concentration (resulting in an increase in the 3HB content with rising NaCl levels), and operational conditions, including the settling stage position in the F/f cycle and OLR increase, leading to an increase in the 3HV content [23,24,28].

In the present study, the valorization of salt-fermented cheese whey, a by-product from the dairy industry, into PHA using MMC is targeted. The primary challenges included enriching an efficient PHA-accumulating MMC and achieving substantial PHA accumulation capacity under conditions of near-seawater salinity (30 g_{NaCl}/L, the highest reported in the literature for MMC) with a real fermented feedstock. Building upon the knowledge gained from the study by Carvalho et al. [28], further process optimization was targeted for the selection stage taking into consideration constraints posed by the use of a real fermented stream. Insights into the tuning potential of the PHA polymer composition of the selected MMC was tackled by supplementing valeric or caproic acid to tune PHA polymer properties.

Overall, this study pioneers sustainability and circularity efforts in the agri-food industry by valorizing CW for PHA production under saline conditions. Additionally, it demonstrates the potential utilization of seawater as a viable alternative to freshwater for PHA production. Furthermore, it marks the first demonstration, to our knowledge, of copolymer production incorporating 3-hydroxyhexanoate (3HHx) using MMC under saline conditions. This research underscores the crucial role of biopolymers in facilitating a transition to a more circular and resource-efficient economy.

2. Materials and methods

2.1. Feedstock

The fermented stream used in this study was obtained by acidogenic fermentation of CW using an upflow anaerobic sludge blanket (UASB) reactor, which was inoculated with anaerobic granular sludge sampled from a brewery waste treatment facility (Porto, Portugal). The CW was sourced from Lactogal (Portugal) and consists of lactose (78.4 % wt.), proteins (13.6 % wt.), and fats (1.2 % wt.). The acidogenic fermentation of CW was not the focus of this study; therefore, no data will be presented on this stage. Additional information on the acidogenic fermentation of CW can be found in [30]. The detailed composition of the fermented streams, including fermentation products (FP) such as volatile fatty acids (VFAs) and ethanol, along with nutrient information, is provided in Table 1.

2.2. Culture selection

A sequencing batch reactor (SBR) with a working volume of 2 L was used for MMC selection. The SBR was inoculated with sediments collected from a saline area of Tagus river (Samouco, Portugal 38°43'50.739'' N, −9°0'29.947'' E) as reported by Carvalho et al. [28]. The SBR was operated under aerobic conditions, where the air was supplied through fine bubble diffusers keeping the dissolved oxygen (DO) as not limited in the reactor. Each F/f cycle lasted 8 h (480 min) and consisted of 6 steps: (i) influent feeding (5 min); (ii) aeration and agitation over 433 min (feast and famine periods); (iii) purge of excess biomass (3 min); (iv) nitrogen supplementation (2 min); (v) settling (30 min); and (vi) supernatant withdrawal (7 min).

Table 1
Fermentation products profile of the fermented cheese whey.

	Parameter [unit]		Selection	ACC-A	ACC-B	ACC-C
Fermentation products	HAc	[% Cmol]	32 ± 3	32	22	24
		[% sCOD]	27 ± 3	28	18	10
	HBu	[% Cmol]	57 ± 3	57	42	44
		[% sCOD]	61 ± 4	61	44	23
	HVa	[% Cmol]	0 ± 0	0	28	0
		[% sCOD]	0 ± 0	0	30	0
	HCa	[% Cmol]	8 ± 1	9	6	26
		[% sCOD]	9 ± 1	10	6	14
	EtOH	[% Cmol]	2 ± 1	0	0	5
		[% sCOD]	2 ± 1	0	0	3
	Sum	[Cmmol/L]	351 ± 11	370	345	226
		[g_{SCOD}/L]	13.3 ± 0.4	14.0	13.4	8.9
Nutrients	Ammonia	[Nmmol/L]	3.4 ± 0.3	4.3	2.8	2.4
	Phosphate	[Pmmol/L]	5.8 ± 0.2	6.4	4.6	5.4

The SBR was controlled at room temperature (19–21 °C), stirred at 150 rpm using a one-blade impeller and at pH 8.5 ± 0.5 through automatic dosing of 0.5 M HCl. Dissolved oxygen (DO) concentration and pH were monitored in real-time. The OLR was set at 60 Cmmol/(L d) (2.3 gCOD/(L d)).

Together with the fermented CW (Table 1), a salt-mineral solution (30 g_{NaCl}/L) was fed to the reactor. This mineral solution included the addition of trace elements in accordance with Huang et al. [31]. The influent consisted of 110 mL of fermented liquid and 890 mL of the mineral solution.

The SBR followed an aerobic dynamic feeding, specifically the F/f regime with partially uncoupled carbon and nitrogen feeding. A C/N/P ratio of 100:5:2 (mol basis) was applied, where 25 % of the required nitrogen fed coupled with the carbon (feast period), and the remaining 75 %, in form of ammonia, fed uncoupled from the carbon after the end of the feast phase (famine period). Phosphorous was provided, in form of phosphate, in excess through addition to the carbon feedstock.

In the first 38 days of operation, the theoretical SRT was 4.5 days (VSS basis). For the remaining operation period the theoretical SRT was set at 3 days (VSS basis). The hydraulic retention time (HRT) was set to 14.5 h for the whole experimentation. The study had a total duration of 148 days.

2.3. PHAs accumulation

The PHA accumulation assays were performed in batch (shake flasks) and in a fed-batch reactor identical to the one used for selection (2 L).

A working volume of 300 mL was used in 500 mL shake flasks. Six experiments were conducted using 233 mL of biomass collected from the selection reactor at the end of the famine phase and fed with salt-fermented CW supplemented with different valeric acid (HVa) concentrations (0 %, 5 %, 15 %, 30 %, 45 %, 60 %, Cmol). The batch tests were conducted in an incubated shaker (Lab companion, IST-4075R) controlled at room temperature (19–21 °C), stirred at 150 rpm, and with a food-to-microorganism (F/M) ratio equal to 45.5 Cmmol/g VSS.

For the fed-batch reactor, a volume of 1 L of biomass was collected from the selection reactor at the end of the famine phase and used as inoculum for the accumulation assays. The accumulation procedure consisted in a pulse-wise feeding using a feed-on-demand strategy. A new pulse was given when the previous one was consumed as identified from the DO profile that was monitored in real-time.

