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Fermentative hydrogen production from cheese whey with *in-line*, concentration gradient-driven butyric acid extraction

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Abstract

Hydrogen (H_2) generation from cheese whey, with simultaneous production and extraction of volatile fatty acids (VFAs), was studied in UASB reactors at two temperatures (20 and 35°C) and pH values (5.0 and 4.5). The extraction module, installed through a recirculation loop, was a silicone tube coil submerged in water, which allows concentration-driven extraction of undissociated VFAs. Operating conditions were selected as a compromise for the recovery of both H_2 and VFAs. Batch experiments showed a higher yield (0.9 mol H_2 mol^{-1} $glucose_{eq.}$) at 35°C and pH 5.0, regardless of the presence of the extraction module, whereas lower yields were obtained at pH 4.5 and 20°C (0.5 and 0.3 mol H_2 mol^{-1} $glucose_{eq.}$, respectively). VFAs crossed the silicone membrane, with a strong preference for butyric over propionic and acetic acid due to its higher hydrophobicity. Sugars, lactic acid and nutrients were retained, resulting in an extracted solution of up to 2.5 g L^{-1} butyric acid with more than 90% purity. Continuous experiment confirmed those results, with production rates up to 2.0 L H_2 L^{-1} d^{-1} and butyric acid extraction both *in-line* (from the UASB recirculation) and *off-line* (from the UASB effluent). *In-line* VFA extraction can reduce the operating costs of fermentation, facilitating downstream processing for the recovery of marketable VFAs without affecting the H_2 production.

Keywords: Biohydrogen; Butyric acid; Dairy wastewater; Pertraction; Selective extraction; Waste biorefinery

1. Introduction

The increasing societal need for energy and materials, along with population growth, fossil fuel depletion and growing interest in environmental issues, are driving a global shift towards a sustainable and circular economy. In 2018, an updated bioeconomy strategy has been adopted by the European Union, along with the Paris Agreement commitments, to achieve the sustainable growth and environmental protection goals included in the 2030 agenda [1]. In this view, biodegradable waste streams are proposed as renewable substrates for energy and chemical production, partially replacing fossil fuels [2,3]. This context encourages production systems to implement the biorefinery concept [4], where waste is considered as an opportunity to diversify the product spectrum while reducing the costs of biomass supply and waste treatment, thereby meeting the increasingly stringent legislation on emissions.

The dairy industry processes 170 billion L of milk per year in Europe [5], generating an average of 2.5 L wastewater per L of milk processed and 9-10 L cheese whey (CW) per kg of cheese produced. When CW is discharged without proper treatment, it can have serious adverse effects on the environment, *i.e.* rising of eutrophication in water bodies or decreased crop yields and oxygen availability in agricultural land [6]. CW management mainly involves whey protein recovery, animal feeding, or treatment in dedicated wastewater treatment plants, depending on the size of the dairy industry and the production context [7]. However, the high concentration of readily degradable compounds (50-100 g_{COD} L^{-1}, 90% of which in the form of lactose) makes CW an outstanding substrate for biological production of energy and chemical commodities [8], not fully exploited so far. Physicochemical and biological processes can be synergically

implemented, according to the waste biorefinery concept, to convert CW to valuable products such as methane [9], hydrogen [10], volatile fatty acids (VFAs) [11], alcohols [12], lactic acid [13], electric energy [14], or bioplastics [15].

Among the suitable processes, dark fermentation is considered the core of a waste biorefinery scheme, as it enables biological simplification and conversion of organic substrates to a carbon-neutral energy carrier (H_2) and building blocks (VFAs) suitable for downstream applications [3,16]. Since sugars are the preferred substrate for fermentative microorganisms, CW is a substrate of particular interest for dark fermentation. CW fermentation results in H_2 yields typically spanning between 1 and 4 mol mol^{-1} lactose (or 0.5 and 2 mol mol^{-1} $glucose_{eq.}$) depending on the operating conditions such as pH, temperature and organic loading rate [10,17–19].

Besides H_2, up to 20-30 g L^{-1} VFAs, mainly acetic, propionic, and butyric acid are produced through CW fermentation, at different mass proportions depending on the operating parameters, pH in particular [10,11,20]. Typically, the operating conditions that foster H_2 production in CW fermentation also favour butyric acid production among the soluble organic fermentation products [10,21]. Butyric acid finds numerous applications in the chemical, pharmaceutical, perfume and animal feed sectors [22], with a market size of about 125 M€ (https://www.marketsandmarkets.com/Market-Reports/butyric-acid-market-76962011.html) which is expected to further increase by 15.1% $year^{-1}$, as a response to its approval as food flavouring agent by the U.S. Food and Drug Administration (FDA) [23]. This already favourable context could further benefit, in the next decade, by the development of the bioplastic sector, as butyric acid

96 is a precursor for polyhydroxyalkanoates (PHA) production [24]. Thus, the
97 development of a process for the combined production of H_2 and butyric acid
98 substantially contributes to a modern and environmentally sustainable CW
99 management.
100
101 Several technologies are available for VFAs extraction, including physical
102 (nanofiltration, liquid-liquid extraction, vapour permeation, membrane contactors, gas
103 stripping and distillation), chemical (adsorption and solvent extraction) and
104 electrochemical (electrodialysis) methods [23,25]. However, the development of a low-
105 cost system to selectively extract the target compound from a VFAs mixture is still a
106 challenge. Outram and Zhang [26] recently showed that concentration-gradient-driven
107 liquid-liquid extraction (pertraction) through a non-porous silicone membrane, using
108 distilled water as the draw solution, can be applied to recover VFAs. Furthermore, it
109 was shown that longer-chain VFAs migrate faster than shorter-chain VFAs through the
110 silicone membrane due to their higher hydrophobicity [26]. This represents a
111 remarkable feature, as it would enable the selective extraction of butyric acid over other
112 typical CW fermentation products (i.e. acetic, propionic and lactic acid).
113
114 The aim of the present study was to study the performance of a novel reactor concept
115 for simultaneous H_2 and butyric acid recovery from CW, where an *in-line* silicone
116 membrane extraction module is implemented into a fermentative UASB reactor through
117 a recirculation loop. The operating conditions were chosen as a compromise between H_2
118 production (optimal pH between 5.5 and 6.0) and VFA extraction (requiring pH below
119 the pK_a of VFA). First, inoculum and up-flow velocity were optimised for H_2 and

butyric acid production. Then, the effects of pH (5.0 *vs.* 4.5) and temperature (35 *vs.* 20 °C) on H_2 production and butyric acid recovery were evaluated in the UASB operated either under batch or continuous mode. Finally, the extraction efficiencies achieved were compared to those obtained by operating an *off-line* butyric acid extraction system fed with the fermentative UASB effluent.

