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Research article

Combined biohydrogen and polyhydroxyalkanoates production from sheep cheese whey by a mixed microbial culture

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ABSTRACT

The present study investigates the combined production of biohydrogen and polyhydroxyalkanoates (PHA) from sheep cheese whey through a 3-stage bioprocess, i.e. dark fermentation, selection of PHA storing microorganisms, and PHA accumulation. Batch dark fermentation tests (Stage I) were performed on raw cheese whey under different pH operating conditions, avoiding either the addition of inoculum or substrate pre-treatment to support the economic and technical feasibility of the proposed process. The performance of the fermentative stage was assessed in terms of biohydrogen and soluble metabolites production yields. The dark fermentation effluent was used as organic acid-rich feedstock either for selecting and harvesting PHA storing microorganisms from a mixed microbial culture without the addition of external nutrient sources (Stage II) or for the PHA accumulation by the selected biomass (Stage III). The results of the study support the possibility of achieving combined recovery yields of 5.3 L biohydrogen and 7.6 g PHA per litre of fed sheep cheese whey in the case of optimal dark fermentation pH setting (pH = 6). Such outcomes underline the untapped potential of sheep cheese whey for the recovery of high-added value bioproducts.

1. Introduction

The European Union's (EU) strategy on circular bioeconomy considers biowaste a widely available and renewable resource to be converted into biofuels and bio-based products via various technologies (European Commission, 2018). To this end, optimising valorisation is necessary both in qualitative and quantitative terms to boost the marketing of recovered products and approximate the zero-waste goal. This goal requires the integration of different processes, which are increasingly made available by advances in biotechnology and other sectors. The type and number of the processes to be implemented, the complexity of their combination, and the targeted outputs depend on numerous local and strategic factors and conditions, such as the availability of waste biomass and market demands. Waste biorefinery is the commonly used definition for such a combination of processes, and the concept is deemed to play a pivotal role in promoting the transition from a fossil-based economy to the more sustainable circular bioeconomy (Alibardi et al., 2020). The implementation of the circular bioeconomy is required on different scales to address either national and supranational strategic needs, or local ones to support resource recovery delocalisation and waste short-chain handling. In this respect, implementing the waste biorefinery concept also requires the ability to identify regional potential and opportunities.

In this framework, the present study deals with the valorisation of sheep cheese whey (SCW), the main biowaste generated by the sheep dairy industry. Sheep milk production - 3 million tons in 2020 in the EU - represents a crucial economic sector in southern European countries such as Greece, Italy, Spain, and France (Eurostat, 2020), where sheep grazing represents a primary source of employment in disadvantaged agricultural areas contexts and a fundamental part of the rural

Abbreviations: CW, Cheese whey; DF, Dark fermentation; DO, Dissolved oxygen; DOC, Dissolved organic carbon; EU, Europe Union; F/F, Feast to famine ratio; FSCW, Fermented sheep cheese whey; HB, Hydroxybutyrate; HRT, Hydraulic retention time; HV, Hydroxivalerate; LA, Lactic acid; LAB, Lactic acid bacteria; MMC, Mixed microbial cultures; OA, Organic acids; OLR, Organic loading rate; PHA, Polyhydroxyalkanoates; SBR, Sequential batch reactor; SCW, Sheep cheese whey; VFA, Volatile fatty acids; SRT, Sludge retention time; WWTP, Waste water treatment plant.

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landscape. However, while the high-quality traditional dairy products contribute to preserving the environment and social cohesion in rural areas (Rossi, 2016; Vagnoni and Franca, 2018), the sheep dairy supply chain experienced painful economic difficulties in recent years mainly due to price volatility and low remuneration of the raw materials and products. In addition, other constraints, such as the small size of dairy farms, ageing of workers, and low generational turnover (Augere--Granier, 2018) adversely affect the sector, aggravating the burden of energy and biowaste management costs and, at the same time, exacerbating the need to innovative approaches that may expand the spectrum of marketable bio-based products.

The high specific production (around 0.9 L per L of processed milk or 9 kg per kg of produced cheese) and compositional characteristics make SCW a problematic biowaste to manage, but also a potential feedstock worth of valorisation (Asunis et al., 2020). In fact, SCW is characterised by high organic carbon content, consisting primarily of soluble carbohydrates, lactose in particular (around 39–60 g L^{-1}, Prazeres et al. (2012)). Other main components are proteins (5.5 % wt) and fats (5.9 % wt), whose content is higher than in cow cheese whey (3.4 and 3.3 % wt, respectively). Citric acid, vitamins and minerals are other compounds of interest (Balthazar et al., 2017).

However, despite the interesting peculiarities, SCW has been much less studied than cow cheese whey (Sánchez-Moya et al., 2020). The reasons can be found in the smaller size of the European sheep dairy industry (only 2% of the European milk production comes from sheep) and its lower attitude towards technological innovation and production processes upgrading (Concu et al., 2020). The SCW management is often limited to use as animal feed or land spreading, which are considered not fully sustainable nowadays. On the other hand, traditional chemical-physical and aerobic biological treatments are costly and do not allow valorisation (Ahmad et al., 2019; Dąbrowski et al., 2017). Anaerobic digestion allows for energy recovery, but the high organic content and low alkalinity of cheese whey may lead to process inhibition by volatile fatty acids (VFA) accumulation (Carvalho et al., 2013; De Gioannis et al., 2014).

Therefore, it is interesting to explore innovative approaches to SCW management, potentially able either to avoid environmental problems or to provide renewable and clean energy, and innovative bioproducts which can strengthen the dairy supply chain (Vagnoni and Franca, 2018).

In this framework, the present study focuses on the combined recovery of biohydrogen and polyhydroxyalkanoates (PHA) biopolymers from SCW through a multi-stage bioprocess. Fermentative production of biohydrogen from cheese whey has been the subject of several studies (Akhlaghi et al., 2017; Blanco et al., 2019; De Gioannis et al., 2014; Dessì et al., 2020; Ferreira Rosa et al., 2014b; Montecchio et al., 2018; Ribeiro et al., 2022). Although the results have demonstrated its technical feasibility, most studies also stressed the need for synergy with other processes to complete the valorisation of the organic substrate (Montecchio et al., 2018; Asunis et al., 2019; Blanco et al., 2019). Therefore, the study of possible combinations of processes that complete and enhance the fermentation production of biohydrogen is considered of great interest by the scientific community. In particular, the concept of integrated valorisation within a waste biorefinery framework pushes towards the combined production of biofuels/energy carriers with that of high-value biomaterials (Alibardi et al., 2020).