Three PHA accumulation assays were performed, named ACC-A, ACC-B, and ACC-C. In ACC-A, the feedstock consisted in the salt-fermented stream used for culture selection. In ACC-B, synthetic HVa was supplemented to the real fermented stream to reach a relative concentration of 30 % Cmol (with respect to the total VFAs concentration), and in ACC-C, synthetic caproic acid (HCa) was supplemented to the real fermented stream to reach a relative concentration of 30 % Cmol.

The assays were conducted under the same controlled conditions of F/M ratio (equal to 9 Cmmol/g VSS), pH, temperature, aeration, and stirring as those employed in each cycle during culture selection.

2.4. Analytical procedures

Total solids (TS), volatile solids (VS), total suspended solids (TSS), and volatile suspended solids (VSS) were determined according to standard methods [32]. Fermentation products were quantified in filtered samples (0.20 μm) using high-performance liquid chromatography (HPLC) on a VWR Hitachi Chromaster chromatographer equipped with a pump 5160, an auto sampler 5260, a column oven 5310, a diode array detector 5430, a RI detector 5450, a Biorad 125–0129 30 × 4.6 mm pre-column, and an Aminex HPX-87 H 300 × 7.8 mm column. The following conditions were used: column temperature 60 °C, 0.01 M H_2SO_4 eluent, flow rate 0.6 mL/min, and injection volume 99 μL. The concentrations of VFAs and ethanol were determined using standard calibration curves (4–1000 mg/L for each compound) [33].

The concentration of nitrogen, in the form of ammonia (N-NH_4), and phosphorus, in the form of phosphate (P-PO_4), were determined in filtered samples (0.20 μm) by colorimetric method implemented in a segmented continuous flow analyser (Skalar SAN++) [33].

For PHA quantification, lyophilized biomass was weighted and incubated with 1 mL chloroform and 1 mL acidic methanol (20 % v/v, H_2SO_4) (for methanolysis) through digestion at 100 °C for 3.5 h. After the digestion, the sample was washed twice with demineralized water to remove salts and other impurities. Afterwards, the organic phase (methylated monomers dissolved in chloroform) was extracted and injected (2 μL) into a gas chromatograph equipped with a flame ionization detector (Perkin Elmer Claurus 590) and a Restek column (60 m, 0.53 mm internal diameter, 1 μm film thickness, Crossbond, Stabilwax), using helium as carrier gas at 1.0 mL/min. Injector and detector temperatures were 280 °C and 250 °C, respectively. The conditions applied were as follows: a rate of 20 °C/min from 0 to 3 min, followed by a rate of 3 °C/min from 3 to 21 min up to 155 °C, and finally, a rate of 20 °C/min from 21 to 32 min up to 230 °C [33].

The concentrations of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3HHx were determined using three distinct calibration curves. Each curve was dedicated to one of the compounds: 3HB, 3HV, and 3HHx. Standards ranging from 0.1 to 1.0 g/L were prepared using a commercial P(3HB-co-3HV) (86:14, % wt., Sigma) and a commercial methyl 3-hydroxyhexanoate (Sigma). The data obtained from these standards were corrected using heptadecane as an internal standard at a concentration of 0.5 g/L.

Intracellular hydrophobic compounds, such as PHA granules, in biomass samples were identified using Nile blue staining [34] and observed with epifluorescence microscope Olympus BX51 equipped with an Olympus XM10 camera (Cell-F software).

Microbial community analyses by means of 16S rRNA gene amplicon

sequencing were conducted on pellets collected from the reactor at two operational conditions: at the end of the selection with sludge retention times (SRT) of 4.5 and 3 days, respectively, and at the end of the study. Additionally, analyses were performed on the inoculum sampled from the saline area of Rio Tejo. DNA extraction, gene sequencing and bioinformatics processing was carried out by DNASense (Aalborg, Denmark) as described by Wang et al. [35]. The exact experimental conditions of the analytical methods can be found in [33].

2.5. Calculations

The F/f ratio was calculated as the ratio between the time duration of feast and famine phases during the F/f cycle. The duration of the feast period was established by monitoring the consumption of fermentation products in the SBR. The PHA content in the biomass was determined in terms of percentage of VSS on mass basis (% wt., gPHA/gVSS), since VSS concentration is assumed to be a proxy of biomass concentration. The generic chemical formula for MMC ($CH_{1.8}O_{0.5}N_{0.2}S_{0.02}P_{0.02}$ [36]), with a resulting molecular weight (MW) of 25.30 g/Cmol, was used to determine cellular growth. The maximum active biomass (X_{max}, gX/L) was determined from the P uptake ($\Delta P\text{-}PO_4$) during a F/f cycle or accumulation batch by considering the stoichiometric P concentration in the biomass (C/P = 50 on molar basis). The amount of stored PHA (ΔPHA) was determined as the maximum PHA content (PHA_{max}, gPHA/L) minus the PHA content at the beginning (PHA_0, gPHA/L) of the experiment.

Stoichiometric and kinetic performance parameters were determined for the enriched cultures, in steady state conditions.

The specific rate of substrate (VFA) consumption (-q_S, $Cmol_S/(Cmol_X$ h)), PHA storage (q_{PHA}, $Cmol_{PHA}/(Cmol_X$ h)) and consumption (-q_{PHA}, $Cmol_{PHA}/(Cmol_X$ h)), and specific growth rate of the biomass (μ, $Cmol_X/(Cmol_X$ h)) were determined from the slope of the linear regression of the experimental data (substrates, PHA, and biomass concentrations over time), divided by the local biomass concentration. The storage yield ($Y_{PHA/S}$, $Cmol_{PHA}/Cmol_S$) was calculated as the ratio between q_{PHAs} and the -q_S. Growth yields on carbon substrate ($Y_{X/S}$, $Cmol_X/Cmol_S$) and stored PHA ($Y_{X/PHA}$, $Cmol_x/Cmol_{PHA}$), were calculated as the ratios between the specific growth rate during the feast phase (μ_{Feast}) and –qS, and the specific growth rate during the famine phase (μ_{famine}) and -qPHA, respectively.

In the accumulation assays, the specific rates were calculated, as described before, for the initial 4 pulses. Volumetric PHA productivity (gPHA/(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 (gPHA/(gX h)) was calculated as the ratio between the volumetric PHA productivity for the accumulation per X at the beginning of the accumulation ($X0_{Acc}$).