2. Materials and methods

2.1 Source of inoculum and pretreatment

The inoculum used in this study was either activated or digested sludge from the wastewater treatment plant of a dairy industry (Dairygold, Mitchelstown, Ireland). The activated and digested sludge had a total solids concentration of 42.7 ± 0.8 and 66.0 ± 3.0 g L^{-1}, and a volatile solids concentration of 24.8 ± 0.4 and 49.8 ± 2.6 g L^{-1}, respectively. Heat pretreatment was done by heating thin tubes containing 5 mL of sludge in a dry bath (Fisher Scientific) at 90°C for 15 minutes.

2.2 Synthetic medium and cheese whey composition

The synthetic medium used for inoculum screening was the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) medium nr. 141 with the following modifications: lactose (10 g L^{-1}) was used instead of glucose as the substrate, and yeast extract, tryptone, resazurine and Na_2S were not added. CW from cow milk processing was collected from the dairy industry (Dairygold), stored at -20°C after transportation to the lab, and defrosted to 4°C 24 hours prior to utilization to prevent acidification. The CW composition was as specified in Table 1.

144 **Table 1.** Cheese whey characterization

Parameter	**Unit**	**Values**
Total Solids (TS)	g L^{-1}	69.98 ± 1.94
Volatile Solids (VS)	g L^{-1}	64.04 ± 1.76
Total suspended solids (TSS)	g L^{-1}	1.18 ± 0.11
Volatile suspended solids (VSS)	g L^{-1}	1.17 ± 0.05
pH	-	6.42
Conductivity	mS cm^{-1}	5.24
COD	g L^{-1}	66.96 ± 4.80
TOC_{sol}	g L^{-1}	20.82 ± 1.08
Total dissolved saccharides	g L^{-1}	41.70 ± 0.91
Acetic acid	mg L^{-1}	262 ± 5
Propionic acid	mg L^{-1}	83 ± 2
Lactic acid	mg L^{-1}	926
Total P	mg L^{-1}	308 ± 22
Anions (Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-})	mg L^{-1}	436 ± 23, <10, <10, 188 ± 3, 23 ± 1
Cations (Ca^{2+}, K^+, Na^+, NH_4^+)	mg L^{-1}	266 ± 56, 1702 ± 177, 441 ± 76 , 83 ± 8
Soluble proteins	g L^{-1}	2.30 ± 0.01

145

146 ***2.3 Inoculum screening***

147 Four inocula, *i.e.* activated or digested sludge with or without heat-shock pretreatment,
148 were compared for H_2 production from lactose in a preliminary batch experiment. The
149 experiment was conducted in triplicate 120 mL serum bottles with 48 mL of synthetic
150 medium and 2 mL of each inoculum. The initial pH was adjusted to 7.0 using 1 M
151 NaOH. Abiotic (without inoculum) and no-substrate (without lactose) controls were
152 also prepared. The bottles were sparged with N_2 for 5 min prior to incubation at 35°C
153 for about 17 days with 150 rpm shaking in an orbital shaker incubator
154 (ThermoScientific MaxQ 8000).

155

156 ***2.4 Effect of up-flow velocity on hydrogen production from CW***

157 The effect of the up-flow velocity on H_2 production was studied in 1 L recirculated
158 UASB reactors operated in batch mode, and maintained at 35°C using a water bath with

159 recirculation (Grant Tc120, UK). A controller (Cole-Parmer 300, USA) connected to a
160 pH probe (VWR, USA) and a peristaltic pump (Verdeflex, The Netherlands) was used
161 to keep the pH above 5.0 in the UASB reactor by addition of 5M NaOH from a bottle
162 under N_2 atmosphere. After sparging with N_2, the reactors were fed with 700 mL CW
163 using a peristaltic pump (Masterflex L/S, Cole-Parmer, USA). Heat-treated digested
164 sludge (4%) was added as inoculum from a sampling port. The CW was recirculated
165 from the top to the bottom of the bioreactor, using a peristaltic pump (Masterflex), to
166 achieve an up-flow velocity of 0.1, 0.5, 1.0 or 2.0 m h^{-1}. The gas produced was collected
167 in a gas bag, and the batch experiments were stopped when no H_2 production was
168 observed anymore for at least 3 consecutive days (after 8-11 days fermentation).
169
170 ***2.5 Batch experiments with in-line VFAs extraction***
171 UASB reactors, with the same configuration as the previous experiment, were used for
172 evaluating the effect of *in-line* VFAs extraction on CW fermentation at different
173 temperature (20 and 35°C) and pH (4.5 and 5.0) using heat-treated digested sludge as
174 inoculum and an up-flow velocity of 1.0 m h^{-1}. The VFAs extraction module included a
175 silicone tube coil (2 and 4 mm internal and external diameter, VWR, The Netherlands)
176 with a total length of 4.2-4.4 m, submerged into 700 mL distilled water (draw solution)
177 in a conical 1 L flask. The flask was sealed at the top with a rubber stopper and
178 connected to the gas line outlet (Fig. 1) to recover the gas diffusing through the silicone
179 membrane. The extraction module was installed to the UASB reactor through a
180 recirculation loop. One UASB reactor was operated without *in-line* VFA extraction as
181 control. Since the working volume increased due to the addition of the extraction unit,

the UASB reactors were fed with 830-850 mL of CW as compared to the 700 mL of the preliminary experiment on the up-flow velocity (section 2.4).

2.6 Continuous experiment with in-line VFA extraction

For the continuous experiments, an influent supply tank, kept at 4-6°C inside a fridge, was connected to two UASB reactors through a pump (Masterflex), and an on-line gas monitoring system composed by a V-count gas counter and H_2 and CO_2 sensors (BlueSens, Germany) was installed (Fig. 1). Two UASB reactors (namely, UASB-A and UASB-B) were run in parallel, according to the experimental stages reported in Table 2. After a 5-day start-up in batch mode, CW was fed continuously at 24 hours hydraulic retention time (HRT) to compare the performance of the UASB reactors in the presence and absence of the extraction module, and then to study the response of the integrated system to pH changes.