Indeed, during dark fermentation, only 30–40% of the organic substrate is converted to gaseous by-products (H_2 + CO_2), while the remaining 60–70% is converted into a pool of soluble metabolites, mainly organic acids (OA), whose composition depends on the prevailing specific metabolic pathways (Sarma et al., 2015). Being the OA characterised by a considerable commercial value, the exploitation of such metabolites may involve direct separation and commercialisation of products such as lactic acid (Luongo et al., 2019) or butyric acid (Dessì et al., 2020). Alternative valorisation routes, rather than requiring specific separation methods of the metabolic products, are based on further processing of the whole OA pool produced during fermentation.

In this respect, PHA granules can be produced from various organic substrates, including OA, and accumulated into bacterial cells of different genera as carbon and energy storage. Nowadays, PHA are of great interest due to its properties comparable to petroleum-based plastics (Palmeiro-Sánchez et al., 2022). The global PHA market is driven mainly by the focus on green procurement policies of the governments, and it is projected to exceed 100 million € by 2025, with an annual growth rate of 14% over the 2020–2025 period (Markets and Markets, 2022). However, the PHA market is currently limited by the high production costs compared to conventional polymers, primarily due to the use of pure cultures or genetically modified bacteria and expensive feedstock (Mannina et al., 2020). The use of a low-cost biowaste as the feedstock could overcome these issues and make PHA competitive with fossil-based plastic (Palmeiro-Sánchez et al., 2022).

Further benefits may be achieved by combining a waste-derived carbon source and mixed microbial cultures (MMC), as the latter do not require sterile conditions (Mannina et al., 2020). Different studies have considered PHA production by MMC from whey-based substrates, such as raw cow cheese whey (Colombo et al., 2016; Valentino et al., 2015), second cheese whey and concentrated cheese whey powder (Colombo et al., 2019), cheese whey powder (Duque et al., 2014; Gouveia et al., 2017; Oliveira et al., 2018) and whey permeate (Carletto, 2014).

PHA production by MMC can be achieved with various process configurations, among which the so-called “three-stage process” is the most used (Valentino et al., 2017), and was applied in the present study.

According to this approach, a fundamental role is played by nutrients, nitrogen in particular. In this respect, compared to the studies available in the literature, a promising and innovative approach involves evaluating whether the process may take advantage from the high nitrogen content of SCW and may be rearranged accordingly.

The present study was prompted by these objectives. The high organic content of SCW was converted through batch dark fermentation (DF) (stage I) into H_2-containing biogas and OA. The obtained OA-rich stream was then used as the feedstock to select and harvest a PHA storing MMC by operating an aerobic sequencing batch reactor (SBR) under a feast and famine regime, i.e. alternating periods of availability and absence of the carbon source (stage II). Finally, the same OA-rich stream was fed to the selected MMC in a batch accumulation reactor to assess the maximum PHA storage capacity (stage III). The results obtained were used as the starting point to develop preliminary considerations on the design of a biorefining plant to be located in Sardinia, which is one of the largest sheep milk production areas in Europe (Concu et al., 2020; Vagnoni and Franca, 2018).

To the best of the authors’ knowledge, no similar studies have been conducted so far on cheese whey of ovine origin. The authors are confident that the present work can provide valuable information for introducing new innovative valorisation routes within the sheep dairy supply chain and in the framework of the circular bioeconomy.

2. Materials and methods

2.1. Feedstock

The raw SCW was obtained from a medium-sized dairy plant in southern Sardinia (Italy) that processes sheep milk to produce Pecorino cheese. As described in a previous study, the collected SCW was stored at −15 °C and thawed before the fermentation tests (Asunis et al., 2019). The most significant parameters of the SCW characterisation are shown in Table 1. All the parameters were measured on 0.45-μm filtered samples, except for total organic carbon (TOC) and total and volatile solids (TS, VS). It was assumed that soluble carbohydrates consist only of lactose ($C_{12}H_{24}O_{11}$), and soluble proteins have an average C content of 0.46 g g^{-1} (Rouwenhorst et al., 1991). The C/N ratio was calculated

Table 1
Main characterisation parameters of raw sheep cheese whey (SCW) and fermented sheep cheese whey (FSCW) under different pH conditions; average values ± standard deviation; < D.L.: below detection limit; UCpH stands for the test performed under uncontrolled pH conditions.

Parameter	Unit of measure	SCW	FSCW-UCpH	FSCW-6	FSCW-7.5
pH	–	6.06 ± 0.63	no pH control	6.0 ± 0.1	7.5 ± 0.1
Total solids (TS)	g L^{-1}	7.74 ± 0.82	7.38 ± 0.78	5.92 ± 0.73	3.31 ± 0.01
Volatile solids (VS)	g L^{-1}	7.17 ± 0.79	6.64 ± 0.73	3.11 ± 0.01	1.51 ± 0.01
Total organic carbon (TOC)	g L^{-1}	32.55 ± 1.11	32.37 ± 0.23	24.03 ± 2.70	24.90 ± 2.50
Dissolved organic carbon (DOC)	g L^{-1}	28.35 ± 1.57	26.36 ± 0.32	19.31 ± 0.40	19.37 ± 0.30
Soluble carbohydrates[a]	g L^{-1}	45.10 ± 3.74	29.82 ± 0.35	< D.L.	< D.L.
Soluble proteins[b]	g L^{-1}	10.08 ± 0.62	9.60 ± 0.04	4.58 ± 0.18	6.90 ± 0.48
Ammonia Nitrogen (NH4+-N)	g L^{-1}	0.41 ± 0.05	0.66 ± 0.01	0.26 ± 0.07	0.76 ± 0.04
Lactic acid	g L^{-1}	4.33 ± 1.05	20.21 ± 0.11	2.55 ± 0.60	4.20 ± 1.30
Total organic acids (OA)	g L^{-1}	3.70 ± 1.20	20.31 ± 0.21	29.23 ± 0.80	24.42 ± 1.40
OA composition Ac/La/Pr/Bu/Va	% molC	0/100/0/0/0	1/99/0/0/0	4/10/25/60/1	42/16/19/21/0
C/N	gC gN^{-1}	16.3	16.0	24.6	13.5

[a] Expressed as lactose.
[b] Expressed as bovine serum albumin (BSA).

considering the TOC and the soluble nitrogen, measured as the sum of N–NH_4^+ and soluble N-proteins, adopting a conversion factor of 6.38 $g_{Protein}$ g_N^{-1}, (Mariotti et al., 2008).