Standard errors associated with the determined parameters were estimated considering standard errors propagation.

3. Results and discussion

3.1. Culture selection

To assess the feasibility of selecting a PHA-accumulating mixed culture using salt-fermented CW, the inoculum was subject to a double selective pressure. This involved applying an F/f regime and a partial uncoupled C/N availability. Regarding the latter, nitrogen, in the form of ammonia, was supplied mostly uncoupled (75 % Nmol) with respect to the feedstock, aiming to separate most of the cellular growth from PHA storage. A theoretical OLR of 60 $Cmmol_S/(L$ d) was applied throughout the study (see Table 1 for feedstock composition).

Two different SRTs were investigated. Initially, an SRT of 4.4 ± 0.1 days was imposed and maintained for a total of 38 days. After 20 days of operation, a steady state was achieved, with the culture exhibiting a stable active biomass concentration of 2.40 ± 0.07 g_X/L. An F/f ratio of 0.09 ± 0.01 h/h was obtained which is indicative of a successful selection of a PHA-accumulating mixed culture. Nevertheless, the selected culture exhibited poor settling capabilities which worsened over time and undesirably biofilm flocculation and fouling of the piping and air diffuser, making the operation difficult. Consequently, the SRT was reduced to a nominal value of 3 days to enhance bioreactor operation, also considering that Carvalho et al. [28] achieved stable operation with a shorter SRT.

Table 2
Main stoichiometric and kinetic performance parameters for the culture selected with the two imposed SRT. Average ± standard deviations are reported for each condition (n = 3 for SRT = 4.5 d and n = 4 for SRT=3 d).

Parameter [unit]	Theoretical SRT [d]	
	4.5	3.0
Experimental SRT [d]	4.4 ± 0.1	2.8 ± 0.4
OLR [$Cmmol_S/(L.d)$]	58.4 ± 2.0	58.9 ± 3.9
Feast/famine [h/h]	0.09 ± 0.01	0.06 ± 0.01
X @ cycle start [g_X/L] / [$Cmmol_X/L$]	2.40 ± 0.07 / 94.8 ± 2.9	1.77 ± 0.04 / 70.1 ± 1.7
PHA_{max} [% wt., VSS basis]	18.0 ± 1.8	35.5 ± 4.9
ΔPHA [% wt., VSS basis]	10.6 ± 0.4	15.4 ± 4.8
3HB:3HHx ratio [%, Cmol]	93.3: 6.7	93.1: 6.9
(-)qS [$Cmol_S/(Cmol_X$. h)]	0.35 ± 0.00	0.65 ± 0.09
qPHA [$Cmol_{PHA}/(Cmol_X$. h)]	0.22 ± 0.01	0.52 ± 0.10
μ_{Feast} [$Cmol_X/(Cmol_X$. h)]	0.03 ± 0.01	0.07 ± 0.02
(-)qPHA [$Cmol_{PHA}/(Cmol_X$.h)]	0.08 ± 0.01	0.15 ± 0.07
μ_{famine} [$Cmol_X/(Cmol_X$. h)]	0.03 ± 0.02	0.06 ± 0.04
$Y_{PHA/S}$ [$Cmol_{PHA}$ / $Cmol_S$]	0.64 ± 0.03	0.80 ± 0.07
$Y_{X/S}$ [$Cmol_X$ / $Cmol_S$]	0.08 ± 0.04	0.11 ± 0.03
$Y_{X/PHAs}$ [$Cmol_X$ / $Cmol_{PHA}$]	0.07 ± 0.05	0.09 ± 0.04

The performance of the culture under the new condition is detailed in Table 2. The shorter SRT applied to the culture led to the selection of a more efficient PHA-accumulation mixed culture as higher ΔPHA was achieved (15.4 ± 4.8 % wt.), along with a faster VFA consumption and consequently a decrease in the F/f value to 0.06 ± 0.01 h/h, despite a reduction in the concentration of biomass (1.35 times lower). The profiles of VFAs, ammonia, phosphorus and DO for a typical SBR cycle are reported in Fig. 1A.

The phosphorus profile indicates that cellular growth occurred only when ammonia was present during both the carbon feed and during the uncoupled nitrogen dosage. In contrast, no P uptake is observed when ammonia was not available in the reactor. When available, ammonia is rapidly depleted by the MMC indicating a fast cellular growth.

Volatile solids, active biomass, and PHA profiles are depicted in Fig. 1B. Solids profiles are consistent with the results observed in Fig. 1A, as the PHA highest concentration was reached approximately at the end of the feast phase, while the active biomass concentration was dependent on cellular growth, thus on nutrients availability. As for performance parameters, the substrate consumption (-qS) increased from 0.35 ± 0.00 $Cmol_S/(Cmol_X$ h) to 0.69 ± 0.09 $Cmol_S/(Cmol_X$ h) by decreasing the theoretical SRT from 4.5 to 3 days. Concurrently, PHA specific storage rate (q_{PHA}) shifted from 0.22 ± 0.01 $Cmol_{PHA}/(Cmol_X$ h) to 0.52 ± 0.10 $Cmol_{PHA}/(Cmol_X$ h). Notably, $Y_{PHA/S}$ displayed a significant increase from 0.64 ± 0.03 $Cmol_{PHA}/Cmol_S$ to 0.80 ± 0.07 $Cmol_{PHA}/Cmol_S$, indicating an enhanced conversion of VFA into PHA.

When operating at an SRT of 4.4 ± 0.1 days, the biomass displayed the ability of accumulating a copolymer of P(3HB-co-3HHX) with a 3HHx content of 6.7 % Cmol, along with good specific PHA production rates and PHA on substrate yields (Table 2). Furthermore, the copolymer P(3HB-co-3HHX) was, to the best of authors' knowledge, never reported in literature for MMC under saline conditions. At an SRT of 2.8 ± 0.4 days, the accumulated PHA comprised a copolymer (3HB-co-3HHx) with 3HHx content of 6.9 % Cmol. This observation aligns with the findings from the culture selected at a higher SRT of 4.4 ± 0.1 days.

