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VFA extraction through silicone membrane fosters PHA production from nutrient-rich biowaste

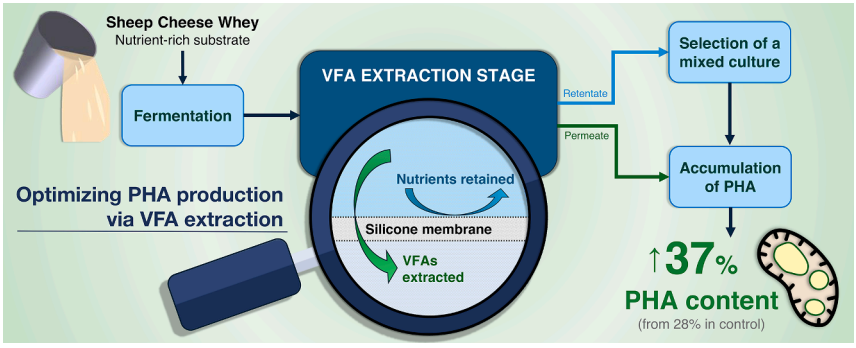
Fabiano Asunis [a,*], Paolo Dessì [b], Giorgia De Gioannis [a,c], Aldo Muntoni [a,c]

[a] Department of Civil, Environmental Engineering and Architecture (DICAAR), University of Cagliari, Piazza d'Armi 1, 09123 Cagliari, Italy
[b] Department of Agricultural Sciences, University of Naples Federico II, Piazza Carlo di Borbone 1, 80055 Portici, Italy
[c] National Research Council of Italy (CNR), Institute of Environmental Geology and Geoengineering (IGAG), Piazza d'Armi 1, 09123 Cagliari, Italy

HIGHLIGHTS

- A novel four-stage process was developed for PHA production from cheese whey.
- Silicone membrane extraction added as a fourth stage to conventional process.
- VFA extraction allowed to optimize C/N ratio for selection and accumulation stages.
- A maximum PHA content of 37% was achieved by implementing the membrane extraction.

GRAPHICAL ABSTRACT



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ABSTRACT

This study presents a novel four-stage process for polyhydroxyalkanoates (PHA) production from nutrient-rich sheep cheese whey (CW). The key advancement was the integration of a volatile fatty acid (VFA) extraction stage into the conventional three-stage PHA production process. Application of membrane separation to fermented cheese whey resulted in the generation of a "retentate" stream containing both organic acids and nutrients, suitable for microbial culture selection, and a VFA-rich but nutrient deprived "permeate" stream, ideal for PHA accumulation. Thus, the carbon-to-nitrogen (C/N) ratio was optimized for both the selection and accumulation stages, which is crucial for efficient PHA production and for eliminating the need for exogenous nitrogen addition. The integrated process resulted in significantly higher yields (0.55 vs 0.26 $g_{C\text{-}PHA}\ g_{C\text{-}OA}^{-1}$) and PHA content (37% vs 28%) than the control, where fermented cheese whey was directly used as feedstock for the

Abbreviations: AS, Activated sludge; COD, Chemical oxygen demand; CW, Cheese whey; DF, Dark fermentation; DO, Dissolved oxygen; DOC, Dissolved organic carbon; ED, Electrodialysis; EU, European Union; F/F, Feast to famine ratio; FCW, Clarified fermented cheese whey; HRT, Hydraulic retention time; MMC, Mixed microbial cultures; OA, Organic acids; OLR, Organic loading rate; pFCW, Permeate FCW; PHA, Polyhydroxyalkanoates; PHBV, Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); rFCW, Retentate FCW; SBR, Sequencing batch reactor; SRT, Sludge retention time; TN, Total nitrogen; TOC, Total organic carbon; VFA, Volatile fatty acids; VSS, Volatile suspended solids; WWTP, Wastewater treatment plant; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate.

* Corresponding author.
E-mail address: fabiano.asunis@unica.it (F. Asunis).

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accumulation stage. The results highlight the potential of this approach for optimizing PHA production from sub-optimal, nutrient-rich substrates.

1. Introduction

Polyhydroxyalkanoates (PHA) are biodegradable, bio-based polymers naturally produced by microorganisms as energy and carbon reserves (Reis et al., 2003). PHA synthesis occurs when specific microorganisms experience nutrient limitations while having an abundant carbon source (Mukherjee and Koller, 2023). Besides acting as carbon storage, intracellular PHA granules enhance microbial stress resistance (Obruca et al., 2020).

PHA production is an attracting investment due to its tailored applications in cosmetic formulations, sustainable packaging materials, drug carriers, controlled-release fertilizers and long-term carbon sources for remediation (Amanat et al., 2021; Estévez-Alonso et al., 2021; Koller, 2018). The PHA market is expected to grow from USD 93 million in 2023 to USD 195 million by 2028 (MarketsandMarkets, 2024). Increasing interest in biowaste-derived PHA aims to lower production costs and improve sustainability.

The conventional PHA production from biowaste using open mixed microbial cultures (MMC) involves three stages: (i) acidogenic fermentation, converting organic feedstock into organic acids (OA) as PHA precursors; (ii) culture selection, enriching PHA-storing microorganisms; and (iii) accumulation, where microorganisms store PHA intracellularly. The accumulated PHA is then extracted and purified for application. In the past decades, the three-stage production process has been used to produce PHA from various biowaste streams, including food waste (Moretto et al., 2020), fruit waste (Matos et al., 2021a; Silva et al., 2022), dairy residues (Asunis et al., 2022; Colombo et al., 2019, 2016), olive mill wastewater (Campanari et al., 2017), mussel processing wastewater (Roibás-Rozas et al., 2021), and sewage sludge (Valentino et al., 2019). Reported PHA contents range from 30% to 60% of the volatile suspended solids (VSS). Successful pilot-scale implementations suggest the feasibility of full-scale market adoption in the near future (Almeida et al., 2024; Mannina and Mineo, 2023; Marreiros et al., 2023; Silva et al., 2022; Sousa et al., 2021).

The overall PHA yield and productivity depend on both operational parameters and substrate characteristics. Easily fermentable substrates are preferred, as insufficient acidification, high solid content, salinity, or excessive nutrients can hinder the PHA accumulation stage (Estévez-Alonso et al., 2021). High nitrogen (N) and phosphorus (P) levels should be avoided, since a C/N ratio above 10 gC gN^{-1} is required for both enriching PHA-storing cultures (stage II) and converting organic acids into PHA (stage III) (Johnson et al., 2010; Sánchez Valencia et al., 2021). However, while the selection stage requires a balanced nitrogen supply to establish a C/N ratio of about 10–15 $g_C\ g_N^{-1}$ (Matos et al., 2021a; Oliveira et al., 2017), PHA storing is optimal under nutrient limiting conditions (15 to 40 $g_C\ g_N^{-1}$).