2.2. Experimental set-up

The adopted experimental set-up consists of 3 lab-scale bioreactors (see Fig. 1): a DF batch reactor (stage I), a Sequencing Batch Reactor (SBR) for PHA-storing biomass selection and enrichment (stage II) and a fed-batch reactor for PHA accumulation (stage III).

2.3. Dark fermentation of sheep cheese whey (stage I)

The dark fermentation tests were carried out in a 2-L stirred glass reactor (BIOFLO 110, New Brunswick Scientific, USA; 1.8 L working volume) operated under mesophilic (39 ± 1 °C) batch conditions. Raw undiluted SCW was used as the substrate. No external inoculum was added as previous studies suggested that the cheese whey indigenous biomass, such as lactic acid bacteria (LAB), is able to sustain the fermentation process (Akhlaghi et al., 2017; De Gioannis et al., 2014). Similarly, no nutrients were added, assuming that these are provided by the SCW components (whey proteins, mineral salts, lipids, and vitamins), as also reported in other studies (Colombo et al., 2016; Duque et al., 2014; Oliveira et al., 2018). The reactor was preliminarily flushed with N_2 gas to displace oxygen from the headspace. Two operating pH (6.0 and 7.5) were adopted based on a previous study since they are expression of two different cheese whey fermentation pathway (Asunis et al., 2019), and the set values were automatically controlled by adding 5 M NaOH. The Fermented SCW (FSCW) samples are identified in the text as FSCW-6 and FSCW-7.5, respectively. Tests with no pH control (UCpH) were also performed for reference purposes. The volume of gas produced was measured using the volume displacement principle. The fermentation tests were stopped after 7 days as both metabolite concentration and cumulative gas production did not show further increases. Each test was run in triplicate, and the results are reported as average values. The FSCW was centrifugated, then the supernatant stored in 2-L bottles at −15 °C and thawed when necessary before analytical characterisation. The supernatant was used as the substrate for the biomass selection and PHA accumulation phases while the residual biomass from fermentation was collected for further studies (data not shown here). The main characterisation parameters for the FSCW are shown in Table 1.

2.3.1. Selection and enrichment of the PHA-storing mixed microbial culture (stage II)

Selection and enrichment of the PHA-storing MMC were carried out

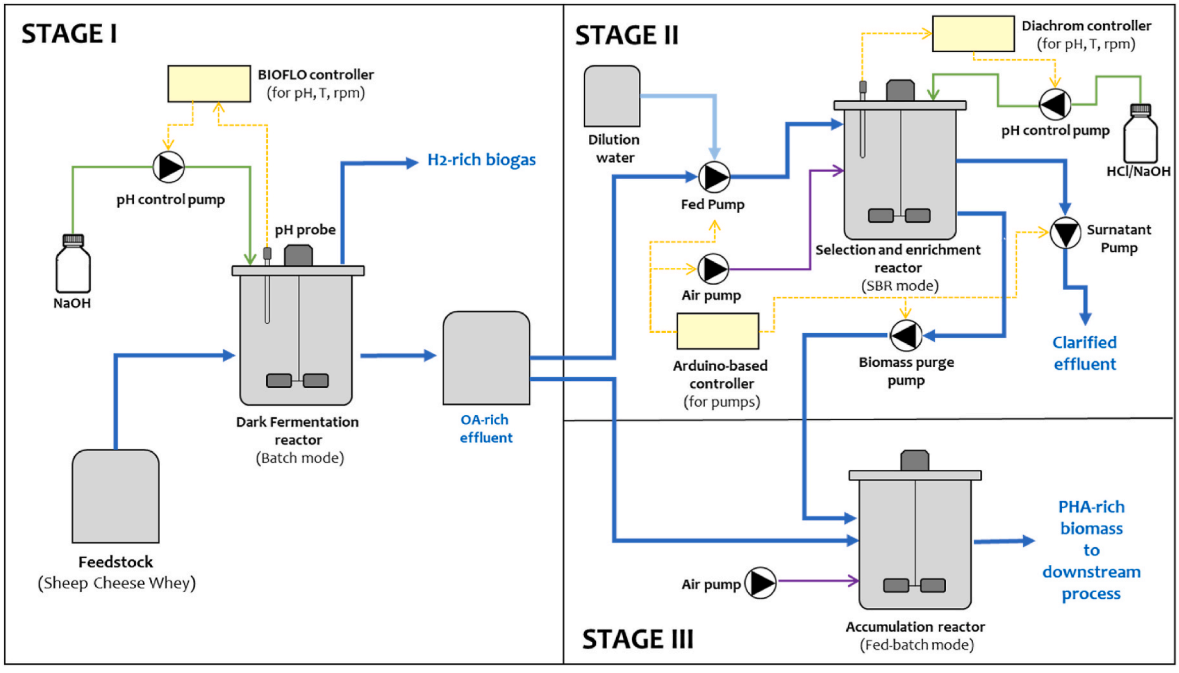

Fig. 1. Experimental set-up used to perform the three-stage process: dark fermentation (DF) (stage I), selection and enrichment of PHA-storing mixed microbial culture (MMC) (stage II) and PHA accumulation (stage III).

in an aerobic SBR (Diachrom Biotechnology, Switzerland; 4 L working volume) by alternating periods of availability (feast) and absence (famine) of the carbon source (feast-famine regime, Duque et al., 2014). The SBR was inoculated with fresh activated sludge (8 g_{TS} L^{-1}) sampled from the aerobic tank of a municipal wastewater treatment plant (WWTP). The SBR cycle lasted 12 h and consisted of five phases: (i) influent feeding (4 min), (ii) aeration (671 min), (iii) biomass purge (1 min), (iv) settling (40 min), and (v) withdrawal of the clarified supernatant (4 min) (Figure S1). The hydraulic retention time (HRT) and the sludge retention time (SRT) were set to 1 and 4 days, respectively. The SBR was operated at 25 °C, and the operating pH was set in the range 7–9, automatically controlled by adding 2 M HCl or 10 M NaOH. Air was supplied through a ceramic diffuser at a rate of 200 NL h^{-1}. The biomass selection process was monitored over time by calculating the ratio between the duration of the feast and famine phases (feast to famine ratio, F/F, h h^{-1}). The dissolved oxygen (DO, mg L^{-1}) concentration profile was used to determine the feast-famine boundaries.