The 16S rRNA gene sequencing revealed the presence of a diverse mixed microbial community in the sediments collected from Samouco, consistent with observations made previously [28]. The selective

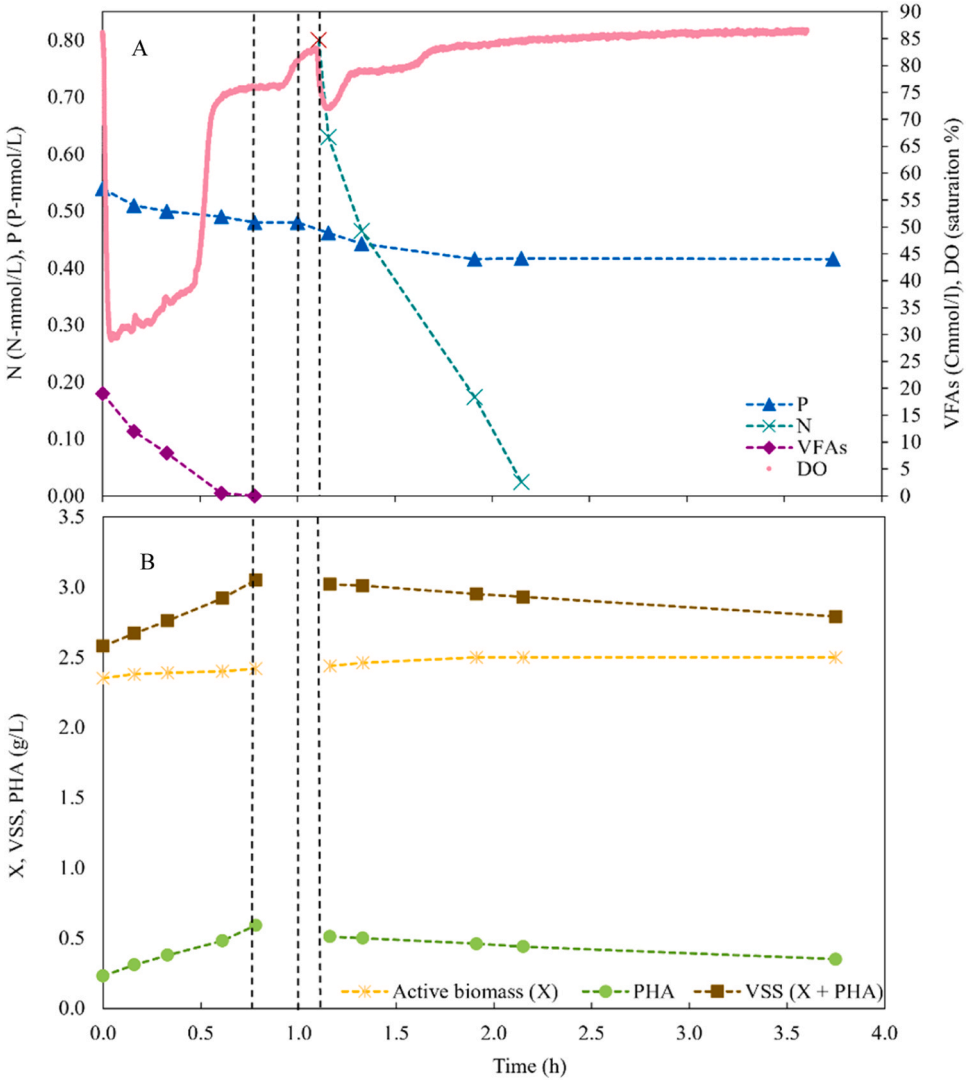

Fig. 1. A representative F/f cycle is shown. Concentration profiles of VFAs, ammonia, phosphorus, DO (Panel A), active biomass, PHA and VSS (Panel B) during the F/f cycle for the culture selected at theoretical SRT of 3 d. Five out of 8 h of the F/f cycle are shown. The end of the famine phase is not reported, as concentrations are constant. The ammonia concentration expected after the feed is highlighted in red as different from the subsequent measured value. The end of the feast phase, the purge time and the ammonia feed are indicated by the vertical black dotted lines.

pressure applied to the MMC led to a shift in the mixed microbial profile (Fig. 2). The culture selected at an SRT of 4.4 ± 0.1 days was enriched with microorganisms from the Alphaproteobacteria phylum, dominated by representatives of the Rhodobacteraceae (69.4 %) and Rhizobiaceae (12.3 %) families.

For the culture selected at an SRT of 2.8 ± 0.4 days, a slight increase in the Rhodobacteraceae family to 71.8 % and a slight decrease in the Rhizobiaceae family to 12.0 % were observed. Carvalho et al. [28] also reported Rhodobacteraceae as the main (74.5 % relative abundance) family in a culture enriched at the same OLR and salinity of this study. Rhodobacteraceae are aquatic bacteria found in a wide range of natural environments, specifically in marine ones. PhaC class I PHAs synthase is known to be present in Rhodobacteraceae bacteria [37,38]. There are three natural PHA biosynthetic pathways, each involving key enzymes associated with precursor molecules. The simplest of these pathways involves three enzymes: PhaA (acetyl-CoA acetyltransferase), PhaB (acetoacetyl-CoA reductase), and PhaC (class I). This pathway is associated with the production of homopolymers such as scl-PHA (e.g., PHB) and copolymers including scl-PHA (e.g., P(3HB-co-3HV)) and scl-mcl-PHA (e.g., P(3HB-co-3HHx)), which supports the results obtained within this study [13].

In the case of the culture enriched under an SRT of 2.8 ± 0.4 days, a large number of organisms belonging to the *Pseudorhodobacter* genus and to an unknown genus referred to as *midas_g_51*, were observed, with a relative abundance of 22.4 % and 11.2 %, respectively. Although no reference to the role of *Pseudorhodobacter* organisms in PHA accumulation was found, a representative of this genus was found in the KEGG database (*Pseudorhodobacter turbinis*), which has three genes coding for enzymes classified as PHA synthases (UniProt: A0A4P8EI94, A0A4P8EKH1, A0A4P8ELA5).