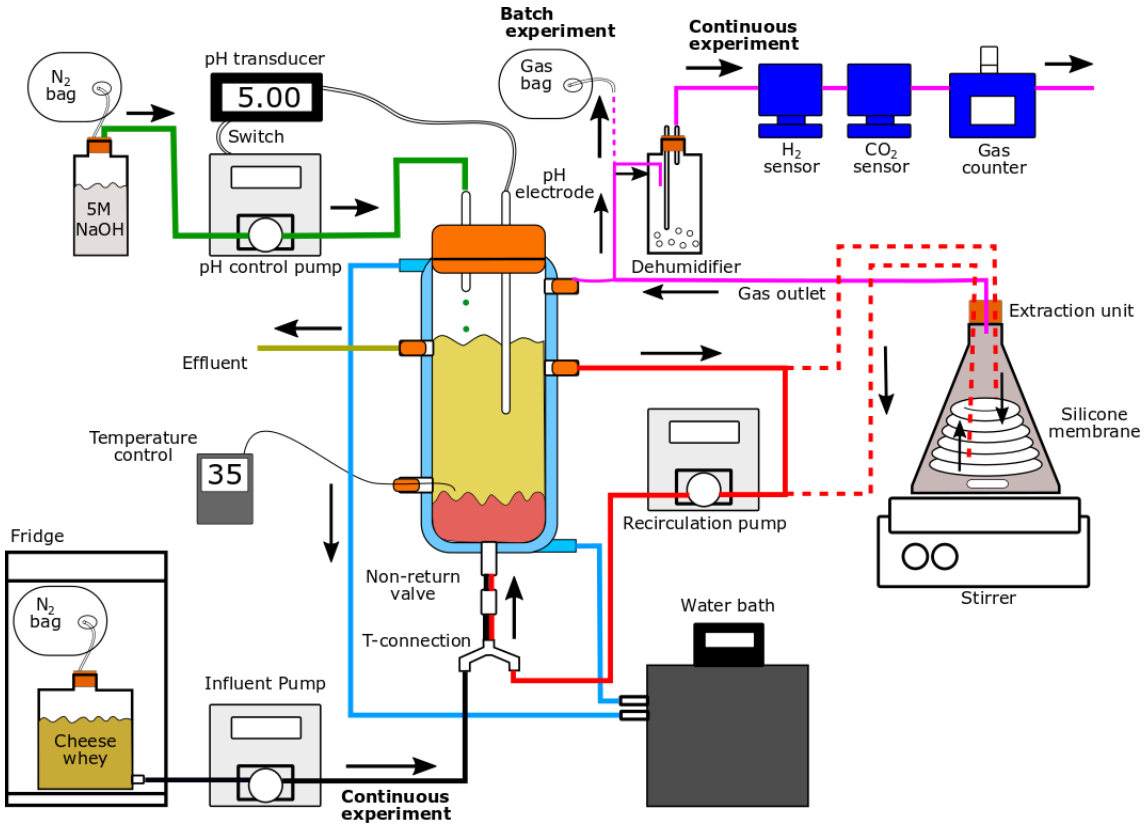


Figure 1. UASB reactor configuration adopted for the experiments in batch and continuous operation mode. The coloured lines represent the influent (black, only for the continuous experiment), recirculation (red), effluent (yellow), pH control (green), water jacket (blue) and gas (magenta) lines.

Table 2. Overview of the UASB reactor operation with the experiments in continuous mode. All experiments were performed at 35°C.

Reactor	Days	Operation mode	pH control	Extraction unit
UASB-A	0-5	Batch (Start-up)	5.0	No
	6-42	Continuous	5.0	No
	43-64	Continuous (restarted with fresh inoculum)	4.5	Yes
	65-84	Continuous	5.0	Yes
UASB-B	0-5	Batch (Start-up)	5.0	No
	6-48	Continuous	5.0	Yes
	49-74	Continuous	4.5	Yes
	75-84	Continuous	5.0	Yes

2.7 Off-line extraction experiment

Fermentate from both UASB reactors, when operated in continuous mode at pH 5.0 and 4.5, respectively, was collected on day 71 and used for *off-line* extraction tests in batch. The fermentate was acidified to pH 3 by HCl addition prior to starting the experiment. A flask containing CW fermentate (500 mL) was connected through a pump to the extraction module containing either 500 mL distilled water or 0.5 M NaOH as the draw solution through a recirculation loop. The recirculation flow was 21 mL min^{-1}, the same applied to the UASB reactor to obtain an up-flow velocity of 1.0 m h^{-1}.

2.8 Monitoring and analytical methods

214 Gas produced during the inoculum screening tests was quantified using a syringe
215 method [27]. Gas produced during the UASB batch tests, including the gas diffusing
216 through the silicone membrane (Fig. 1), was collected in 5 L gas bags and measured
217 using the water displacement method. For all the batch experiments, gas samples (5 mL)
218 were collected either from the headspace of the serum bottles or from the gas bags and
219 stored in 5.9 mL gas collection vials (Exetainer®, Labco, UK) at ambient temperature
220 for analysis. Gas composition (H_2, CH_4 and CO_2) was analysed using a gas
221 chromatograph (GC) (Agilent 7890A, USA) equipped with a thermal conductivity
222 detector (TCD) and a 80/100 Hayesep Q column. Argon was the carrier gas with a flow
223 of 24 mL min^{-1}, and oven, injector and detector were kept at 90, 90 and 200°C,
224 respectively. For the continuous experiment, both gas lines were connected to the on-
225 line monitoring sensors and gas counter (BlueSens, Germany).
226
227 Liquid samples were collected from the serum bottles (2 mL), from a sampling port in
228 the recirculation tube of the UASB reactors (4 mL), as well as from a sampling tube
229 submerged in the draw solution (2 mL), and stored at -20°C in plastic tubes for analysis.
230 Sugars, carboxylic acid and alcohol concentrations in liquid samples were analysed
231 using a liquid chromatograph (LC) (1260 Infinity II, Agilent, USA) equipped with a
232 refractive index detector (RID) and a Hi-Plex H column (300×7.7 mm) held at 60°C.
233 The mobile phase was H_2SO_4 (5 mM) at a flow rate of 0.7 mL min^{-1}. Total dissolved
234 saccharides were measured using a phenol-sulphuric colorimetric method [28] with a
235 spectrophotometer (Shimadzu UV-1900, Japan) at 485 nm.
236

Total solids (TS), total suspended solids (TSS), volatile solids (VS), volatile suspended solids (VSS) and chemical oxygen demand (COD) were measured according to the APHA procedures [29]. Total organic carbon (TOC) was analysed using a TOC analyser (TOC-L CSN Analyser, Shimadzu, Japan). Conductivity and pH were measured with a conductivity meter (Mettler Toledo, USA) and with a pH controller (Cole Parmer 300, UK) connected to a pH probe (SlimTrode, Hamilton, Switzerland), respectively. Cations and anions were measured via ionising coupled plasma - optical emission spectroscopy (ICP-OES 5110, Agilent, USA) and ion chromatography (IC AS-DV, Thermo Scientific, USA), respectively. Total phosphorus, ammonium and soluble proteins were measured using a Nutrient analyser (Gallery Plus, Thermo Scientific, USA).

2.9 Calculations

The modified Gompetz model was applied as reported in Asunis et al. [10]. Carbon balances were made based on the carbon content of liquid and gas products detected. A carbon content of 46% was assumed for proteins [30]. The organic loading rate was calculated based on COD. The acidification degree was calculated according to Bengtsson et al. [31]. Fluxes and mass transfer coefficients (K_{OV}) were calculated according to Outram and Zhang [26].