Three different selection and enrichment runs were carried out. The first one, aimed at a preliminary feasibility assessment, was performed by feeding a synthetic medium. The second and third runs used the fermented whey FSCW-6 and FSCW-7.5, respectively, as the influent. Distilled water was used to dilute FSCW according to the desired OLR.

The synthetic medium used for the first run contained CH_3COONa (1.395 g L^{-1}), NH_4Cl (0.214 g L^{-1}) and KH_2PO_4 (0.054 g L^{-1}), and was supplemented by a mineral medium prepared according to Silva et al. (2017). During the first run, the organic loading rate (OLR) was 34.0 $mmol_C$ L^{-1} d^{-1} and the C/N molar ratio was equal to 10.

The second and third runs were carried out adopting OLR values of 40.6 ± 6.7 $mmol_C$ L^{-1} d^{-1} (FSCW-6) and 40.0 ± 1.6 $mmol_C$ L^{-1} d^{-1} (FSCW-7.5), respectively.

Allylthiourea (20 mg L^{-1}) was added to inhibit nitrification (Colombo et al., 2016; Duque et al., 2014).

2.3.2. PHA accumulation (stage III)

Similarly to the biomass selection phase, the process of PHA accumulation was carried out in a 1-L working volume fed-batch reactor, by feeding first the synthetic medium and subsequently the fermented SCW with 500 mL of enriched culture drawn from the SBR, according to a pulse-wise method controlled by the observed DO evolution (Colombo et al., 2016). The synthetic medium was prepared according to Silva et al. (2017), and it did not contain nitrogen nor phosphorus to ensure nutrient-limiting conditions. Air was supplied through a ceramic diffuser, and the DO was continuously acquired by a polarographic probe (InPro 6800, Mettler Toledo). The total fed C was set to apply the same carbon-to-microorganism ratio adopted in the biomass selection reactor (Colombo et al., 2016). All the tests were carried out in duplicate, at room temperature (25 °C), with no pH control, adopting magnetic stirring (300 rpm), and stopped when no DO variation was observed after substrate feeding.

2.4. Analytical methods

The analytical methods for the determination of total, volatile and volatile suspended solids (TS, VS and VSS), total organic carbon and its dissolved fraction (TOC and DOC), soluble carbohydrates and proteins, ammonia, as well as for the assessment of biohydrogen and OA production, are reported in Asunis et al. (2019).

Each mixed liquor sample (5 mL) was treated immediately with 1 mL of a NaClO solution (7% active Cl_2), according to Silva et al. (2017), and stored at −4 °C until the assessment of the PHA content was carried out. PHA were extracted, hydrolysed, and determined by gas chromatography (Serafim et al., 2004). The sample was centrifuged twice (11 000 rpm, 15 min), and the supernatant was discharged. Afterwards, the remaining solid material underwent methanolysis in a 20% vol. H_2SO_4 in methanol solution (1 mL), and extracted with chloroform (1 mL) at 100 °C for 3.5 h. A sample of 1 μL containing the methylated monomers dissolved in chloroform was injected into a gas chromatograph (model 7890 B, Agilent Technology, USA) equipped with a flame ionisation detector and a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology, USA) using helium as the 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} up to 100 °C, then 3 °C min^{-1} up to 175 °C and then 20 °C up to a final temperature of 220 °C (4-min holding time). The concentration of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) were quantified using a commercial P(HB/HV) copolymer with 12% w/w HV content (Sigma-Aldrich, CAS number 80181-31-3). Benzoic acid (50 mg L^{-1}) was used as an internal standard and added before the methanolysis step. All the analyses were performed in triplicate and the results are presented as the average values of the replicates with the associated standard deviation.

2.5. Calculations

The biohydrogen production yield ($Y_{H2/SCW}$) was normalised per unit of initial TOC in SCW and expressed as mol_{H2} $mol_{C\text{-}SCW}^{-1}$. The fermentation yield ($Y_{OA/SCW}$) was calculated as the ratio between the OA produced and the initial TOC content, both expressed on a carbon basis. The OA concentration was assumed as the of lactic acid (LA) and VFA concentrations.

The PHA content was determined on a dry mass basis and expressed as a percentage of VSS. The concentration of active biomass (X) was estimated from the difference between the VSS and PHA concentrations (Duque et al., 2014). A biomass carbon content of 44.2 $mmol_{C\text{-}X}$ g_X^{-1} was assumed on the basis of the chemical formula $C_5H_7NO_2$ (Valentino et al., 2014).

Protein consumption (Δ_{PROTE}) in stage II was calculated as the percent difference between the initial and final mass of soluble proteins divided by the initial mass.

The OA uptake rate (-q_{OA}, as $mol_{C\text{-}OA}$ $mol_{C\text{-}X}^{-1}$ h^{-1}), PHA storage (q_{PHA}, as $mol_{C\text{-}PHA}$ $mol_{C\text{-}X}^{-1}$ h^{-1}), and protein uptake by biomass (-q_{PROTE}, as $mol_{C\text{-}PROTE}$ $mol_{C\text{-}X}^{-1}$ h^{-1}) were derived from linear regression of the evolution over time of OA (as $mol_{C\text{-}OA}$ $mol_{C\text{-}X}^{-1}$), PHA (as $mol_{C\text{-}PHA}$ $mol_{C\text{-}X}^{-1}$), and protein (as $mol_{C\text{-}PROTE}$ $mol_{C\text{-}X}^{-1}$) concentrations. The PHA storage yield ($Y_{PHA/OA}$) was calculated as the ratio between q_{PHA} and -q_{OA}.

Rates and yields were assessed for each of the pulses performed during the accumulation test, and the average values were calculated considering the first three pulses (Colombo et al., 2016).

The overall PHA yield ($Y_{PHA/SCW}$, kg_{PHA} t_{SCW}^{-1}) was derived from the mass balance of the process, as explained in detail in Asunis et al. (2021). Finally, the comments reported in section 3.3 focusing on the possible energy recovery associated with the process are based on the lower heating value of H_2 and CH_4, equal to 12.74 MJ Nm^{-3} and 35.16 MJ Nm^{-3}, respectively.

3. Results and discussion

3.1. Stage I – dark fermentation of sheep cheese whey

The performance of the SCW dark fermentation stage was evaluated in terms of OA and H_2 yield (Table 2). As far as the production of soluble

Table 2
Performance data for sheep cheese whey (SCW) dark fermentation (stage I) (average values ± standard deviation).