3.2. PHA accumulation

During the final period of the feast phase of each F/f cycle, the SBR underwent automatic purging with a pump to remove excess biomass. The purged flow, enriched with PHA-accumulating organisms, was then stored before being utilized for PHA accumulation tests. The biomass selected to evaluate the maximum PHA storage capacity and productivity was the culture enriched with the SRT of 2.8 ± 0.4 days, given that the stoichiometric and kinetic parameters indicated a more efficient

A

Domain	Phylum	Class	Order	Family	Inocullum	Selected Culture #3	Selected Culture #10
Bacteria	Actinobacteriota	-	-	-	6.8	2.2	2.0
	Bacteroidota	Bacteroidia	Flavobacteriales	Flavobacteriaceae	11.7	1.9	0.7
	Bacteroidota	Bacteroidia	Bacteroidales	-	0.8	0.0	0.0
	Desulfobacterota	-	-	-	10.0	0.0	0.0
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	7.3	12.3	12.0
				Stappiaceae	0.0	1.2	3.4
			Rhodobacterales	Rhodobacteraceae	13.7	69.4	71.8
			Sphingomonadales	Sphingomonadaceae	1.6	0.0	0.0
		Gammaproteobacteria	-	-	21.3	0.0	0.0
	Verrucomicrobiota	Verrucomicrobiae	Verrucomicrobiales	-	0.7	5.5	0.0
	Others	-	-	-	11.7	2.1	1.3
	Unclassified	-	-	-	14.4	5.4	8.8
					100.0	100.0	100.0

B

Family	Genus	Inocullum	Selected Culture #3	Selected Culture #10
-	-	6.8	2.2	2.0
Flavobacteriaceae	-	11.7	1.9	0.7
-	-	0.8	0.0	0.0
-	-	10.0	0.0	0.0
Rhizobiaceae	*Mesorhizobium*	0.1	5.6	5.9
	Pseudochrobactrum	0.0	5.6	4.7
	Others	7.2	1.0	1.4
Stappiaceae	*Stappia*	0.0	1.2	3.4
Rhodobacteraceae	*Albirhodobacter*	0.0	16.0	8.2
	Aliiroseovarius	0.7	3.9	0.2
	Citreicella	0.0	0.4	8.3
	midas_g_51	0.1	1.2	11.2
	Paracoccus	0.0	2.5	0.7
	Pontibaca	0.0	13.4	0.9
	Pseudorhodobacter	0.1	2.3	22.4
	Roseovarius	0.5	4.9	4.6
	Ruegeria	0.0	1.2	7.7
	Sulfitobacter	1.2	13.8	5.7
	Others	10.2	3.6	1.5
	Unclassified	1.0	6.1	0.6
Sphingomonadaceae	*Novosphingobium*	1.6	0.0	0.0
-	-	21.3	0.0	0.0
-	-	0.7	5.5	0.0
-	-	11.7	2.1	1.3
-	-	14.4	5.4	8.8
		100.0	100.0	100.0

Fig. 2. Heatmaps illustrating the most abundant microbial communities at the family level (A) and genus level (B) in both the inoculum and the selected culture for SRTs of 4.5 days and 3 days. Values are presented as a normalized fraction of total sequences (%).

Table 3
Main stoichiometric and kinetic performance parameters for the accumulation tests ACC-A (fermented cheese whey) and ACC-B (fermented cheese whey + 30 % HVa) evaluated in the first 4 feed pulses.

	ACC-A	ACC-B
PHA @ACC start [% wt., VSS]	27.6	20.2
PHA max [% wt, VSS]	56.4	62.0
3HB:3HV:3HHx [%, Cmol]	93.5: 0: 6.5	65.4: 29.8: 4.8
qPHA [$Cmol_{PHA}/(Cmol_X.h)$]	0.45 ± 0.17	0.53 ± 0.05
qHB [$Cmol_{PHA}/(Cmol_X.h)$]	0.42 ± 0.15	0.30 ± 0.03
qHV [$Cmol_{PHA}/(Cmol_X.h)$]	-	0.21 ± 0.02
qHHx [$Cmol_{PHA}/(Cmol_X.h)$]	0.03 ± 0.01	0.03 ± 0.00
-qS [$Cmol_S/(Cmol_X.h)$]	0.74 ± 0.05	0.70 ± 0.05
-qHAc [$Cmol/(Cmol_X.h)$]	0.20 ± 0.03	0.18 ± 0.03
-qHBu [$Cmol/(Cmol_X.h)$]	0.44 ± 0.04	0.30 ± 0.06
-qHVa [$Cmol/(Cmol_X.h)$]	-	0.25 ± 0.04
-qHCap [$Cmol/(Cmol_X.h)$]	0.08 ± 0.01	0.03 ± 0.00
$Y_{X/S}$ [$Cmol_X/Cmol_S$]	0.07 ± 0.02	0.11 ± 0.05
$Y_{PHA/S}$ [$Cmol_{PHA}/Cmol_S$]	0.61 ± 0.23	0.76 ± 0.09
Volumetric PHA productivity [gPHA/(L. h)]	0.68	0.83
Specific PHA productivity [$gPHA/(g_X.h)$]	0.42	0.45

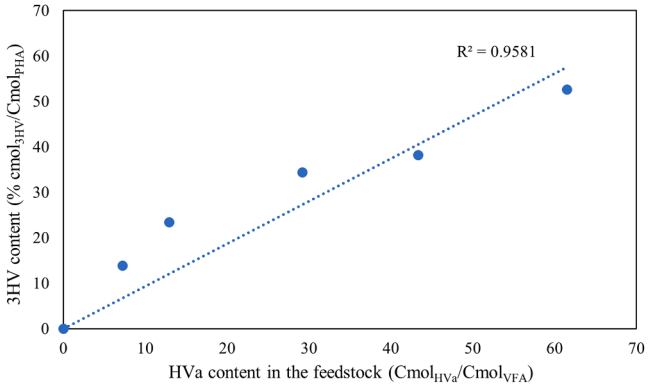

Fig. 3. Polymers produced in a batch test using the salt-fermented stream supplemented with various relative contents of valeric acid (measured, 0 %, 7 %, 13 %, 29 %, 43 %, 62 %, Cmol). A linear regression was applied to the experimental data, resulting in an R^2 value of 0.9581.

culture.

A feed-on-demand strategy was applied, involving the addition of another VFA pulse when the previous pulse was entirely consumed, as indicated by a sudden increase in the DO values. The same F/M ratio of the SBR was used for each pulse fed to the accumulation reactor.