3. Results and discussion

3.1 Inoculum screening and optimal up-flow velocity

When incubated in batch with the lactose-containing synthetic medium, heat-treated digested sludge gave a significantly higher H_2 production rate (0.66 L L^{-1} d^{-1}) and yield

(0.92 ± 0.38 mol mol^{-1} $glucose_{eq.}$), as well as a higher butyric acid yield (0.27 ± 0.12 mol mol^{-1} $glucose_{eq.}$) than the other inocula tested, *i.e.* non-treated digested sludge, and both treated and non-treated activated sludge (Table S1). Thus, heat-treated digested sludge was selected as the inoculum for all follow-up experiments.

A remarkable effect on the H_2 production from cheese whey was observed for the different up-flow velocities tested (0.1, 0.5, 1.0 and 2.0 m h^{-1}) in recirculated UASB reactors operated in batch mode when controlling the pH at 5.0. Up-flow velocities of 1.0 and 2.0 m h^{-1} resulted in a H_2 yield of about 1.0-1.1 mol mol^{-1} $glucose_{eq.}$, 40 and 60% higher than the yields obtained at 0.5 and 0.1 m h^{-1}, respectively (Fig. S1, Table S2), as a result of the higher mixing and gas stripping from the fermentation broth. Methane was not detected at any condition tested, due to the quick acidification of the medium, with pH dropping from 6.4 to 5.0 within one operation day. Based on these results, an up-flow velocity of 1.0 m h^{-1} was selected for further experiments.

3.2 Batch cheese whey fermentation in UASB reactors and VFA extraction

3.2.1 Effect of in-line VFAs extraction, pH and temperature on H_2 production

Similar yields of about 0.9 mol H_2 mol^{-1} $glucose_{eq.}$ and maximum production rates of about 0.26 mol H_2 mol^{-1} $glucose_{eq.}$ d^{-1} (Fig. 2; Table 3) were observed in the UASB reactors operated in batch at 35°C and pH 5.0 with and without the *in-line* VFA separation module. Therefore, the VFAs extraction module had a minimum impact on CW fermentation in UASB reactors. The results were also similar to those obtained in the preliminary test at up-flow velocities of 1.0 and 2.0 m h^{-1} (Fig. S1), confirming the replicability of the fermentation process. This is further confirmed by the fact that the

obtained H_2 yield was comparable to the results achieved in previous studies on CW fermentation [10,32].

Decreasing the operating temperature to 20°C, or pH to 4.5, resulted in a lower H_2 yield of 0.36 and 0.49 mol H_2 mol^{-1} $glucose_{eq.}$, respectively. The fermentation kinetics of CW, and in particular lactose hydrolysis, are indeed slower at low temperature [33]. At pH 4.5, despite the relatively fast kinetics (0.35 mol H_2 mol^{-1} $glucose_{eq.}$ d^{-1}) and short lag-phase (1.0 d) (Table 3), H_2 production was likely inhibited by the accumulation of butyric acid in its undissociated form (7.6 g L^{-1}, 68% of the total), which can penetrate the bacterial cells suppressing growth and metabolic activity [34]. H_2 yields obtained from CW-based substrates using mixed cultures at pH below 5.0 are typically low, although a H_2 yield of 1.83 mol H_2 mol^{-1} $glucose_{eq.}$ was reported from a diluted CW powder solution (4.9 g lactose L^{-1}) in a fluidized bed reactor operated at pH 4.0-4.5 under thermophilic conditions (55°C) [19].

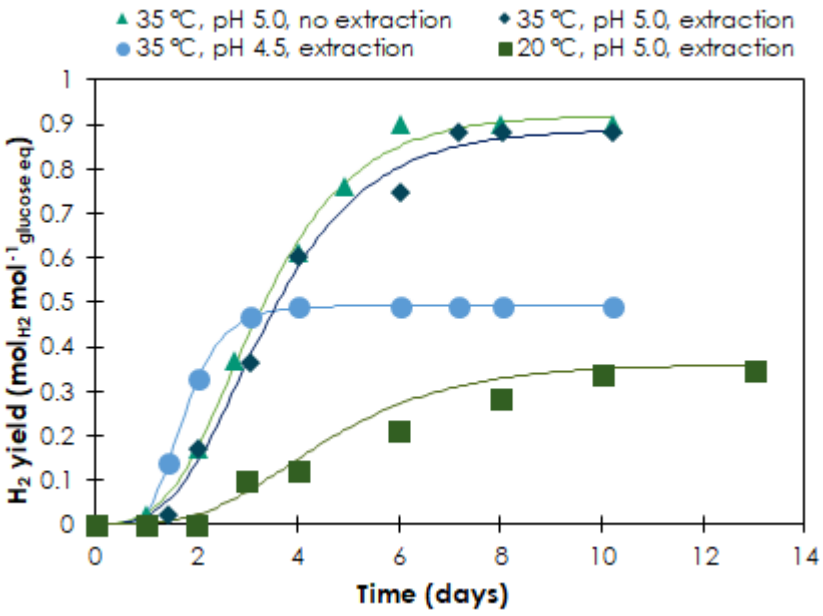


Figure 2. Evolution of H_2 yields over time for the UASB fermentation tests performed at 20 and 35°C, pH 5.0 and 4.5, with or without *in-line* VFA extraction. Scatter plots represent the experimental data, and continuous lines represent the Gompertz model fitting.

Table 3. Gompertz model parameters as calculated for the fermentation tests performed at different operating conditions.

Parameter	Measure unit	35°C, pH 5.0, no extraction	35°C, pH 5.0, extraction	35°C, pH 4.5, extraction	20°C, pH 5.0, extraction
H_2 $yield_{max}$	mol H_2 mol^{-1} $glucose_{eq.}$	0.921	0.888	0.491	0.360
R_{max}	mol H_2 mol^{-1} $glucose_{eq.}$ d^{-1}	0.259	0.238	0.354	0.075
λ	d	1.379	1.474	1.049	2.041
$t_{95\text{-}H2}$	d	7.214	6.908	3.106	9.100
R^2	-	0.996	0.994	1.000	0.981

3.2.2 Effect of in-line VFA extraction, pH and temperature on fermentation pathways

In all conditions tested, fermentation evolved according to three subsequent degradation stages. Lactose was first hydrolysed to glucose at different rates depending on the operating conditions, and then converted to lactic acid via homolactic fermentation. Galactose, the other monomeric sugar formed during lactose hydrolysis, was always below detection, suggesting its rapid conversion to glucose 6-phosphate, since there is no catabolic pathway to metabolize it [35]. Lactic acid was then converted to H_2, CO_2 and VFAs, with a prevalence of butyric acid which was produced up to 15 and 20 g L^{-1} regardless of temperature and pH. The highest butyrate production rate of 5.7 g L^{-1} d^{-1} was obtained at pH 5 in the UASB without extraction module, after about two days of lag-phase (Fig. 3). The full conversion of lactic acid to VFAs was achieved at pH 5.0, within 6-8 days at 35°C and around 10 days at 20°C, whilst the same fate was not observed at pH 4.5, likely due to inhibition of the fermentative microorganisms [34].