In case of nutrient-deficient substrates, nitrogen (e.g., ammonium sulfate) is often added to support biomass growth in the selection stage. In contrast, protein-rich substrates such as whey may not require supplementation, as microorganisms can metabolize nitrogen from whey protein, although organic nitrogen uptake efficiency depends on the specific microorganism and requires further investigation (Asunis et al., 2022; Oliveira et al., 2018). When using nitrogen-rich substrates, it is vital to avoid high nutrient concentrations in the accumulation stage since microorganisms may shift their metabolism from PHA production and storage to cell growth.

Different pretreatment strategies have been proposed to optimize PHA accumulation by removing excess nutrients. For instance, phosphorous precipitation using iron chloride was proposed by Bengsston et al. (2017), while Domingos et al. (2017) explored electrodialysis (ED) to concentrate acidic streams from fermented cheese whey for PHA production using *Cupriavidus necator*. Since ED alone cannot selectively separate nutrients from dissociated OA, it is often combined with membrane processes or chemical precipitation. Tao et al. (2016) combined microfiltration, ED, and struvite precipitation to generate an OA-rich stream from fermented sludge for PHA accumulation. Alternatively, membranes alone can extract carbon-rich OA while retaining nutrients, though few studies have tailored this approach for PHA production.

Outram & Zhang (2018) reported a low-cost method for undissociated VFA extraction from fermented effluents, using a non-porous silicone membrane and distilled water as the extractant. This method was applied to cheese whey fermentation, demonstrating that VFAs such as butyric acid can be selectively extracted from the fermentation broth while retaining N and P (Dessì et al., 2020). Thus, this approach allows to split whey fermentate into a *N*-rich retentate and a VFA rich but nutrient deprived permeate, suitable for the culture selection and for the PHA accumulation stages, respectively.

Based on this hypothesis, this study introduces a novel four-stage PHA production process for PHA production from nutrient rich sheep cheese whey. A VFA extraction unit, based on silicone membrane pertraction, was added to the conventional "three stage process" to produce optimal feed streams for both the selection and accumulation stages, improving PHA yields. The simplicity, low cost, flexibility and energy efficiency of the innovative process proposed could contribute to the development of industry scale PHA production.

2. Materials and methods

2.1. Substrate and inoculum

The substrate for PHA production was raw sheep cheese whey (CW) from a local dairy industry in Sardinia, Italy, processing around 20 million liters of milk per year. The CW was frozen at −15 °C in 2 L bottles and thawed at room temperature for 12 h when needed. The CW

Table 1
Characterisation of the raw cheese whey (CW), clarified fermented CW (FCW), retentate fermented CW (rFCW) and permeate fermented CW (pFCW).

Parameter	Measure unit	Raw CW	FCW	rFCW	pFCW
pH	–	6.13 ±0.49	6.00 ±0.00	2.91 ±0.26	3.05 ±0.06
Total organic carbon (TOC)	$g_C\ L^{-1}$	27.92 ±2.22	20.29 ±2.75	15.38 ±1.86	2.63 ±0.50
Dissolved organic carbon (DOC)	$g_C\ L^{-1}$	25.44 ±2.14	17.53 ±1.53	15.38 ±1.86	2.63 ±0.50
Soluble carbohydrates	$g\ L^{-1}$	42.04 ±4.83	< DL	< DL	< DL
Soluble proteins	$g\ L^{-1}$	10.55 ±0.56	5.62 ±0.90	5.41 ±0.02	< DL
Lactic acid	$g\ L^{-1}$	3.87 ±0.16	13.08 ±1.88	14.58 ±2.10	< DL
Total VFA	$g\ L^{-1}$	< DL	21.04 ±0.84	12.80 ±1.41	4.53 ±0.37
OA composition La/Ac/Pr/Bu	% of C	100/0/0/0	33/12/38/17	45/12/9/31	0/6/54/40
Total Nitrogen (TN)	$g_N\ L^{-1}$	1.90 ±0.08	1.57 ±0.06	1.35 ±0.12	< DL
Ammonium	$g_{N\text{-}NH4}\ L^{-1}$	0.06 ±0.01	0.04 ±0.01	0.05 ±0.03	< DL
C/N*	$g_C\ g_N^{-1}$	14.7	12.9	11.4	n.a.**

*as TOC/TN.
**"n.a." indicates that the C/N ratio is not applicable for the pFCW due to the absence of a measurable nitrogen value.
< DL = Less than detection limits.

characterisation before the experimental trials is reported in Table 1. Sheep CW is both a carbon- and nitrogen-rich residue (27.9 and 1.9 g L^{-1} of total organic carbon, TOC and total nitrogen, TN, respectively). Most of the carbon (up to 66% of the TOC) was in the form of lactose, while the nitrogen mostly derives from whey proteins (up to 90% of the TN).

Fermentation was carried out by exploiting the natural CW microflora, without the addition of an external inoculum. The selection and enrichment of PHA-storing MMC was performed by using activated sludge (AS) inoculum, sampled from the aeration tank of a municipal wastewater treatment plant located in Cagliari (Italy). The volatile suspended solid (VSS) content of AS was around 7 g_{VSS} L^{-1}.

2.2. Experimental set-up

A four-stage experimental set-up (Fig. 1) was implemented to produce PHA from CW, based on: a dark fermentation (DF) batch reactor for CW acidification (stage I); a silicone membrane VFA extraction unit (stage II); a sequencing batch reactor (SBR) for the selection and enrichment of PHA-storing MMC (stage III); and a fed-batch reactor for the accumulation of PHA (stage IV).

2.3. Stage I: Fermentation

Five batch fermentation tests were carried out in a stirred glass reactor (BioFlo 110, New Brunswich Scientific, USA) with a working volume of 1.8 L. The reactor was operated for six days under anaerobic controlled conditions (39°C, pH 6, 200 rpm agitation), using raw CW as the substrate. After fermentation, the fermented CW (FCW) was clarified through sedimentation for 12 h at 4 °C. The clarified FCW from the five fermentation tests, (8 L in total, representing around 90–92% v/v of the whole fermented cheese whey) was mixed and frozen at −15 °C into 2 L bottles until being used in Stage II.