Parameter	Unit of measure	UCpH	pH 6	pH 7.5
$Y_{H2/SCW}$	$mmol_{H2}$ $mol_{C\text{-}SCW}^{-1}$	0.00	87 ± 6	36 ± 2
	L_{H2} L_{SCW}^{-1}	0.00	5.30 ± 0.37	2.22 ± 0.14
H_2	%vol.	n.d.	42 ± 1	67 ± 7
$Y_{OA/SCW}$	$mol_{C\text{-}OA}$ $mol_{C\text{-}SCW}^{-1}$	0.242 ± 0.001	0.534 ± 0.019	0.510 ± 0.105

metabolic products is concerned, the evolution over time shows that the fermentation of SCW occurred in two distinct phases, as already discussed in Asunis et al. (2019) and also mentioned by García-Depraect et al. (2021). First, the original lactose content was converted by LAB into lactic acid, and then the latter was used by the lactate-fermenting and hydrogen-producing bacteria to produce gaseous H_2 and a pool of VFA. As expected, the operating pH affected the DF process significantly. The highest $Y_{H2/SCW}$ (87 $mmol_{H2}$ $mol^{-1}_{C\text{-}SCW}$ or 0.85 mol_{H2} mol^{-1}_{hexose}, corresponding to 5.3 L_{H2} L^{-1}_{SCW}) was observed at pH 6, whilst the performance dropped by 60% at pH 7.5. The best performance attained is consistent with data available in the literature for CW (0.7–1.5 mol_{H2} mol^{-1}_{hexose}), though it is worth stressing that most of the studies focused on CW of different origin (Antonopoulou et al., 2008; Carrillo-Reyes et al., 2014; Castelló et al., 2020; Davila-Vazquez et al., 2008; Dessì et al., 2020; Ferreira Rosa et al., 2014a; Ribeiro et al., 2022; Venetsaneas et al., 2009).

On the other hand, the overall OA yield ($Y_{OA/SCW}$), spanning the range 0.51–0.53 $mol_{C\text{-}OA}$ $mol^{-1}_{C\text{-}SCW}$, was more similar at the two pH values. However, it is worth mentioning that when fermentation was performed without any pH control, the OA production was much lower (0.24 $mol_{C\text{-}OA}$ $mol^{-1}_{C\text{-}SCW}$). By comparison, Colombo et al. (2016) reported similar OA yields using raw and sterilised CW in mesophilic batch fermentation using autochthonous LAB and heat-shocked digestate as the inoculum (0.4 and 0.6 $mol_{C\text{-}OA}$ $mol^{-1}_{C\text{-}soluble\ substrates}$, respectively). Similarly, Duque et al. (2014) reported a yield of 0.64 $g_{COD\text{-}OA}$ $g_{COD\text{-}soluble\ substrates}^{-1}$ using CW powder in an anaerobic membrane bioreactor.

The operating pH influenced the composition of the pool of soluble by-products. Butyrate was the most abundant (60% on a mol C-basis) at pH 6, while acetate was predominant in FSCW-7.5 (42% on a mol C-basis). The residual lactate fell in the range 10–16% on a mol C-basis, with the notable exception of the test performed without pH control as, in this case, the DF process stopped at lactose conversion to lactic acid, confirming the inhibitory effect of adverse pH on hydrogen-producing bacteria (García-Depraect et al., 2020). For the pH-controlled tests, the composition of the OA pool (% on a mol C-basis) was Ac/La/Pr/Bu/Va = 4/10/25/60/1 (FSCW-6) and Ac/La/Pr/Bu/Va = 42/16/19/21/0 (FSCW-7.5), respectively. The results are in line with Regueira et al. (2020), who found that fermentation at acidic pH favours butyrate production, whilst basic pH values foster acetate production.

The C/N ratio is a critical parameter for both the selection of biomass and the accumulation of PHA (Silva et al., 2017). A higher C/N ratio was observed for FSCW-6 (24.6) than for FSCW-7.5 (13.5) (Table 1). Since the fermentation yield ($Y_{OA/SCW}$) was very similar for both the investigated pH values and no residual carbohydrates were detected, the observed difference in the C/N ratios was due to the different concentrations of soluble nitrogen measured at the end of the fermentation tests. In anaerobic fermentation, whey proteins are hydrolysed to soluble proteins, then converted into amino acids, and finally to VFA with associated ammonia release, based on the widely accepted Stickland reaction (Tang et al., 2005). Therefore, the soluble nitrogen concentration at the end of the fermentative process was the sum of the residual soluble proteinaceous N and ammonia N from protein degradation, net of the N incorporated in biomass cells. The higher final concentration of soluble N at pH 7.5 could be ascribed to a faster protein hydrolysis compared to pH 6 (Table 1), as also observed by Duong et al. (2021, 2019). Although the transformation of proteins during DF is raising increasing interest (Bevilacqua et al., 2021; Duong et al., 2019; Roibás-Rozas et al., 2021), further details are required to understand the dynamics of the process thoroughly.

3.2. Stage II – selection and enrichment of the PHA-storing mixed microbial culture

The possibility of harvesting a PHA-storing biomass from the MMC sampled at a WWTP was preliminarily assessed by feeding a synthetic medium composed of acetate as the main carbon source. During the preliminary selection assays, an average biomass concentration of 0.98 ± 0.26 g_{VSS} L^{-1} and a feast to famine ratio (F/F) of 0.15 ± 0.06 were achieved (Fig. 2a); the attained F/F agreed with values reported as optimal by other Authors for the selection of PHA-storing bacteria (Colombo et al., 2016; Valentino et al., 2017).

Further selection runs were carried out by feeding the real fermented cheese whey (FSCW-6 and FSCW-7.5). These runs were characterised by an average biomass concentration of 1.32 ± 0.22 g_{VSS} L^{-1} (FSCW-6) and 0.99 ± 0.10 g_{VSS} L^{-1} (FSCW-7.5), and an overall F/F ratio of 0.16 ± 0.08 (FSCW-6) and 0.16 ± 0.06 (FSCW-7.5) (Fig. 2a), respectively. Since no external nutrient source was added, the cellular metabolism proved to be fully supported by the CW nutrient content, as underlined by protein consumption (Δ_{PROTE}) of 62% (FSCW-6) and 90% (FSCW-7.5), respectively (Table 3). Interestingly, the soluble nitrogen removed during test FSCW-6 would theoretically sustain a biomass concentration of 1.62 g_{VSS} L^{-1} according to the general biomass formula ($CH_5H_7NO_2$), a figure that is very close to the measured value (see Table 3). Conversely, the biomass concentration observed for test FSCW-7.5 (2.84 g_{VSS} L^{-1}) was much lower than the theoretical value based on the nitrogen uptake. This figure may be tentatively explained by ammonia volatilization occurring during biomass selection due to the higher pH compared to test FSCW-6 (pH 9 vs pH 8, data not shown in paper). Indeed, at 25 °C and pH 9, assuming a value for the dissociation constant of 1.8×10^{-5}, about 36% of ammonia nitrogen is expected to be in the gaseous form, as opposed to only 5% at pH 8.