In both UASB reactor tests at 35°C and pH 5.0, the acetic acid concentration increased after 6-8 operation days (Fig. 3), suggesting the onset of homoacetogenic pathways with related negative effects on the H_2 yields [36,37]. Although the optimum growth pH of propionate producing microorganisms is typically around 7 [38] , propionic acid was detected in all tests at pH 5.0, with a final concentration of 4-5 g L^{-1}, and even at pH 4.5 with a final concentration of 2.3 g L^{-1}. Significant ethanol production, up to 5 g L^{-1}, was obtained only at 20°C, suggesting a shift from homolactic to heterolactic fermentation. In this case, the overlapping pathways may have been caused by the slower sugar consumption rates due to the lower temperature [39].

Under all conditions investigated, the pH dropped from the initial value of 6.3 to either 5.0 or 4.5 within 1-2 days (at 35°C) or 3 days (at 20°C), and a further decrease was avoided only by automatic NaOH dosing. In the UASB reactors operated at pH 5.0, once the sugars were fully consumed, the pH raised again likely due to protein hydrolysis [40], and the consequent ammonium release. Indeed, about 430 and 460 mg L^{-1} ammonium was found upon CW fermentation at 20 and 35°C, respectively, against the 83 mg L^{-1} detected in the CW prior to fermentation (Table 1). The pH increase was more evident in the UASB reactor with the extraction module, due to the VFAs crossover through the silicone membrane, resulting in a final pH of 6.5 as compared to a pH of 6.2 observed in the UASB reactor without extraction module (Fig. 3). As an interesting consequence, a 25% lower NaOH dosage (9.0 g L^{-1} CW) was required in the UASB reactor provided with the extraction module than in the UASB reactor where

VFAs were not extracted (12.0 g L^{-1} CW) in order to maintain pH values above 5.0, which, in turn, significantly reduces the operating costs in full-scale application.

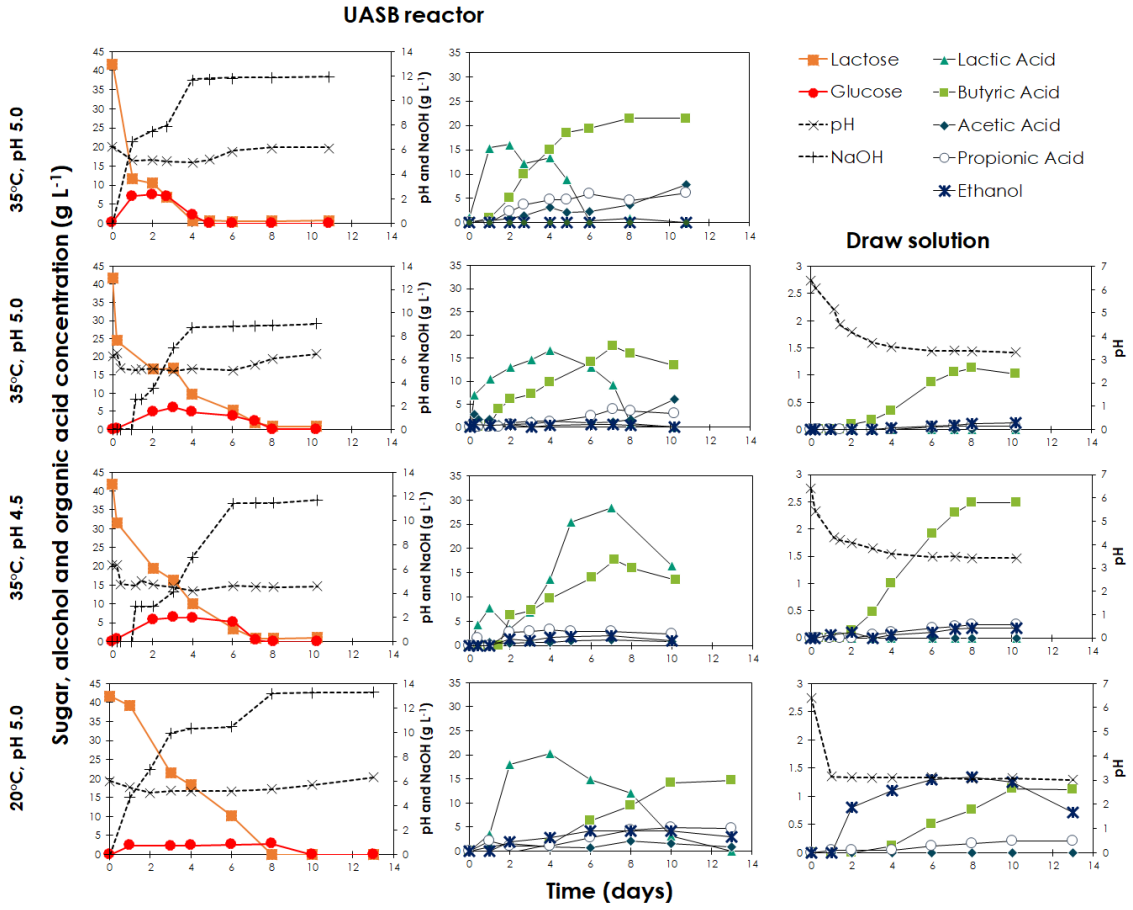


Figure 3. Sugar, alcohol and VFAs concentration profiles, pH and NaOH dosage for the UASB fermentation tests at different temperature (20 or 35°C), pH (4.5 or 5.0) with or without the silicone membrane extraction module. The column "Draw solution" refers to the VFAs and alcohol extracted from the UASB reactors through the silicone membrane, and the resulting pH profiles.

3.2.3 Effect of pH and temperature on VFAs and alcohol extraction through silicone membrane

357 At 35°C, in the UASB reactor equipped with in-line extraction, irrespectively of the
358 operating pH values (5.0 or 4.5), butyric acid was the main metabolite extracted,
359 accounting for more than 90% of the carbon content (Table 4). Indeed, butyric acid
360 migrates faster than shorter chain acids through the silicone membrane matrix due to its
361 higher hydrophobicity [26]. Sugars, lactic acid, and nutrient sources such as proteins, P,
362 anions (Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}) and cations (Ca^{2+}, K^+, Na^+, and NH_4^+) were,
363 however, retained in the UASB reactor mixed liquor. This confirms that the extraction
364 module prevents the migration of substrates and nutrients, which could inhibit the
365 fermentation process, besides reducing costs for pH control, and allows recovery of
366 butyric acid with more than 90% purity (on carbon content basis), simplifying
367 downstream processing. However, when the extraction module was installed into the
368 UASB, 16.6–19.9% of the inlet carbon was not detected as fermentation product,
369 against only 3.1% unaccounted carbon in the control UASB reactor (Table 4),
370 suggesting VFA adsorption on the silicone membrane. This hypothesis is supported by
371 the higher butyric acid concentration obtained at pH 5 and 35°C without, rather than
372 with, the extraction module (Table 4), despite the similar butyric acid yield expected
373 based on the similar H_2 yield obtained in the two UASBs (Fig. 2). This would be,
374 nevertheless, a minor issue in continuous operation, since the membrane will be quickly
375 saturated with VFAs, after which a further loss will not occur.
376
377 In the test at 20°C, it is worth underlining that also ethanol was produced and extracted
378 through the silicone membrane, opening up further fermentation-related applications.
379 However, from day 8 onwards, ethanol concentrations in the draw solution decreased

(Fig. 3), likely due to its volatilization, a feature to be considered when ethanol is the target of the separation process, which was not the case in this study.