2.4. Stage II: Silicone membrane extraction

The experimental setup for VFA extraction stage was adapted from previous studies (Dessì et al., 2020; Ravishankar et al., 2020) and is shown in Supplementary Material. Briefly, a glass bottle (namely feed tank) was filled with 1.7 L of FCW and connected to a tubular silicone membrane coil with 3.2 mm internal diameter, 1.6 mm wall thickness and 4.9 m length (VWR, The Netherlands) submerged in a second glass bottle containing 1.7 L of distilled water (namely draw tank). The pH of FCW was adjusted to around 3 with H_2SO_4 to obtain undissociated OAs that can diffuse through the membrane (Ravishankar et al., 2020). The acidified FCW was continuously recirculated on the inner part of the tubular membrane at 200 mL min^{-1} flow rate using a peristaltic pump (SP 311/12, VELP Scientifica, Italy). The draw tank was mixed by magnetic stirring. The VFA extraction tests were performed in duplicate at room temperature (25 °C) for 14 days.

The extraction stage resulted in the production of two different streams: (i) retentate FCW (rFCW), containing both carbon (i.e., OA) and nitrogen (i.e., proteins and ammonia), suitable for selecting PHA accumulators (stage III); and (ii) permeate FCW (pFCW) containing VFAs but deprived from nutrients, ideal for the PHA accumulation stage (Stage IV).

2.5. Stage III: Selection and enrichment of the PHA-storing MMC

The selection stage was carried out in a sequencing batch reactor (SBR) (Diachrom Biotechnology, Switzerland; 4 L working volume) inoculated with activated sludge (AS) and using the rFCW as substrate. The reactor was operated at 25 °C, with hydraulic retention time (HRT) and sludge retention time (SRT) of 1 and 4 days, respectively. The feast-famine regime was applied, which involves alternating periods of carbon source availability and absence (Huang et al., 2018; Oliveira et al., 2017). The pH was not controlled and spanned between 8 and 9. The rFCW was diluted to apply an organic loading rate (OLR) of 0.62±5 g_C L^{-1} d^{-1} (equivalent to 52 $mmol_C$ L^{-1} d^{-1} or 2.1 g_{COD} L^{-1} d^{-1}). It was supplemented with 20 mg L^{-1} of allylthiourea to inhibit nitrification (Colombo et al., 2016).

The SBR cycle length was 12 h, consisting of four discrete phases: (i) influent filling (4 min), (ii) aeration (671 min), (iii) biomass purge (1 min), settling (40 min), and (iv) withdrawal of the clarified supernatant (4 min) (Asunis et al., 2022). Aeration was provided at a flow rate of 200

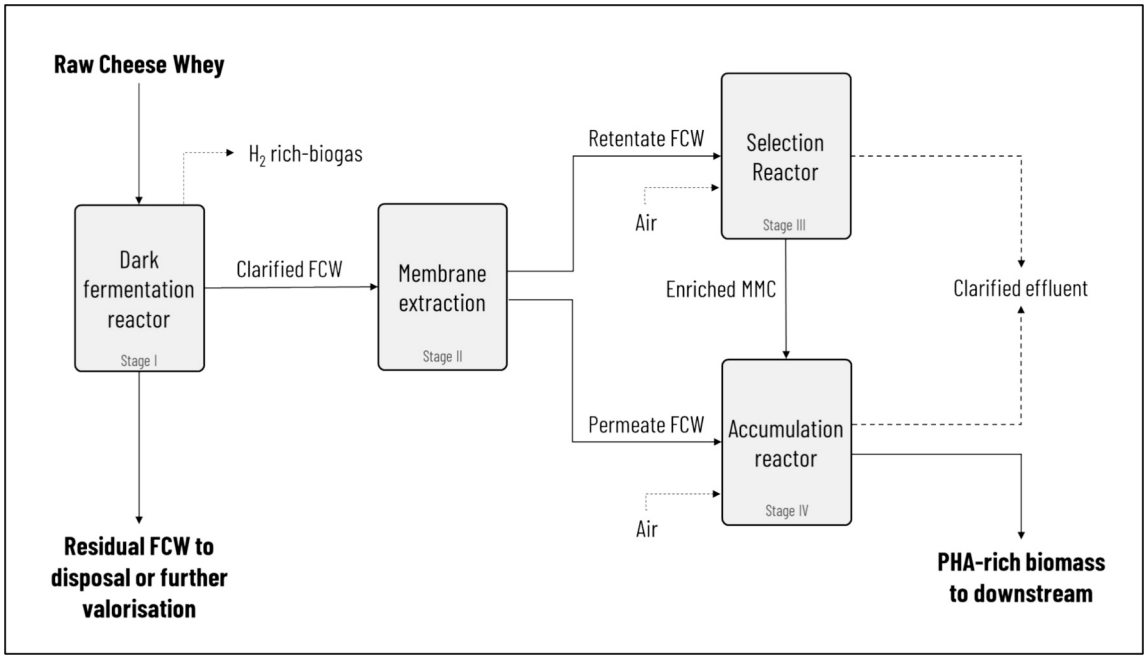

Fig. 1. Experimental set-up of the four-stage process proposed for PHA production by mixed microbial cultures (MMC) using raw sheep cheese whey (CW) as the starting substrate: fermentation (stage I), silicone membrane extraction (stage II), selection and enrichment of PHA-storing MMC (stage III) and PHA accumulation (stage IV). FCW stands for fermented CW.

L h^{-1}, automatically controlled by an Arduino-based software developed by our research group. DO concentration was monitored throughout the experiment.

2.6. Stage IV: PHA accumulation

The PHA storage capacity of the selected MMC was assessed in fed-batch mode in a 1 L glass reactor using either pFCW (nitrogen-free, containing only VFA as the C source) or FCW (as a control, C/N ratio of 12.9) as the substrate. The accumulation tests were conducted in duplicate between days 24 and 36 (corresponding to 6–9 SRTs), during which a stable feast-famine (F/F) regime was observed. Stability was defined by an F/F ratio with a standard deviation of less than 10%. The biomass was collected from the SBR reactor at the end of the famine phase. The reactor was operated at room temperature (25 °C) with oxygen supplied by air sparging, and the pH was uncontrolled and spanning in the range 7.5–9. The reactor was fed manually every two hours to ensure carbon excess, for a total duration of 8 h (Rossi et al., 2021).