The salt-fermented CW was used in ACC-A as carbon source. The accumulation performance of the culture is detailed in Table 3. The maximum PHA content (56.4 % wt. VSS basis) was reached after 4 pulses; two additional pulses were supplied, resulting in a negligible increase in PHA content. Similarly, to what observed in the culture selection stage, and consistently with the feedstock composition, the PHA produced consisted of 3HB and 3HHx monomers, in a 93.5:6.5 ratio (%, Cmol).

As there are indications that the incorporation of 3HV monomer usually improves thermal and mechanical characteristics of the polymer [39,40], making it more suitable for downstream applications such as in packaging [39,41] and biomedical industries [42–44], the enriched culture's capability to utilize HVa for the production of PHA with different compositions was assessed, eventually allowing to obtain co-polymers with a broader range of applications.

To investigate the relationship between the HVa concentration in the feed and the 3HV content in the final PHA copolymer monomeric composition, batch tests using feeds of salt-fermented CW supplemented with different HVa concentrations were carried out. Fig. 3 depicts the

relationship between the HVa fed to the culture and the polymer's 3HV content. Through linear regression, an R^2 value of 0.958 was obtained, indicating a significant linear relation, consistent with what was reported by Carvalheira et al. [40]. After confirming the capability of the selected culture to incorporate the 3HV monomer into its polymer synthesis, the accumulation in reactor (ACC-B) to assess the maximum productivity of PHA was carried out. The ACC-B was conducted using salt-fermented CW supplemented with HVa at a relative content of 30 % (Cmol basis), based on the understanding that a 20 % to 30 % (wt.) 3HV content in copolymers is considered optimal for packaging applications [40].

In ACC-B, a maximum PHA content of 62.0 % wt. (VSS basis) was achieved after 5 pulses, surpassing the observed content in ACC-A. Meanwhile, qPHA and (-)qS values remained similar in the two accumulations, as detailed in Table 3. In the case of ACC-B, it is noteworthy that (-)q_{HVa} was significantly lower than (-)q_{HBu} during the initial pulse. However, after the culture acclimatized to HVa exposure during one pulse, the two parameters reached similar values (Fig. 4). Furthermore, ACC-B showed a higher substrate conversion into PHA (0.83 ± 0.03 $Cmol_{PHA}/Cmol_S$ vs 0.69 ± 0.07 $Cmol_{PHA}/Cmol_S$) and a higher PHA volumetric productivity (0.83 $g_{PHA}/(L\ h)$ vs 0.68 $g_{PHA}/(L\ h)$). Regarding the polymer composition, a terpolymer with a composition of 65.4:29.8:4.8 (% Cmol, 3HB:3HV:3HHx) was obtained, confirming the batch test results.

With the same rational applied to the production of a copolymer with a 3HV content, the increase in the 3HHx content in the copolymer by conducting an accumulation in reactor (ACC-C) using salt-fermented CW supplemented with higher content of HCa (30 % Cmol) was eveluated. Although the previously demonstrated capacity of the selected culture to incorporate 3HHx into its copolymer, no significant enrichment of the 3HHx content in the accumulated copolymer (6.5 % in ACC-A and 7.5 % in ACC-C) was observed, despite higher concentrations of HCa fed to the culture. Additionally, accumulation of HCa was observed throughout the assay, resulting in a lower maximum PHA content of 34 % wt.

3.3. PHA production from CW under near-seawater salinity conditions

The study successfully demonstrated the feasibility of selecting a PHA-accumulating mixed culture using salt-fermented cheese whey as a substrate under near-seawater salinity. The double selective pressure applied for PHA-accumulating organisms at both SRTs (4.5 and 3 days) led to the enrichment of mixed cultures capable of accumulating a copolymer of P(3HB-co-3HHx) with an approximate 7 % Cmol 3HHx content. However, challenges such as high flocculation and biofilm formation on the reactor's walls led to settling issues observed with the culture selected under SRT of 4.4 ± 0.1 days. Conversely, when a lower SRT was applied, improved reactor performance, enhanced PHA accumulation, and increased conversion of VFAs to PHA was observed. Concerning studies that investigated the influence of SRT on the culture selection, some authors reported a better performance of the culture as well in case of short SRTs [45,46] while others reported a higher PHA productivity for longer SRTs [21,47]. Nevertheless, the different operational parameters such as OLR and coupled/uncoupled carbon and nitrogen feed seem to play a fundamental role in the MMC response to the SRT change.

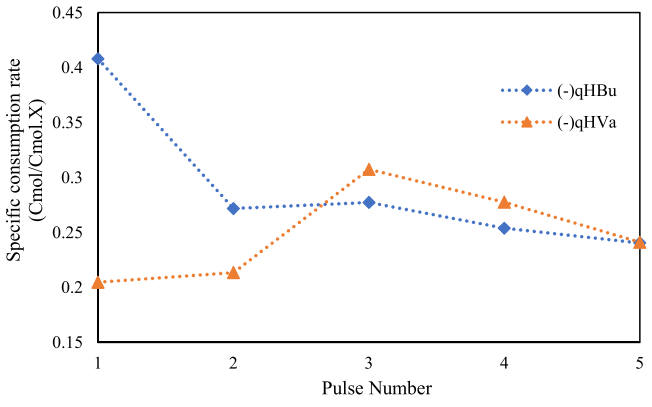

Fig. 4. The specific consumption rates for butyric acid (-qHBu) and valeric acid (-qHVa) during the accumulation B (ACC-B).

In Table 4 literature studies regarding the selection of PHA-accumulating MMC in saline conditions are reported, describing the substrate used, operative conditions, key stochiometric and kinetic parameters, and microbiological characterization.

It is worth noting that the salinity tested in this and Carvalho et al. [28] studies are the highest assessed so far using MMC. However, it is important to highlight that while this study utilized real feedstock, the previous investigation utilized synthetic feedstock. Additionally, it is notable that many of the cited studies were conducted using synthetic feedstocks [24,26,28,29].

Only Wen et al. [26] found a conversion yield ($Y_{PHA/S}$) higher than this study (up to 0.79 vs 0.61 $gCOD_{PHA}/gCOD_{VFA}$) that was, however, obtained with a salinity of 15 g_{NaCl}/L and with a synthetic feedstock. In accordance with what highlighted for $Y_{PHA/S}$, the specific PHA production (q_{PHA}) is also significantly higher than what found in studies with real fermented feedstocks, such as fermented fish canning wastewater [25], waste fish oil [27] and fermented cooking wastewater [23] where the maximum value obtained was 0.22 $Cmol_{PHA}/(Cmol_X\ h)$ (vs 0.52 $Cmol_{PHA}/(Cmol_X\ h)$ reported in this study). Furthermore, as already mentioned, the accumulation of the copolymer P(3HB-co-3HHx) was never reported for MMC in saline conditions. This copolymer is characterized by more desirable mechanical properties than P(3HB) such as rubber-like elastomeric properties [49].