In the UASB reactor operated at 35°C and pH 5.0 with in-line VFAs extraction, a total of 14 g L^{-1} butyric acid was produced, 1 g L^{-1} of which was recovered in the draw solution upon extraction (Fig. 3). The butyric acid flux through the silicone membrane reached a maximum of 0.41 g m^{-2} h^{-1} on day 6 (Fig. 4). At pH 5.0, only 45% of the butyric acid (pK_a=4.82), *i.e.* about 6.3 g L^{-1}, was in the undissociated form, which is a requisite for crossing the silicone membrane [26]. Furthermore, from day 6, the concentration of undissociated acid further decreased to <10% (*i.e.*, < 1.4 g L^{-1}) due to the pH raise above 6.0, whereas the pH of the draw solution dropped to 3.5 (Fig. 3). The decreasing concentration gradient between fermentate and draw solution caused a decrease of the butyric acid flux, which became even negative on day 10 (Fig. 4), suggesting that a small amount of butyric acid was diffusing back towards the fermentation compartment. This issue can be mitigated in continuous operation, since continuous carbohydrate fermentation would prevent a pH raise, and butyric acid migration would thus continue as long as a concentration gradient is kept between the fermentate and the draw solution.

To maintain the concentration gradient between the fermentation broth and draw solution as high as possible, VFAs can be periodically or continuously extracted from the draw solution, e.g. using electrodialysis technology [41]. Electrodialysis has previously been applied to extract VFAs directly from a fermentation broth [41,42], but its application is limited by the fact that all the anions are unselectively extracted, and

by biofouling. Both issues are avoided, or at least mitigated, if a silicone membrane separation module is installed prior to the electrodialysis unit. Jones et al. (2017) reported also a 3.75 higher H_2 production in a bioreactor operated with *in-line* VFAs extraction *via* electrodialysis, with respect to a control reactor without extraction unit. Such a beneficial effect was, nevertheless, not evident in this study (Fig. 2), likely due to the substantially higher VFA concentrations (20-35 g L^{-1} total VFAs) in the fermentation broth (Fig. 3) compared to those (3-4 g L^{-1} total VFAs) reported in Jones et al. [42]. Indeed, although lower VFA concentrations were measured in the presence, than in the absence, of the extraction unit (Fig. 3), the mitigation effect of the *in-line* VFA extraction was not enough to impact the H_2 yield.

Fermentation at pH 4.5, which led to about 70% of the produced butyric acid in undissociated form, resulted in a 240% higher butyric acid extraction (2.5 g L^{-1}, 40% of the theoretical maximum value) through the silicone membrane than at pH 5.0 (Fig. 3). The butyric acid flux reached a peak of 0.51 g m^{-2} h^{-1} on day 4, higher than the maximum flux of 0.41 g m^{-2} h^{-1} obtained at pH 5.0 (Fig. 4). Ultimately, the butyric acid extraction process can be facilitated by lowering the pH in the fermentation reactor, although this would be detrimental to the H_2 production (Fig. 2). An acidification-neutralization step could also be included into the extraction loop, but this would result in higher operation costs.

424 **Table 4.** Carbon balances (in g L^{-1}) of the fermentation tests performed at different temperature (20 or 35°C) and pH (4.5 or 5.0) using

425 UASB reactors with or without silicone membrane extraction unit.

Compound (g C L^{-1})	**Cheese whey**	**35°C, pH 5.0, no extraction**	**35°C, pH 5.0, extraction**		**35°C, pH 4.5, extraction**		**20°C pH 5.0, extraction**	
		Fermentate	**Fermentate**	**Draw solution**	**Fermentate**	**Draw solution**	**Fermentate**	**Draw solution**
Lactose	17.54	-	-	-	-	-	-	-
Lactic acid	0.37	-	-	-	6.54	-	-	-
Acetic acid	0.10	1.46	2.44	-	0.34	-	0.41	-
Propionic acid	0.04	2.18	1.47	0.07	1.12	0.12	1.94	0.10
Butyric acid	-	11.69	7.32	1.03	3.61	1.36	8.03	0.61
Ethanol	-	-	0.27	0.13	0.55	0.10	1.55	0.21
CO_2	-	3.11	1.68	1.22	0.76	0.74	0.81	0.99
Proteins[a]	1.06	0.07	0.30	-	0.07	-	0.46	-
Total	**19.11**	**18.51**	**15.93**		**15.31**		**15.11**	
Balance	**100%**	**96.9%**	**83.4%**		**80.1%**		**79.1%**	

426 [a] Calculated assuming that 46% of the protein weight is carbon [30]

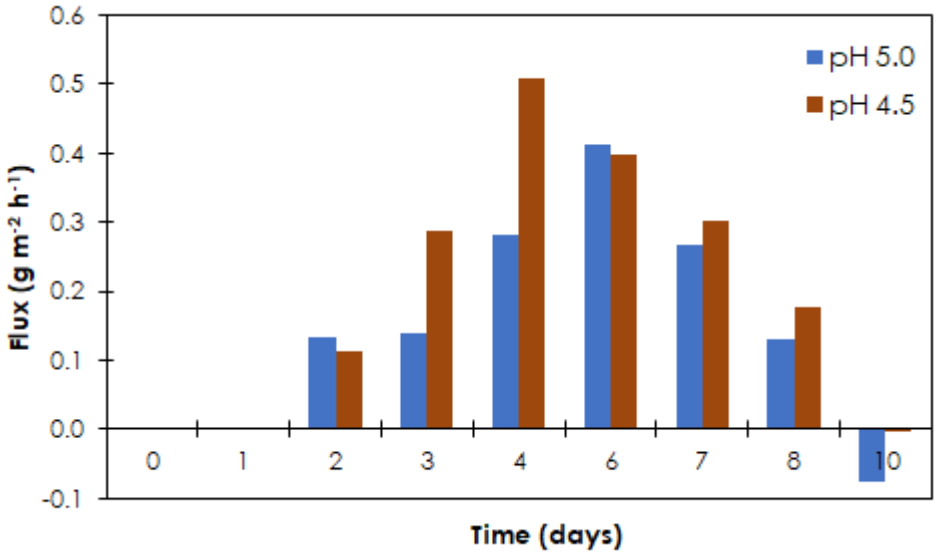


Figure 4. Evolution of the butyric acid flux through the silicone membrane over time during the UASB batch experiments performed at 35°C and at different pH.