2.7. Analytical methods

Total, volatile and volatile suspended solids (TS, VS and VSS), total organic carbon (TOC), dissolved organic fraction (DOC), soluble carbohydrates and proteins, lactic acid, VFAs (acetic, butyric, propionic) and ammonia were analysed as reported in Asunis et al. (2019). The elemental analysis in terms of carbon, hydrogen and nitrogen content was carried out with a CHN analyser (CHN 628, Leco, USA). The dissolved oxygen (DO) concentration was measured by a polarographic probe (InPro 6800, Mettler Toledo).

PHA content and speciation were determined by gas chromatography (GC) using a method adapted from Serafim et al. (2004). Biomass was lyophilized for 24 h at −55 °C and 0.05 mbar using a Lyovapor L-200 (BUCHI) freeze-dryer. Lyophilized biomass (around 4 mg) was incubated in a 20% vol. H_2SO_4 in methanol solution (1 mL) for methanolysis, with benzoic acid (50 mg/L) added as internal standard, and extracted with chloroform (1 mL). The mixture was then digested at 100 °C for 3.5 h. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) was separated and injected (1 μL) into a GC equipped with a flame ionization detector (model 7890B, Agilent Technology, USA) using split injection mode (10:1 split ratio) and a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology, USA) using helium as carrier gas at constant pressure (14.5 psi). The injector and the detector temperatures were 280 °C and 230 °C, respectively. The oven temperature was initially set at 40 °C, followed by a ramp of 20 °C min^{-1} until 100 °C, then 3 °C min^{-1} until 175 and 20 °C min^{-1} until a final temperature of 220 °C (4 min holding time). The 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) concentrations were calibrated using a commercial polymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV) (88%/12% by weight) (Sigma-Aldrich, CAS number 80181–31-3).

2.8. Performance parameters calculation

The fermentation yield ($Y_{OA/CW}$ or $Y_{VFA/CW}$, $g_C\ g_{C\text{-}CW}^{-1}$) was calculated by dividing the mass of OA produced (either lactic acid + VFA and VFA only, respectively) by the initial TOC of CW, expressed in terms of carbon mass.

For the extraction stage, the recovery efficiency R_e (%) was calculated as the amount of VFA extracted through the silicone membrane (draw tank) divided by the initial amount in the fed tank, expressed in terms of carbon mass:

$$R_e = \frac{gC_{VFA}^{Draw\ tank}}{gC_{VFA}^{Fed\ tank}} \bullet 100$$

The PHA content of the biomass was determined on a dry mass basis (wt %) and expressed as a percentage of the measured VSS:

$$PHAcontent = \frac{g_{PHA}}{g_{VSS}} \bullet 100$$

The concentration of active biomass (X_a) was calculated as the difference between the concentration of VSS and PHA (Valentino et al., 2015).

The F/F ratio was calculated as the ratio between the length (in hours) of the two phases.

The carbon content in protein was estimated based on an empirical C content of 0.52 g g^{-1} (Rouwenhorst et al., 1991).

Specific substrate uptake rates ($-q_{TOC}$, $-q_{OA}$ and $-q_{PROTE}$; $mg_C\ mg_{C\text{-}Xi}^{-1}\ h^{-1}$), PHA production rate ($q_{PHA}$; $mg_C\ mg_{C\text{-}Xi}^{-1}\ h^{-1}$) and maximum specific growth rate (μ_{max}; $mg_{C\text{-}X}\ mg_{C\text{-}Xi}^{-1}\ h^{-1}$) were calculated from the slope of the linear regression fittings (at least $R^2 > 0.80$, (Matos et al., 2021c)) of TOC, OA, proteins, PHA and X_a specific concentrations over time. PHA storage yields ($Y_{PHA/TOC}$ or $Y_{PHA/OA}$) were calculated by dividing q_{PHA} by $-q_{TOC}$ or $-q_{OA}$. Biomass growth yield ($Y_{X/TOC}$) was calculated by dividing μ_{max} by $-q_{TOC}$.

The overall PHA yield ($Y_{PHA/CW}$; $g_{C\text{-}PHA}\ g_{C\text{-}CW}^{-1}$) was calculated from a carbon mass balance. Key assumptions are based on the work of Asunis et al. (2021) and Valentino et al. (2019).

Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) post-hoc test to determine significant differences between groups. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Sheep cheese whey fermentation

The fermentation stage aims to convert the organic compounds present in the cheese whey (CW), primarily carbohydrates, into a pool of organic acids (OA), precursors of polyhydroxyalkanoates (PHA) production (Matos et al., 2021a). In this study, CW fermentation occurred in to two steps. Within the first 24 h, the carbohydrates were entirely (>99%) converted into lactic acid through the homolactic fermentation pathway. Subsequently, the lactic acid was further converted into a mixture of volatile fatty acids (VFA), predominantly propionic, butyric and acetic acids (Fig. 2), along with biohydrogen (see the Supplementary Material) and carbon dioxide as byproducts. Lactic acid fermentation to VFA is typically mediated by lactate-consuming bacteria, primarily *Clostridium spp*, and it has been reported as "lactate-driven" fermentation metabolic pathway in previous studies on fermentation of carbohydrate-rich waste, such as CW and dairy residues (Aranda-Jaramillo et al., 2023; Asunis et al., 2019; Bian et al., 2024; Blanco et al., 2019), sugarcane vinasse (Fuess et al., 2019), tequila vinasse (García-Depraect et al., 2020a), and it has been extensively reviewed by García-Depraect et al., (2020b).

The fermentation yields obtained in this study, expressed as $Y_{OA/CW}$ and $Y_{VFA/CW}$, were 0.71 $g_{C\text{-}OA}\ g_{C\text{-}CW}^{-1}$ and 0.51 $g_{C\text{-}VFA}\ g_{C\text{-}CW}^{-1}$, respectively. Thus, 71 % of the carbon mass in the CW was converted into OAs, mostly consisting of VFAs. In particular, the soluble carbon content of the fermented CW (FCW) comprised lactic acid, acetic acid, propionic acid and butyric acid in a proportion of 33/12/38/17 (as $\%g_{C\text{-}OA}$). The remaining carbon fraction was detected as carbon dioxide. Although the presence of lactic acid (11.7 g L^{-1}) underlines its incomplete conversion into OAs, the obtained fermentation yields are in line with those reported for PHA production from several agro-industrial wastes, including CW (0.40–0.80 $g_C\ g_C^{-1}$, (Asunis et al., 2022; Colombo et al., 2016; Duque et al., 2014; Lagoa-Costa et al., 2020; Oliveira et al., 2018)), fruit waste (0.74 $g_{COD}\ g_{COD}^{-1}$, (Matos et al., 2021b)) and olive mill wastewater (0.57 $g_{COD}\ g_{COD}^{-1}$, (Campanari et al., 2017)).