In general, the diversity of the dominant microbial culture shows how the selection of the MMC is critical and dependent on the feedstock used and the operative conditions such as OLR and C/N feeding strategy.

Table 5 reports PHA accumulation results of the MMC selected in the above mentioned studies (Table 4). Some cited works report a maximum PHA content significantly lower than what obtained in this research [22,25,48], others similar values [23,24,26,29] while only Carvalho et al. [28] found a maximum PHA content significantly higher (84 % vs 62 %). The latter was, however, achieved with a MMC selected with a double OLR and using a synthetic VFAs mixture with no ammonia.

Furthermore, this work found volumetric and specific PHA productivities (up to 0.83 $g_{PHA}/(L\ h)$ and 0.45 $g_{PHA}/(X\ h)$) overcoming results reported in the cited studies.

Regarding PHA production from fermented CW in non-saline conditions, Colombo et al. [50] reported a qPHA in the range 0.2 - 0.4 $Cmmol_{PHA}/(Cmol_X\ h)$ while the yield $Y_{PHA/VFA}$ was in the range 0.7 - 0.9 $Cmmol_{PHA}/Cmmol_{VFA}$. Oliveira et al. [20] using fermented cheese whey as feedstock and uncoupling carbon and nitrogen dosage, found a qPHA of 0.069 $Cmmol_{PHA}/(Cmol_X\ h)$ and a $Y_{PHA/VFA}$ of 0.63 $Cmmol_{PHA}/Cmmol_{VFA}$. Carvalheira et al. [40], working with a PHA-accumulating mixed culture selected using fermented CW investigated PHA accumulation supplementing the feedstock with different contents of 3HV precursors. The resulting copolymers of P(3HB-co-3HV) exhibited different 3HV content, ranging from 11 % to 63 % (wt.); the maximum PHA content achieved was approximately 50 % wt. (VSS basis).

Therefore, the high salinity conditions of this study were not inhibitory for the selection of an efficient PHA-accumulating mixed culture, as the stoichiometric and kinetic performance parameters were actually among the best reported in literature.

Overall, PHA production under saline conditions not only promotes environmental sustainability but also offers economic advantages. By converting a saline organic side stream into PHA, the process simultaneously treats the side stream while valorizing its carbon content, thereby avoiding higher costs associated with difficult treatment

Table 4
Culture Selection: Overview of achieved performance parameters and comparison with previous studies associated with saline conditions.

II. Culture Selection Parameter / Units		This study	[22]	[23]	[25]	[26]	[27]	[28]	[29]	[48]
Reactor	-	SBR	SBR	SBR	SBR	SBR	SBR	SBR	SBR	SBR
HRT	d	16	1	1	1	1	1	0.67	1	1
SRT	d	3	1	1	1	10	1	3	1	1; 2
T	°C	21	30	30	30	21	30	20	30	30
Feedstock	-	Fermented CW	Fermented cooked mussel processing WW	Fermented cooked mussel processing WW	Fermented fish-canning WW	Syntetic waste fermentation leachate	Waste fish oil	Syntetic mixture of VFAs	Syntetic mixture of VFAs	Fermented cooking mussel processing wastewater
Salinity	g_{NaCl}/L	30	4	4-12	1.2-3.2	0; 5; 10; 15	10	30	2	17.5
OLR	gCOD/(L d)	2.3	2.5	0.8−1.5	12.3	1.4; 8.4	2.6[#2]	~ 2.3; 4.5[#1]	~ 3.7[#1]	N.A.
Time of F/f cycle	h	8	12	12	12	3; 12	12	8	12	N.A.
Time Feast	h	0.45	2.9	1.5	2.6	~ 0.66-4.1[#1]	3.72; 1.32	< 1.3	1.85	N.A.
F/f	h/h	0.06 ± 0.01	0.28	0.14	0.27	0.018; 0.029	0.45; 0.12	< 0.2	0.15	0.3; 0.6
C/N feeding strategy	Nmol	25 % coupled / 75 % uncoupled	coupled	coupled	coupled	coupled	coupled / uncoupled	uncoupled	coupled	N.A.
C/N ratio	$g_{COD/gN}$	100:1.8	6.5	3−6	N in excess	100:9	N limitation			N.A.
	Cmol/Nmol	20						100:7.5; 100:10; 100:5	6-13	
C:N:P	g_{COD}:gN:gP	100:1.8:1.6	N.A.	N.A.	N.A.	100:9:1	N.A.		N.A.	N.A.
	Cmol:Nmol:Pmol	100:5:2						100:7.5:1; 100:10:1; 100:5:1		
PHA_{max}	% wt., VSS basis	35.5 ± 4.9	12.8 ± 0.8	18.3	8.4	14.3; 34.6	39.0	35.1; 49.2	N.A.	3.5; 18.8
Monomer ratio [3HB: 3HV: 3HHx]	% Cmol	93.1:0:6.9	90:10:0	70:30:0	N.A.	N.A.	100:0:0	46:54:0; 33:67:0	73:27:0	N.A.
$-q_S$	$Cmol_{VFA,S}$/($Cmol_X$ h)	0.65 ± 0.09	N.A.	0.27	0.39	0.69-1.07[#2]	N.A.	0.40; 0.60	0.904	N.A.
q_{PHA}	$Cmol_{PHA}$/($Cmol_X$ h)	0.52 ± 0.10	N.A.	0.22	0.045	0.51-0.68[#2]	0.002; 0.042	0.20; 0.46	0.29	N.A.
μ_{Feast}	$Cmol_X$/($Cmol_X$ h)	0.07 ± 0.02	N.A.	N.A.	0.14	0.10-0.17[#2]	N.A.	N.A.	N.A.	N.A.
$-q_{PHA}$	$Cmol_{PHA}$/($Cmol_X$ h)	0.15 ± 0.07	N.A.	N.A.	N.A.	N.A.	N.A.	0.10; 0.36	N.A.	N.A.
μ_{famine}	$Cmol_X$/($Cmol_X$ h)	0.06 ± 0.04	N.A.	N.A.	N.A.	N.A.	N.A.	0.06; 0.15	N.A.	N.A.
$Y_{PHA/S}$	$Cmol_{PHA}$/$Cmol_{S,VFA}$	0.80 ± 0.07	0.24	0.8	0.12	0.64-0.79[#2]	0.006; 0.09	0.60; 0.75	0.314	N.A.
$Y_{X/S}$	$Cmol_X$/$Cmol_{S,VFA}$	0.11 ± 0.03	N.A.	N.A.	0.38	0.07-0.16[#2]	0.012; 0.765	N.A.	0.288	N.A.
$Y_{X/PHA}$	$Cmol_X$/$Cmol_{PHA}$	0.09 ± 0.04	N.A.	N.A.	N.A.	N.A.	N.A.	0.70; 0.44	N.A.	N.A.
Dominant microbial culture	Class[a], family[b], genus[c], species[d]	Rhodobacteraceae[b]; *Pseudorhodobacter*[c]	*Azoarcus*[c]; *Thauera*[c]	*Comamonas*[c]; *Azoarcus*[c]; *Thauera*[c]	N.A.	*Paracoccus; Hydrogenophaga; Azoarcus; Thauera; Halomonas*[c]	*Acinetobacter*[c]; *Rhizobium*[c]	Rhodobacteraceae[b]	N.A.	N.A.