3.3 Continuous cheese whey fermentation in UASB reactors and VFA extraction

3.3.1 Effect of in-line VFA extraction on H_2 production

After a 5-day start-up in batch, both UASB-A (without extraction module) and UASB-B (with extraction module) were operated in continuous mode, with a HRT of 24 hours, reaching the same maximum HPR of 1.9-2.0 L L^{-1} d^{-1} within 20 and 37 days operation, respectively (Fig. 5). This confirms that the use of the *in-line* VFA extraction module implemented in this study had a minimum impact on the achievable H_2 production. The presence of the long silicone spiral (4.2-4.4 m in this study) in the recirculation line may, however, impact the contact time between the substrates and microorganisms, particularly during continuous operation, resulting in a slower onset of the H_2 production (Fig. 5). The gas produced by CW fermentation was mainly composed of H_2 and CO_2, with a H_2 concentration of 35-37% in UASB-A and 27-28% in UASB-B. The

observed difference in gas composition was attributed to the lower solubility of CO_2 (< 10^{-4} g kg^{-1}) as compared to H_2 (1.6×10^{-3} g kg^{-1}) in distilled water (the draw solution) at the low pH (<4) caused by the extracted VFAs. In line with a waste biorefinery approach, the produced CO_2 can be converted to value-added products through algae- or cyanobacteria-based processes [43,44], or microbial electrosynthesis [45].

Providing a UASB reactor with an extraction module also enabled a more stable process, particularly on days 39-48, as underlined by the HPR values which spanned between 0.8-1.1 L L^{-1} d^{-1} in UASB-B, as compared to UASB-A (0.6 and 1.6 L L^{-1} d^{-1}). The performance of both UASB reactors (average HPR of 1.0-1.3 L L^{-1} d^{-1}, with peaks of about 2.0 L L^{-1} d^{-1}, and a highest yield of 0.7-0.8 mol H_2 mol^{-1} $glucose_{eq.}$) was remarkable, since it fairly compares with the highest HPR obtained through continuous dark fermentation of CW. Castelló et al. [46] operated a UASB reactor at 30°C and average pH 5, reporting a low HPR of only 0.12 L L^{-1} d^{-1} due to the onset of methanogenesis, an issue which did not occur at any stage in the present study. However, methane production cannot be fully excluded over extended operation periods, due to the acclimation of the consortium and the possible presence of favourable micro-environments [37]. A slightly higher average HPR of 1.6 L L^{-1} d^{-1} was obtained by Blanco et al. [21], who used a novel structured-bed reactor configuration, operated at pH 5, 25°C and an OLR of 24 g COD L^{-1} d^{-1}, though fed with synthetic cheese whey. Higher HPRs can be potentially obtained at a pH close to 6 (Fig. 5), but this will result in a much lower VFA recovery through the silicone membrane due to acid dissociation. For instance, high HPRs (28 and 25 L L^{-1} d^{-1}) have been obtained by CW powder fermentation at pH 5.9, when applying an OLR of 150-160 g_{COD} L^{-1} d^{-1}

[20,47], more than three times higher than the OLR applied in this study. However, at pH 5.9, only a small fraction (8%) of the butyric acid is undissociated, making *in-line* extraction through the silicone membrane ineffective.

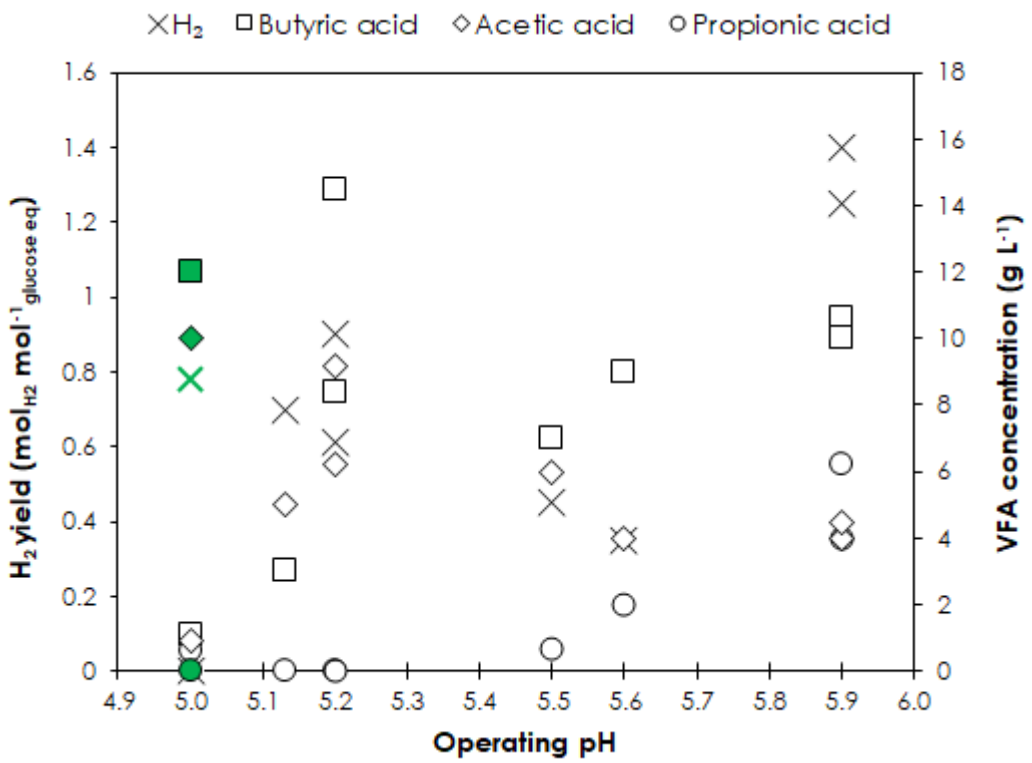


Figure 5. Highest H_2 yield and VFA concentrations obtained by fermentation of cheese whey or cheese whey powder under mesophilic conditions at different controlled pH, as reported in references [20,21,32,46–50]. The green values refer to the present study.

3.3.2 Effect of pH on H_2 production

Since a low pH is preferable for VFAs extraction through the silicone membrane (Fig. 4), two different strategies were attempted to adapt the microorganisms to ferment CW at low pH. On day 42, operation of UASB-A was stopped, and the reactor was restarted at pH 4.5 with fresh inoculum, whereas the pH of UASB-B was decreased from 5.0 to 4.5 on day 49. Both UASB reactors were operated with in-line VFAs extraction during

this stage (Table 2). In both reactors, the low pH caused a HPR below 0.2 L L^{-1} d^{-1}, substantially lower than those obtained at pH 5.0 (Fig. 6). Furthermore, in UASB-A, a consistent H_2 production was not achieved even after raising the pH to 5.0 (on day 65), whereas the H_2 production was resumed in UASB-B, though with an average HPR of only 0.4 L L^{-1} d^{-1} during days 75-84 (Fig. 6). This suggests that the microbial community enriched at pH 5.0 was resilient, and able to resume the H_2 production after a pH shock, but was unable to fully restore its productivity in the short term.