During the fermentation process, protein concentration decreased by 45% (from 10.5 to 5.72 g L^{-1}). This resulted in a slight reduction of the C/N ratio, from an initial value of 14.7 to a final value of 12.9.

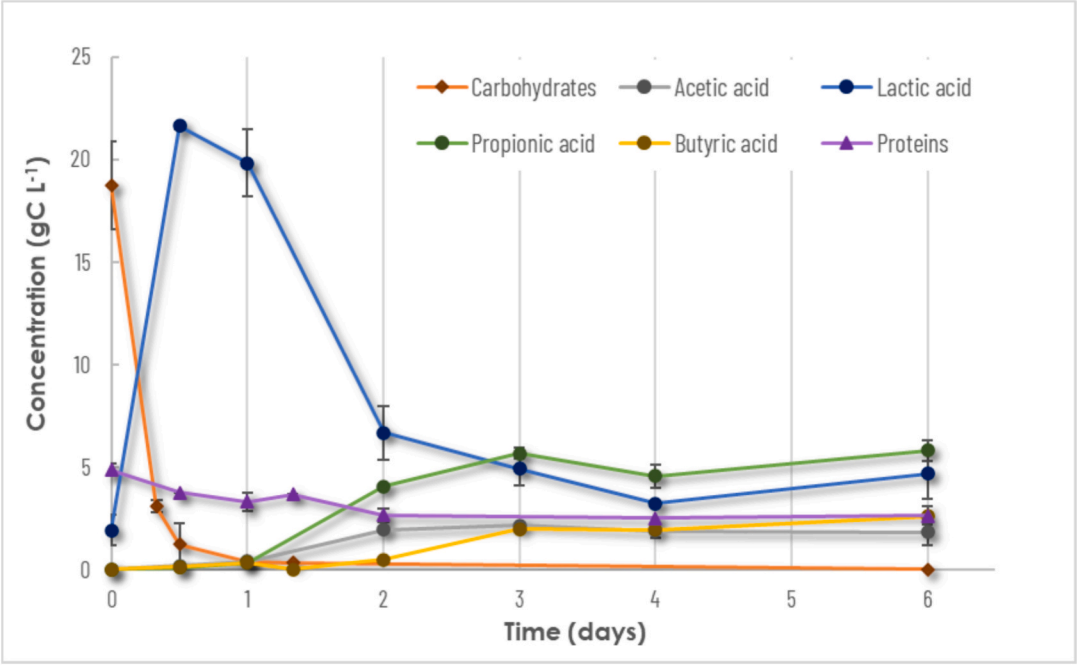

Fig. 2. Carbohydrates, proteins and OA (lactic, acetic, propionic and butyric acid) concentration over time during the batch fermentation tests carried out with raw CW as substrate (Stage I).

A FCW characterization is reported in Table 1. Overall, significant concentrations of PHA precursors (approximately 32 g_{OA} L^{-1}) were obtained by CW fermentation. The generation of highly concentrated OA streams is crucial to minimise the capital and operational costs of PHA production (Matos et al., 2021a).

3.2. VFA extraction through silicone membrane

Recirculation of the FCW on the inner part of the silicone membrane resulted in the concentration-driven diffusion of VFAs towards the draw solution with a preference for longer chain acids (butyric and propionic acids) over acetic acid (Fig. 3). After 14 days, 2.27 $g_{C\text{-}VFA}$ L^{-1} (equivalent to 4.53 g_{VFA} L^{-1}), were extracted and accumulated in the draw solution, with an average flux of 10.56 $g_{C\text{-}VFA}$ m^{-2} d^{-1}. The recovery efficiencies (R_e) for VFA and OA were 30% and 18% on a carbon basis, respectively. Butyric acid had the highest recovery (46%), followed by propionic acid (28%) and acetic acid (10%). These differences are due to the increasing hydrophobicity of VFA in relation to their carbon chain length, which results on a greater affinity on the non-porous silicone membrane (Outram and Zhang, 2018). This result aligns with previous studies on VFA diffusion through silicone membrane (Dessì et al., 2024, 2020; Ravishankar et al., 2020) and other types of hydrophobic membranes, such as polyethylene membrane contactor (Kotoka et al., 2024). The use of a porous membrane, such as a polyethene membrane, would not offer a comparable selectivity for VFA recovery, as it allows also nutrients to permeate (Aydin et al., 2018, Outram & Zhang, 2018).

The extraction stage resulted in the production of two different streams: permeate FCW (pFCW) and retentate FCW (rFCW). The pFCW,

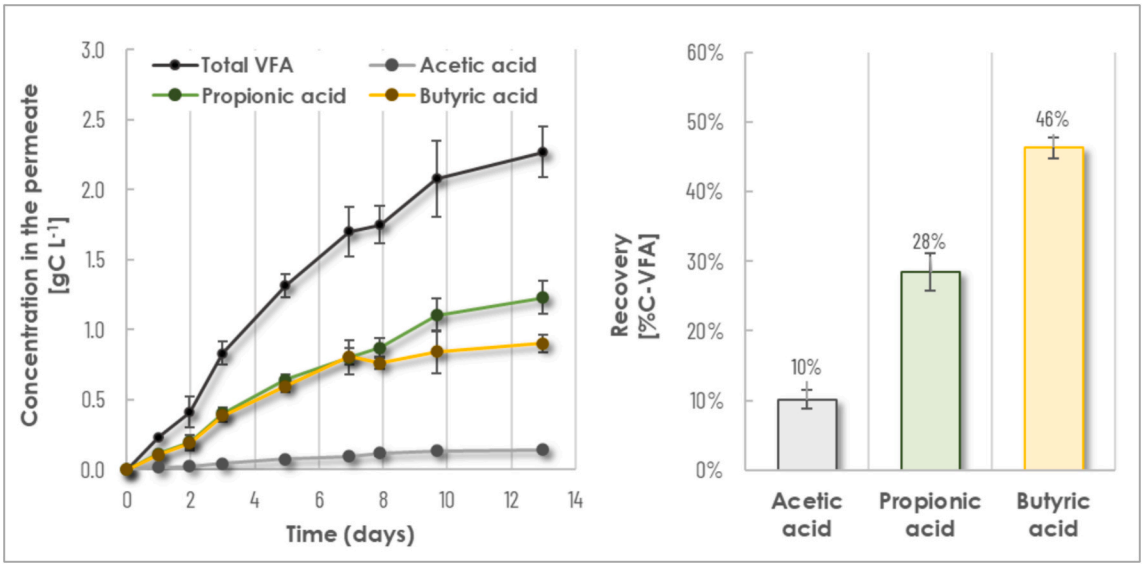

Fig. 3. Time evolution of VFA concentration in the permeate (pFCW) (Stage II) (left); percentage of VFA recovered with respect to the initial concentrations in the FCW (right).