The capability of the selected PHA-storing biomass of using the N content of CW is worth of interest, as it would avoid the need for an external N source. In PHA production, N addition is a widely adopted strategy in the case of nutrient-deficient feedstocks (e.g., sugarcane molasses, paper mill effluent, olive oil mill effluent). However, it may also be required for substrates that are characterised, despite their good protein content, by limited N availability resulting from poor protein hydrolysis (Colombo et al., 2019; Duque et al., 2014; Oliveira et al., 2018; Valentino et al., 2015). Oliveria et al. (2018) failed to select a biomass capable of metabolizing N from proteins in cow CW through the progressive reduction of the external supply of readily available N.

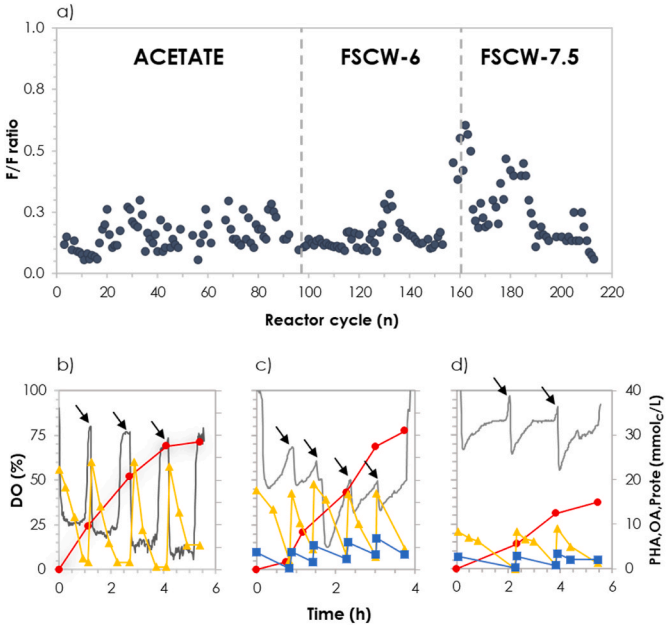

Fig. 2. Feast and famine ratio (F/F) during biomass selection performed using acetate and fermented sheep cheese whey (FSCW-6 and FSCW.7.5) as substrates (a). Time evolution of DO (—), substrate (acetate or OA, ▲), protein consumption (■), and PHA accumulation (●) during PHA accumulation using acetate (b), FSCW-6 (c), and FSCW-7.5 (d). Black arrows (→) in (b, c, d) indicate the pulse feed.

Table 3
Performance data for the PHA storing microorganism selection process (stage II) (average values ± standard deviation).

	Parameter	Unit of measure	Acetate	FSCW-6	FSCW-7.5
Operating conditions	OLR	$mmol_C$ L^{-1} d^{-1}	34.0 ± 0.0	40.6 ± 6.7	40.0 ± 1.6
	F/F ratio	h h^{-1}	0.15 ± 0.06	0.16 ± 0.08	0.16 ± 0.06
Performance parameters	Biomass concentration	g VSS L^{-1}	0.98 ± 0.26	1.32 ± 0.22	0.99 ± 0.10
	PHA content[a]	% wt	17 ± 2	18 ± 1	16 ± 1
	Polymer composition[a]	% wt_{HB} % wt_{HV}^{-1}	100:0	76:24	89:11
	$Y_{PHA/OA}$[a]	$mol_{C\text{-}PHA}$ $mol_{C\text{-}OA}^{-1}$	0.45	0.45	0.42
	Δ_{PROTE}	%	n.a.	90 ± 3	62 ± 0

n.a.: not available.
[a] End of the feast phase.

Possibly, the availability of external N at the beginning of the test harvested biomass species unable to hydrolyse CW proteins. On the contrary, the encouraging results in terms of protein uptake obtained in the present study could be due both to the use of ovine FCW, more protein-rich than the bovine one (about 10 g L^{-1} vs 1.7 g L^{-1} reported by Oliveira et al. (2018)), and having avoided the use of external N sources since the beginning of the selection process.

The successful selection and harvesting of a PHA-storing biomass are also supported by the observed accumulation capacity, which was comparable to that obtained when acetate was used as the substrate (Table 3). The maximum PHA content observed ad the end of the feast phase was always in the range 16–18% wt, while the PHA yield was 0.42–0.45 $mol_{C\text{-}PHA}$ $mol_{C\text{-}OA}^{-1}$.

3.3. Stage III – PHA accumulation

The accumulation tests confirmed that an active PHA-accumulating biomass was selected from the MMC sampled at a WWTP and using the FSCW as the substrate, without any external nutrient supply. In particular, the use of FSCW-6 resulted in a maximum PHA content of 35% wt, a storage yield $Y_{PHA/OA}$ of 0.52 $mol_{C\text{-}PHA}$ $mol_{C\text{-}OA}^{-1}$ and a specific PHA uptake rate (q_{PHA}) of 0.221 $mol_{C\text{-}PHA}$ $mol_{C\text{-}X}^{-1}$ h^{-1}, whilst the use of FSCW-7.5 yielded a maximum PHA content of 34% wt, 0.41 $mol_{C\text{-}PHA}$ $mol_{C\text{-}OA}^{-1}$ and 0.136 $mol_{C\text{-}PHA}^{-1}$ $mol_{C\text{-}X}$ h^{-1} (Table 4). The evolution over time of the typical accumulation process is summarised in Fig. 2 for both the substrates FSCW-6 and FSCW-7.5. Process monitoring showed that, despite a similar maximum storage capacity, a noticeable difference in terms of substrate conversion (i.e. $Y_{PHA/OA}$) was observed. The worse performance observed when using FSCW-7.5 could be ascribed to its higher N content at the end of the fermentation phase. In this respect, it is worth underlining that the lower N uptake rate compared to that of C

Table 4
Performance data for the PHA-accumulation process (stage III) (average values ± standard deviation).