#1 *OLR was determined through calculation. In some cases, Cmmol/L was converted to gCOD using the provided substrate profile and the theoretical Cmol/gCOD ratio. The duration of the feast period and PHA composition were derived from graphical representations*

#2 *Yields and specific rates are given in gCOD basis.*

Table 5
PHA production: Overview of achieved performance parameters and comparison with previous studies associated with saline conditions.

III. PHA production: Parameter / Units		This study	[22]	[23]	[25]	[26]	[27]	[28]	[29]	[48]
Reactor operation	-	Fed-Batch	Fed-batch	Fed-batch	Batch; Fed-Batch	Batch	Fed-Batch	Fed-Batch	Fed-Batch	Fed-Batch
C feeding strategy	-	Pulse-wise	Pulse-wise	Pulse-wise	Batch; Pulse-wise	Batch	Pulse-wise	Pulse-wise	Pulse-wise	Pulse-wise
T	°C	21	30	30	30	21	30	20	30	23-25
Feedstock	-	Fermented CW	Fermented cooked mussel processing wastewater	Synthetic fermented cooked mussel processing wastewater	Fermented fish-canning wastewater	Syntetic waste fermentation leachate	Waste fish oil	Syntetic mixture of VFAs	Syntetic mixture of VFAs	Fermented cooking mussel processing wastewater
Salinity	g_{NaCl}/L	30	4-14	4-12	2-12.3	0; 2; 5; 10; 15	10	30	0; 7; 13; 20	17.5
PHA_{max}	$g_{PHA}/g_{VSS;TSS}$	56.4; 62	24.9	59.9	5.7; 8.35	60.9; 30.6	54.2	55.3; 84.1	53-~10	6.9; 41.5
Monomer ratio [3HB:3HV:3HHx]	% Cmol	93.5:0:6.5; 65.4:29.8:4.8	83:17:0	70:30:0	93.6:6.4:0	~ 86.5:13.5:0[#1]	100:0:0	47:53:0; 35:65:0	73:27:0-86:14:0	N.A.
$Y_{PHA/S}$	$Cmol_{PHA}/Cmol_{S,VFA}$	0.61 ± 0.23; 0.76 ± 0.09	0.24	0.72	0.69; 0.35	N.A.	0.006; 0.303	0.60; 0.67	N.A.	N.A.
$Y_{X/S}$	$Cmol_{X}/Cmol_{S,VFA}$	0.07 ± 0.02; 0.11 ± 0.05	0.52	N.A.	N.A.	N.A.	0.130; 0.353	N.A.	N.A.	N.A.
PHA productivity	$g_{PHA}/(L\ h)$	0.68; 0.83	0.28	N.A.	0.055; 0.0103	N.A.	N.A.	0.30; 0.77	N.A.	N.A.
Specific PHA productivity	$g_{PHA}/(gX\ h)$	0.42; 0.45	N.A.	N.A.	0.006; 0.001[#3]	N.A.	N.A.	0.11; 0.21	N.A.	0.007; 0.068

#1 OLR was determined through calculation. In some cases, Cmmol/L was converted to gCOD using the provided substrate profile and the theoretical Cmol/gCOD ratio. The duration of the feast period and PHA composition were derived from graphical representations
#2 Yields and specific rates are given in gCOD basis.
#3 specific PHA productivity is given in CmolPHA/(Cmol X.h)

methods. Additionally, substituting freshwater, a scarce resource, with seawater as a washing and diluting agent in the PHA production process enables industries to significantly reduce their freshwater consumption and operational costs.

4. Conclusions

This study successfully demonstrated the feasibility of producing PHA by a MMC using fermented CW under near-seawater salinity conditions. Indeed, the utilization of 30 g_{NaCl}/L did not inhibit the selection of an efficient PHA-accumulating mixed culture, dominated by Rhodobacteraceae, under an SRT of 2.8 days. PHA accumulation tests achieved a maximum PHA content of 56.4 % wt., which increased to 62.0 % wt. when supplemented with 30 % Cmol of valeric acid. Additionally, polymers with varied monomeric content could be produced, expanding the potential for broader applications. Moreover, the stoichiometric and kinetic parameters of the selected culture, including specific PHA production rates and PHA on substrate yields, in most cases, superior to those reported for some degree of salinity.

This study presents a sustainable process for treating and valorizing saline organic side streams, traditionally difficult to manage, through PHA production. Furthermore, it introduces the potential use of seawater as an alternative to freshwater in the process, which could also be applied to PHA production processes using non-saline organic side streams, as process performance parameters were comparable to those reported in the literature for similar substrates under non-saline conditions.

CRediT authorship contribution statement

Matteo Grana: Writing – original draft, Methodology, Investigation, Data curation. **Maria A.M. Reis:** Writing – review & editing, Supervision, Project administration, Methodology. **Elena Ficara:** Writing – review & editing, Supervision, Funding acquisition. **Mónica Carvalheira:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Bruno C. Marreiros:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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