containing about 20% of the total carbon, consisted of VFA (54% butyric acid, 40% propionic acid, and 6% acetic acid, on C% basis), with undetectable levels of lactic acid, nitrogen, and phosphorus (Table 1). Such composition makes pFCW ideal for the PHA accumulation stage, where nutrient-limiting conditions and a high carbon-to-nitrogen ratio (C/N) are required to promote PHA-storing rather over cell growth (Zhou et al., 2022). Interestingly, the composition of the pFCW can be controlled by fine-tuning the diffusion of the single VFAs through the silicone material, which depends on parameters such as feed pH, temperature, silicone membrane thickness and flow regime (i.e.: Reynolds number) inside the tube (Dessì et al., 2024; Outram and Zhang, 2018). Attaining different mutual ratios between the VFAs in the pFCW (and in particular the ratio between even and odd-chain VFA) is of interest as it affects the composition of the PHA monomers, as well as their thermal and mechanical properties and biodegradability (Duque et al., 2014). This potentially allows the production of tailored PHA for a wide range of industrial and environmental applications.

The extraction process showed significant room for improvement, as 80% of the carbon contained in the FCW was retained in the rFCW stream, consisting of 45% lactic acid, 31% butyric acid, 12% propionic acid and 9% acetic acid. Nevertheless, the rFCW comprised the nitrogen deriving from whey proteins and ammonia, resulting in a C/N ratio of 11.4, optimal for the selection and enrichment of PHA-storing microorganisms (Johnson et al., 2010; Silva et al., 2017).

3.3. Selection and enrichment of PHA-storing MMC

The selection of a PHA-storing MMC was carried out in an SBR using rFCW as the feed. The F/F regime, required for selecting PHA-storing microorganisms (Huang et al., 2018), was applied over a period of 36 days, which corresponds to approximately 9 SRTs. The key process parameters during the accumulation stage are summarised in Table 2. The observed F/F ratio was 0.17±0.1, which is within the reported suitable range for inducing the selection of microorganisms with a high PHA-storage capacity (Dionisi et al., 2006; Valentino et al., 2017). After an acclimation period, the average biomass concentration reached approximately 1.4 g_{VSS} L^{-1} (see Supplementary Material).

Approximately 16% of the initial carbon content of the rFCW was in the form of protein. Recent studies have emphasized the beneficial role of proteins in the enrichment process, as far as they do not exceed 30% of the COD content (Roibás-Rozas et al., 2021). In fact, higher protein concentrations can lead to the competitive growth of undesired microorganisms that do not accumulate PHA (Argiz et al., 2020). In this study, during each 12 h-cycle, the protein concentration decreased by an average of 42%, with a specific consumption rate of 2 $mg_{C\text{-}PROTE}$ $g_{C\text{-}X}$ h^{-1}, mainly during the famine phase. These findings suggest the capability of the selected PHA-storing MMC to utilize the nitrogen content of rFCW, in contrast with other studies stating the need to provide readily available nutrients during the selection process (Oliveira et al., 2018).

Table 2
Main operation and performance parameters from the selection/enrichment stage (Stage III).

Parameters	Unit of measure	Value
Feast-to-famine ratio (F/F)	h h^{-1}	0.17±0.01
Biomass	g_{VSS} L^{-1}	1.39±0.29
X_a*	g L^{-1}	1.31±0.27
PHA content*	%wt	14 ± 0
3HV fraction*	%wt	59±1
$-q_{TOC}$*	$mg_{C\text{-}TOC}$ $g^{-1}_{C\text{-}X}h^{-1}$	71±36
$-q_{OA}$*	$mg_{C\text{-}OA}$ $g^{-1}_{C\text{-}X}h^{-1}$	69±36
q_{PHA}*	$mg_{C\text{-}PHA}$ $g^{-1}_{C\text{-}X}h^{-1}$	32±6
$-q_{PROTE}$	$mg_{C\text{-}PROTE}$ $g^{-1}_{C\text{-}X}h^{-1}$	2±0
$Y_{PHA/TOC}$*	$g_{C\text{-}PHA}$ $g^{-1}_{C\text{-}TOC}$	0.45
$Y_{PHA/OA}$*	$g_{C\text{-}PHA}$ $g^{-1}_{C\text{-}OA}$	0.46
$Y_{X/OA}$	$g_{C\text{-}X}$ $g^{-1}_{C\text{-}OA}$	0.29

* calculated at the end of the feast phase.

The successful selection and harvesting of a PHA-storing MMC was confirmed by a 14% PHA content observed at the end of the feast phase. The PHA obtained was a copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), with HV comprising 59% of the polymer.

A typical profile of organic acids (OA), intracellular PHA, proteins, and dissolved oxygen (DO) observed during a representative SBR cycle (cycle 43, SRT 6) in the selection and enrichment of the PHA-storing MMC is provided in Supplementary Material.

The effluent from the selection/enrichment reactor exhibited a DOC concentration of approximately 100 mg_C L^{-1}, partially consisting of residual whey proteins. Therefore, it can be potentially recirculated to decrease freshwater use for diluting the rFCW, resulting in higher process efficiency and sustainability.

3.4. PHA accumulation stage

To evaluate the PHA accumulation potential of the selected MMC, biomass samples were taken at the end of a feast and famine cycle, between days 24 and 36. Accumulation fed-batch tests were conducted using both FCW and pFCW as substrates. The main performance parameters for each substrate are summarized in Table 3. The maximum PHA content and storage yield ($Y_{PHA/TOC}$) obtained from pFCW were 37%±1 wt and 0.53±0.11 $g_{C\text{-}PHA}$ $g^{-1}_{C\text{-}TOC}$, respectively (Fig. 4). These values were significantly ($p < 0.05$) higher than those obtained from FCW, which resulted in a PHA content of 28%±3 wt and a storage yield of 0.25±0.09 $g_{C\text{-}PHA}$ $g^{-1}_{C\text{-}TOC}$, respectively. The maximum PHA content achieved in this study falls within the range reported in other studies performed using fermented CW as the substrate (Asunis et al., 2022; Colombo et al., 2016; Lagoa-Costa et al., 2023; Oliveira et al., 2018).