Parameters	Unit of measure	Acetate	FSCW-6	FSCW-7.5
PHA_{max}	% wt	50 ± 0	35 ± 1	34 ± 5
Polymer composition	% wt_{HB} % wt_{HV}^{-1}	100:0	66:34	88:12
$-q_{OA}$	$mol_{C\text{-}OA}$ $mol_{C\text{-}X}^{-1}$ h^{-1}	0.496 ± 0.036	0.417 ± 0.048	0.327 ± 0.052
q_{PHA}	$mol_{C\text{-}PHA}$ $mol_{C\text{-}X}^{-1}$ h^{-1}	0.292 ± 0.087	0.221 ± 0.025	0.136 ± 0.01
$Y_{PHA/OA}$	$mol_{C\text{-}PHA}$ $mol_{C\text{-}OA}^{-1}$	0.59	0.52	0.41
$-q_{PROTE}$	$mol_{C\text{-}PROTE}$ $mol_{C\text{-}X}^{-1}$ h^{-1}	n.a.	0.071 ± 0.001	0.078 ± 0.001

n.a.: not applicable.

implies a concentration build-up in the reactor fed with a pulse mode. The progressively increasing N concentration may have driven the system towards cell growth rather than PHA accumulation. This feature seems to be confirmed by the higher biomass growth yield $Y_{X/OA}$ (0.37 $mol_{C\text{-}X}$ $mol_{C\text{-}OA}$) and specific protein uptake rate ($-q_{PROTE}$) observed for FCSCW-7.5 compared to FSCW-6 (Table 4). To avoid this, a feeding strategy could be tested that involves periodic biomass settling followed by supernatant removal as proposed also by Argiz et al. (2020).

The results achieved in terms of maximum PHA content are in line with Oliveira et al. (2018), who reported content of 43% wt achieved under similar process conditions, though using cow FCW and an external N source during the selection stage. In that study, when the external N supply was reduced to rely on indigenous organic N, the accumulation performance significantly decreased (2–5 wt%). However, it is worth underlying that other Authors reported higher PHA contents when using cow CW and providing nutrient supply during the selection stage (PHA content of 55–81%, Table 5).

The accumulated PHA were composed of HB and HV, for both FSCW-6 and FSCW-7.5 as the substrate, although in different proportions. In particular, the HV fraction in the final polymers produced using FSCW-6 and FSCW-7.5 was 34% and 12%, respectively. Considering the common precursors of HB (acetate, butyrate, and lactate) and HV (propionate and valerate), the composition of the VFA pool used as the feedstock (Ac/La/Pr/Bu/Va = 4/10/25/60/1 for FSCW-6, and Ac/La/Pr/Bu/Va = 42/16/19/21/0 for FSCW-7.5) was not found to have a clear effect on the nature of the PHA produced. The correlation between the composition of the biopolymers accumulated by an MMC and that of the fed substrate is a topic of great interest, which has also been studied through prediction models (Pardelha et al., 2012, 2014; Tamis et al., 2014). Most models are based on the assumption that all the available VFA are degraded simultaneously; however, recent studies have suggested that in the presence of a range of organic acids, the PHA-accumulating biomass uses them according to an order of preference (Wang et al., 2018, 2020). As shown by the results of previous studies (see Table 5 and Fig. 3), while a correlation between the polymer type obtained and the nature of the precursors can be recognized, it is relatively low. This suggests that complex transformations are involved during the accumulation of PHA species, and the underlying mechanisms still need to be fully elucidated.

It is worth mentioning that the production of HV is interesting on account of the benefits achievable in terms of final physical characteristics such as crystallinity, brittleness and flexibility. Indeed, PHBV owns superior thermal and mechanical proprieties compared to PHB and it is believed to play a key role as a substitute for traditional fossil-based plastics (Palmeiro-Sánchez et al., 2022).

3.4. Preliminary considerations on the implementation of a plant for the valorisation of sheep cheese whey

As stated above, the sheep dairy sector is essential in several European rural areas, especially in countries like Italy, Greece, Spain, and France. The sector, and in general the agro-industrial context, is characterised by multiple needs: modernize the system, appropriately manage the residues generated by the production activities, expand and diversify the range of products on the market, strengthen the resilience towards the fluctuations of the economic situation and the impacts of climate change.

In this framework, the Italian region of Sardinia, being one of the most important sheep milk-producing areas in the EU (Concu et al., 2020; Vagnoni and Franca, 2018), can be considered as a case study for some considerations that, though general, may be indicative of the perspectives inherent in the proposed approach for an innovative bio-waste management system.

The sheep milk production in Sardinia accounts for 330 000 ty^{-1} (around 15% of the overall EU sheep production) (Vagnoni and Franca, 2018), which produces approximately 280 000 t of SCW (assuming a conversion factor of 0.9 (Carvalho et al., 2013)). Assuming that about

Table 5
Performances observed for PHA production from cheese whey (CW) (* on COD basis; ** of total OA; *** as $mgCOD_{PHA}$ mg COD_{OA}^{-1}).

Substrate	pH value (DF)	VFA profile (Ac/La/Pr/Bu/Va)	Max. PHA content	Polymer composition	$-q_{OA}$	q_{PHA}	$Y_{PHA/OA}$	PHA productivity	Refs.
		%, on molC basis	% wt	% wt_{HB}:% wt_{HV}^{-1}	$mol_{C\text{-}OA}$ $mol_{C\text{-}X}$ h^{-1}	$mol_{C\text{-}PHA}$ $mol_{C\text{-}X}$ h^{-1}	$mol_{C\text{-}PHA}$ $mol_{C\text{-}OA}^{-1}$	g PHA L^{-1} d^{-1}	
Fermented sheep CW	6.0	4/10/25/60/1	35 ± 1	66:34	0.417 ± 0.048	0.221 ± 0.025	0.52	4.67	This study
Fermented sheep CW	7.5	42/16/19/21/0	34 ± 5	88:12	0.327 ± 0.052	0.136 ± 0.01	0.41	2.05	
Fermented fresh CW	5.5	16/58/0/26/0	66	100:0	0.3 ± 0.0	0.2 ± 0.0	0.7 ± 0.0	10.9 ± 0.8	Colombo et al. (2016)
Fermented sterilised CW	5.5	58/6/19/13/4	81	60:40	0.5 ± 0.1	0.4 ± 0.0	0.8 ± 0.1	28.2 ± 2	
Fermented sweet whey powder	6.0	52/8/9/15/4	n.a.	87:13	0.42 ± 0.0	0.40 ± 0.03	0.96 ± 0.07	6,09	Oliveira et al. (2017)
Fermented sweet whey powder	6.0	46/0/4/44/5	43	89:11	n.a.	0.17 ± 0.02	0.85 ± 0.12	4,8	Oliveira et al. (2018)
Fermented whey powder	6.0	68/3/8/21/0	65	81:19	0.45 ± 0.07	0.30 ± 0.06	0.66 ± 0.14	0,56	Duque et al. (2014)
Fermented whey permeate	6.0	44/0/2/50/0*	60	85:15	n.a.	n.a.	0.4***	n.a.	Valentino et al. (2015)
Fermented second CW	5.5–5.8	50/23/0/27**	62 ± 4	100:0	40.55 ± 0.09	90.4 ± 0.08	0.88 ± 0.30	n.a.	Colombo et al. (2019)
Fermented concentrated CW permeate	5.5–5.8	55/0/0/45/0**	55 ± 1	100:0	0.37 ± 0.07	90.31 ± 0.06	0.87 ± 0.14	n.a.	
Fermented whey powder	4,5	18/80/0/1/0	n.a.	95:5	n.a.	n.a.	n.a.	n.a.	Gouveia et al. (2017)
	5.0	30/67/2/0/0	n.a.	83:17	n.a.	n.a.	n.a.	n.a.	
	6.0	40/48/10/2/0	n.a.	70:30	n.a.	n.a.	n.a.	n.a.	
	7.0	37/56/6/0/0	n.a.	75:25	n.a.	n.a.	n.a.	n.a.	
Fermented CW	5.0	36/0/4/52/8*	50	88:12	n.a.	n.a.	n.a.	n.a.	Lagoa-Costa et al. (2022)