The nutrient-limiting conditions and high VFA concentration of pFCW promoted PHA accumulation. In contrast, when the nutrients-containing substrate FCW (C/N ratio of 12.9) was fed, competitive and simultaneous growth and storage occurred, reducing the carbon available for PHA storage (Johnson et al., 2009; Matos et al., 2021c; Oliveira et al., 2017). Specifically, a growth rate of 46 $mg_{C\text{-}X}$ $mg_{C\text{-}Xi}$ h^{-1} was observed with FCW (Table 1). This was approximately two times higher than the specific growth rates obtained with pFCW (26 $mg_{C\text{-}X}$ $mg_{C\text{-}Xi}$ h^{-1}) suggesting microbial growth over PHA storage and possible proliferation of competing microorganisms on the FCW. The profiles of OA, PHA and proteins concentration during the accumulation stage with pFCW are shown in Fig. 4.

The produced PHA was a copolymer of 3HB and 3HV monomers, with HV content of 46% and 59% for pFCW and FCW, respectively. Depending on the number of carbon atoms in their backbone, VFAs can be classified as even-numbered or odd-numbered. Even-numbered VFAs are suitable as precursors for 3HB, while odd-numbered VFAs are precursors for 3HV (Sekoai et al., 2022). The higher proportion of 3HB when using pFCW is attributable to the higher butyric acid ratio (40% of

Table 3
Main operational and performance parameters obtained in the accumulation stage.

Parameters	Unit of measure	Fermented CW FCW	Permeate FCW pFCW
Test duration	h	8	8
PHA content	%wt	28±1	37±1
HB:HV**	%wt	41:59	54:46
$-q_{TOC}$	$mg_{C\text{-}TOC}$ $g^{-1}_{C\text{-}X}h^{-1}$	353±99	209±20
$-q_{OA}$	$mg_{C\text{-}OA}$ $g^{-1}_{C\text{-}X}h^{-1}$	325±106	202±12
q_{PHA}	$mg_{C\text{-}PHA}$ $g^{-1}_{C\text{-}X}h^{-1}$	84±6	110±12
$-q_{PROTE}$	$g_{C\text{-}PROTE}$ $g^{-1}_{C\text{-}X}h^{-1}$	28±6	2±1
μ_{max}	$mg_{C\text{-}X}$ $mg_{C\text{-}Xi}$ h^{-1}	46±20	26±6
$Y_{PHA/TOC}$	$g_{C\text{-}PHA}$ $g^{-1}_{C\text{-}TOC}$	0.25	0.53
$Y_{PHA/OA}$	$g_{C\text{-}PHA}$ $g^{-1}_{C\text{-}OA}$	0.26	0.55
$Y_{X/TOC}$	$g_{C\text{-}X}$ $g^{-1}_{C\text{-}TOC}$	0.25	0.13

n.a. not applicable.
** Measured at the end of the test.

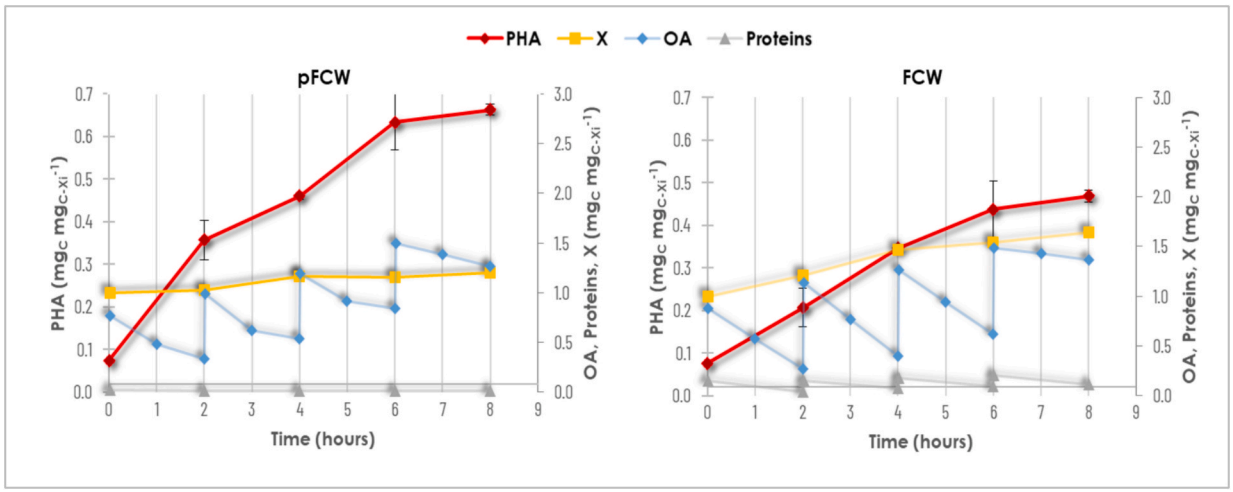

Fig. 4. Specific concentrations of PHA, OA, protein, and active biomass (X) during the PHA accumulation tests performed with permeate fermented cheese whey (pFCW) (left) and fermented cheese whey (FCW) (right). All concentrations are expressed as specific concentrations).

the total carbon), as compared to FCW, which contains only 17% butyric acid. These considerations underline the inherent potential of the silicon membrane to fine-tune the composition of the VFAs pool for the PHA accumulation phase.

3.5. Overall performances and future research directions

A mass balance was conducted to define the amount of sheep cheese whey necessary to produce 1 kg of PHA (with an 3HV fraction of 46%), and define the carbon flow over the different process steps (see sections 3.3 and 3.4).

Considering the PHA content and storage yield achieved with pFCW (37% wt, 0.55 $g_{C\text{-}PHA}\ g^{-1}_{C\text{-}OA}$) and the biomass growth yield observed with rFCW (0.29 $g_{C\text{-}X}\ g^{-1}_{C\text{-}OA}$), the amount of OA required to produce 1 kg of PHA was estimated as 3.11 $kg_{C\text{-}OA}$ for the selection stage and 1.05 $kg_{C\text{-}OA}$ for the accumulation stage (more details on the assumptions used are provided in the Supplementary Material). Thus, up to 75% of the organic acids generated during fermentation are required to grow and select MMCs capable of PHA production, while the remaining 25% is utilized to synthesize the final PHA product. The OA partitioning observed in the extraction stage was 80/20 split between rFCV and pFCW (on a C-OA basis), which closely aligns with the calculated ratio of 75/25. Overall, 1 kg of PHA requires 213 kg of cheese whey (CW), which corresponds to 4.7 g of PHA per kg of CW (Fig. 5).

Despite such near-optimal partitioning, the results suggest that the silicone membrane extraction process still requires further optimization to ensure that enough OA are available for maximizing PHA accumulation in the accumulation reactor and attaining good process efficiency. Specifically, increasing the membrane surface area or utilizing thinner membranes (up to 0.5 mm wall thickness) could enhance the flux of OA

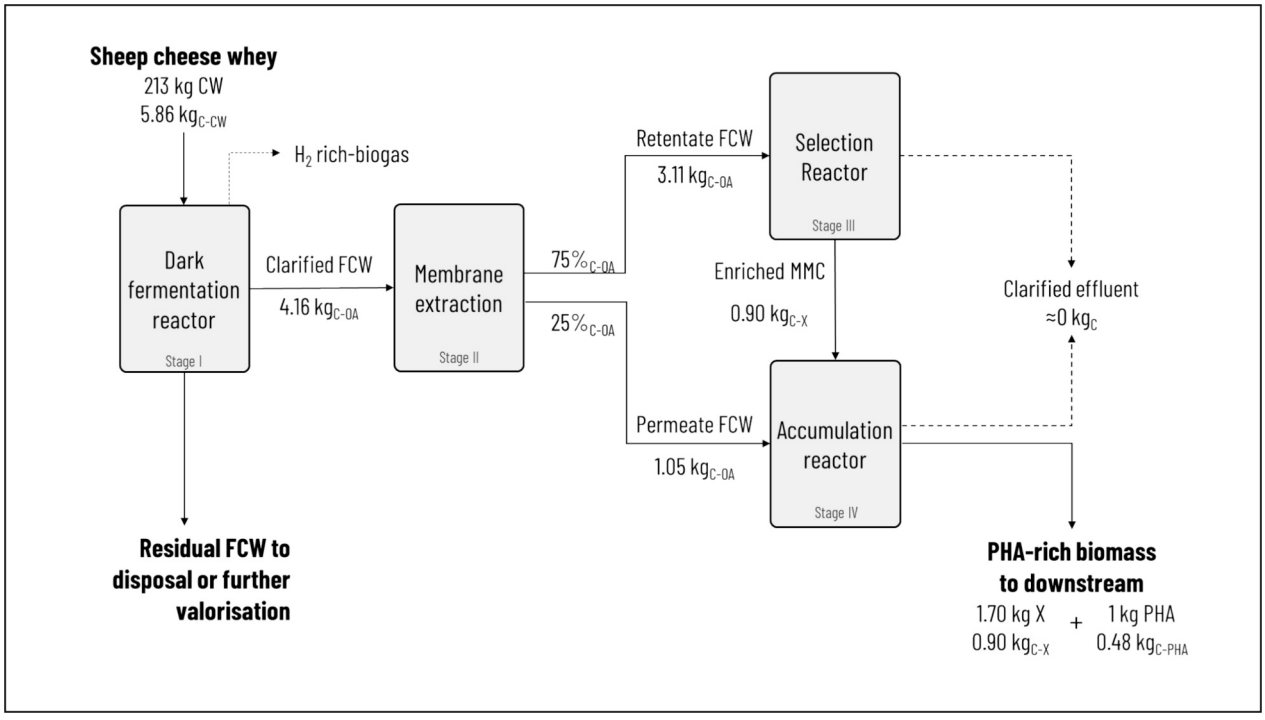

Fig. 5. Overall carbon mass balance and flow sheet of the proposed 4-stage process for the production of 1 kg of PHA. Further details about the calculation have been included in the Supplementary Material.

through the membrane, providing a greater quantity of OA per time unit to the accumulation reactor (Adeel, 2024). These adjustments would help meet the OA demand for optimal PHA production.

Furthermore, the implementation of the silicone membrane extraction stage shows promising prospects in the PHA production process. The results obtained in this study show a good separation efficiency of VFA, which reached 30% (as $g^{pFCW}_{C\text{-}VFA}$ $g^{FCW\text{-}1}_{C\text{-}VFA}$). For butyric acid, the separation efficiency was fairly close to the theoretical maximum obtainable (50%, due to the requirement for concentration gradient). On the contrary, lactic acid does not diffuse through the membrane. Thus, the process would benefit from the optimization of the fermentation phase aimed at the total conversion of lactic acid into VFAs, as previously reported (Asunis et al., 2019; Duque et al., 2014).

Finally, the PHA productivity strongly depends on the specific type of substrate. Thus, further experiments must be conducted with other nutrient-rich substrates, including other dairy residues (cow and goat cheese whey), molasses or food waste. The integration of silicone membranes offers a promising route for optimizing the feed streams for both biomass selection and PHA accumulation. Additionally, the cost-effectiveness and energy efficiency of silicone membranes, not requiring energy investment for VFA diffusion, provide a flexible and scalable solution for enhancing the economic viability of waste-to-PHA processes, paving the way for more sustainable bioplastic production systems in the future.

4. Conclusions

This study introduces a novel four-stage process for PHA production from sheep cheese whey (CW), integrating a VFA extraction step through silicone membrane to optimize the C/N ratio for both the selection and accumulation stages. Using the carbon deprived, fermented CW permeate (pFCW) resulted in a maximum PHA content of 37%, significantly higher than the 28% achieved when using raw FCW. The integration of the silicone membrane may also enable tuning of the PHA polymer composition by modifying the VFA distribution. These findings highlight the potential of this method for sustainable biopolymer production; however, further studies are needed to assess its performance on a larger scale and with different substrates.

CRediT authorship contribution statement

Fabiano Asunis: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Paolo Dessì:** Writing – review & editing, Conceptualization. **Giorgia De Gioannis:** Writing – review & editing, Resources, Conceptualization. **Aldo Muntoni:** Writing – review & editing, Resources, 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132314.

Data availability

Data will be made available on request.

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