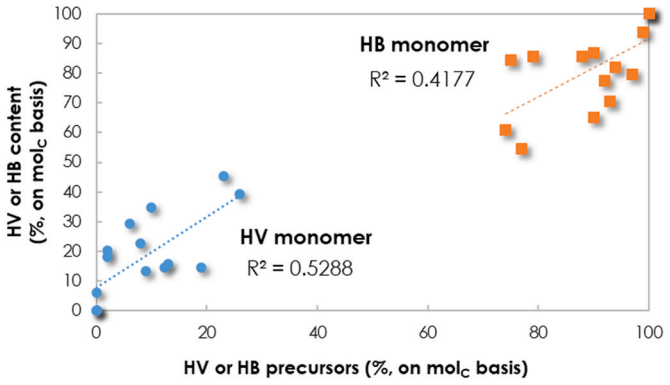

Fig. 3. Correlation between polymer composition in terms of HB (% on a mol C basis) and related precursors (acetate, butyrate, and lactate, % on mol C basis) (■), and in terms of HV (% on a mol C basis) and related precursors (propionate and valerate, % on a mol C basis) (●). Note: data from Table 5.

50% of SCW is further processed to produce ricotta cheese, the remaining 148 500 t y^{-1} would need proper management. According to the results of this study, a multi-stage bioprocess could potentially produce 5.3 Nm^3_{H2} and 7.6 kg_{PHA} per t of SCW (for more details about the calculations, see (Asunis et al., 2021)). Therefore, the annual regional potential would consist of some 787 000 Nm^3 of green H_2 and 1132 t of PHA. The latter slightly exceeds the value of 1000 t y^{-1}, often indicated as the minimum plant size for an economically feasible MMC-PHA production process (Bengtsson et al., 2017; Roibás-Rozas et al., 2020), and suggests the possibility of implementing a centralised regional facility. Furthermore, it is worth mentioning that the ricotta cheese whey (also known as scotta or secondary cheese whey) could integrate the availability of whey-based substrates for PHA production, increasing it up to 285 000 t y^{-1}, and leading to a PHA potential production of 2170 t y^{-1}. These prospects are worthy of interest, even acknowledging that the PHA production from MMC technology is still in the development stage (TRL of 4–5) and that the first pilot plant in the EU has only recently started operation (Matos et al., 2021; Silva et al., 2022; Valentino et al., 2019).

Finally, these prospects could be further integrated by the production of biomethane achievable through the anaerobic digestion of the residual biomass coming from the fermentation phase (stage I), as proposed and investigated by Roibás-Rozas et al. (2020). This further possibility was recently evaluated in the context of a life cycle assessment (LCA) applied to the multi-stage process under concern. According to the performed LCA, implementing anaerobic digestion may be an essential improvement for the environmental sustainability of the process (Asunis et al., 2021). Taking into account the anaerobic digestion of the residual biomass obtained in this study, the methane recovery could account for some 13.4 Nm^3_{CH4} per t SCW, that is 3.8 × 10^6 Nm^3_{CH4} y^{-1}. Therefore, considering the assessed bio-hydrogen production, the overall energy output would be 42 660 MWh y^{-1}.

These preliminary and general evaluations need in-depth economic and environmental assessments, but they can hopefully stimulate discussion and, above all, further studies, possibly on a pilot scale.

4. Conclusions

The present study focuses on a multi-stage process for the combined production of bio-hydrogen and PHA from sheep cheese whey. The yields, 5.3 L H_2 and 7.6 g PHA per L of sheep cheese whey obtained under optimal fermentation conditions (operating pH = 6), as well as some application considerations, are encouraging and represent a solid basis for further investigations.

The specific characteristics of the investigated substrate were found to affect the process features at various levels. In particular, the higher protein content as compared to the most-studied bovine CW on the one hand allows the selection of a PHA-accumulating biomass with no need for external nitrogen sources, on the other hand seems to limit the biopolymer accumulation yield. Some process modifications, such as, for example, a feeding strategy that limits nitrogen build-up in the reactor, or alternatively the implementation of an upstream separation

system to reduce the nitrogen content of fermented CW, may be proposed to make the best out of the peculiarities of sheep cheese whey, offering new perspectives to an economic sector that is strategic in some rural European areas, and increasing its economic and environmental resilience.

Credit author statement

Fabiano Asunis: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing – Review and Editing, Visualization. **Giorgia De Gioannis:** Conceptualization, Validation, Formal analysis, Supervision, Writing - Review and Editing, Funding acquisition. **Aldo Muntoni:** Conceptualization, Supervision, Writing - Review and Editing, Funding acquisition. **Alessandra Carucci:** Supervision, Writing - Review and Editing. **Daniela Spiga:** Conceptualization, Writing – Review and Editing. **Alessandra Polettini:** Writing - Review and Editing. **Andreina Rossi**: Writing – Review and Editing. **Gianluigi Farru:** Writing - Review and Editing. **Raffaella Pomi:** Writing - Review and Editing.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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