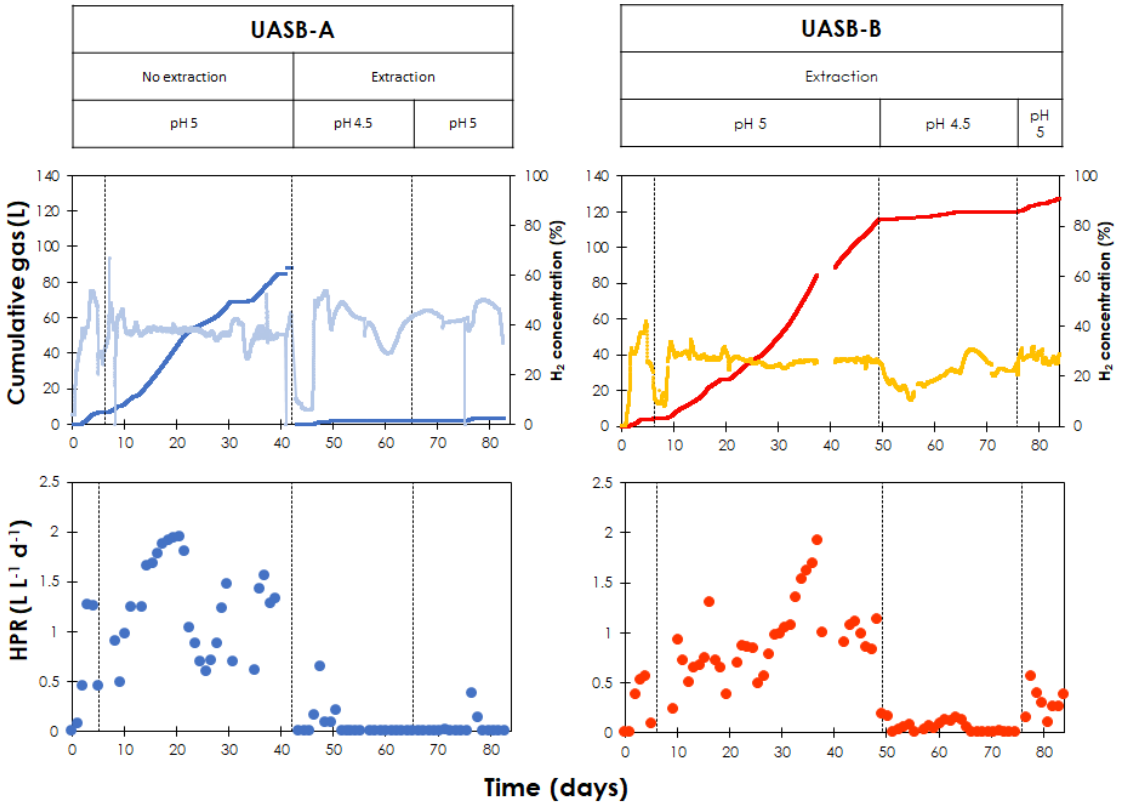


Figure 6. Cumulative gas production (primary axis, blue or red), H_2 concentration (secondary axis, light blue or orange) and daily average H_2 production rate (HPR) of the two UASB reactors throughout the experiment. The experimental stages, separated by the vertical dotted lines, refer to Table 2. In UASB-A, gas production stopped on days

31-34 due to influent pump failure. For UASB-B, data are missing on days 37-39 due to a sensor failure.

3.3.3 Cheese whey fermentation pathways under different operation conditions

During continuous operation, a partial conversion of lactose to lactic acid occurred already in the supply tank, despite it was regularly cleaned, re-supplied with fresh CW and maintained at 4-6°C. This led to an influent lactose concentration of 10-30 g L^{-1} or even lower (Fig. 7), against 41.7 g L^{-1} in the fresh CW (Table 1). Partial acidification of the CW (average acidification degree of 20%) resulted in an average pH of 4.5 ± 0.3, and an OLR ranging between 40 and 80 g_{COD} L^{-1} d^{-1}, or even lower (Fig. 7). Ethanol was produced from day 28 in the supply tank, and its concentration reached 10-15 g L^{-1} on day 65, suggesting the onset of heterolactic fermentation. Although the variability of the influent characteristics affected the execution of the experimental tests, it is unavoidable and also occurs in full-scale applications. This issue can be mitigated by minimising preliminary storage prior to fermentation and keeping the distance between the dairy factories where CW is produced and the treatment plant as short as possible.

During the first ten days of continuous operation at pH 5.0 (day 6-16), acetic acid was the main metabolite produced in both UASB reactors, reaching concentrations up to 25 g L^{-1} (Fig. 7), which subsequently decreased to <10 g L^{-1}. After this initial stage, butyric acid was the main VFA produced at pH 5.0 in both UASB reactors, with fluctuating concentrations (between 5 and 12 g L^{-1}) due to the unstable influent lactose concentration (Fig. 7). The achieved butyric acid concentration of 12 g L^{-1} is among the highest reported in literature (Fig. 5). The trend of butyric acid concentrations reflects

519 that of the H_2 production (Fig. 6), suggesting that H_2 was mainly produced via lactic
520 acid conversion to butyric acid, as previously reported [10,20]. In both UASB reactors,
521 when operated under continuous mode at pH 5.0, over 80% of the influent sugars were
522 consumed, whereas lactic acid conversion was incomplete resulting in an average
523 residual concentration of 5.9 and 8.7 g L^{-1} in UASB-A and UASB-B, respectively (Fig.
524 7). Longer HRTs may allow a full sugar and lactic acid conversion to VFAs, but this
525 will cause an increase of the required reactor volume, resulting in higher costs, probably
526 not balanced by the advantages that can be obtained.
527
528 A drastic decrease in butyric acid production, along with H_2 production, was observed
529 when both UASB reactors were operated at pH 4.5, although the sugar consumption
530 remained over 80% (Fig. 7). Most sugars were indeed converted to lactic acid, which
531 accumulated up to 25-30 g L^{-1}, but further conversion of lactic acid to VFAs was
532 inhibited by the low pH, resulting in VFAs concentrations below 2 g L^{-1}. Interestingly,
533 at pH 4.5, the ethanol concentration increased up to 15 g L^{-1} in both UASB reactors,
534 besides being produced already in the supply tank (Fig. 7). When the pH was increased
535 back from 4.5 to 5.0, the lactic acid concentration immediately decreased to < 10 g L^{-1}
536 in both UASB reactors, and the butyric acid concentration increased back to about 5 g
537 L^{-1}.

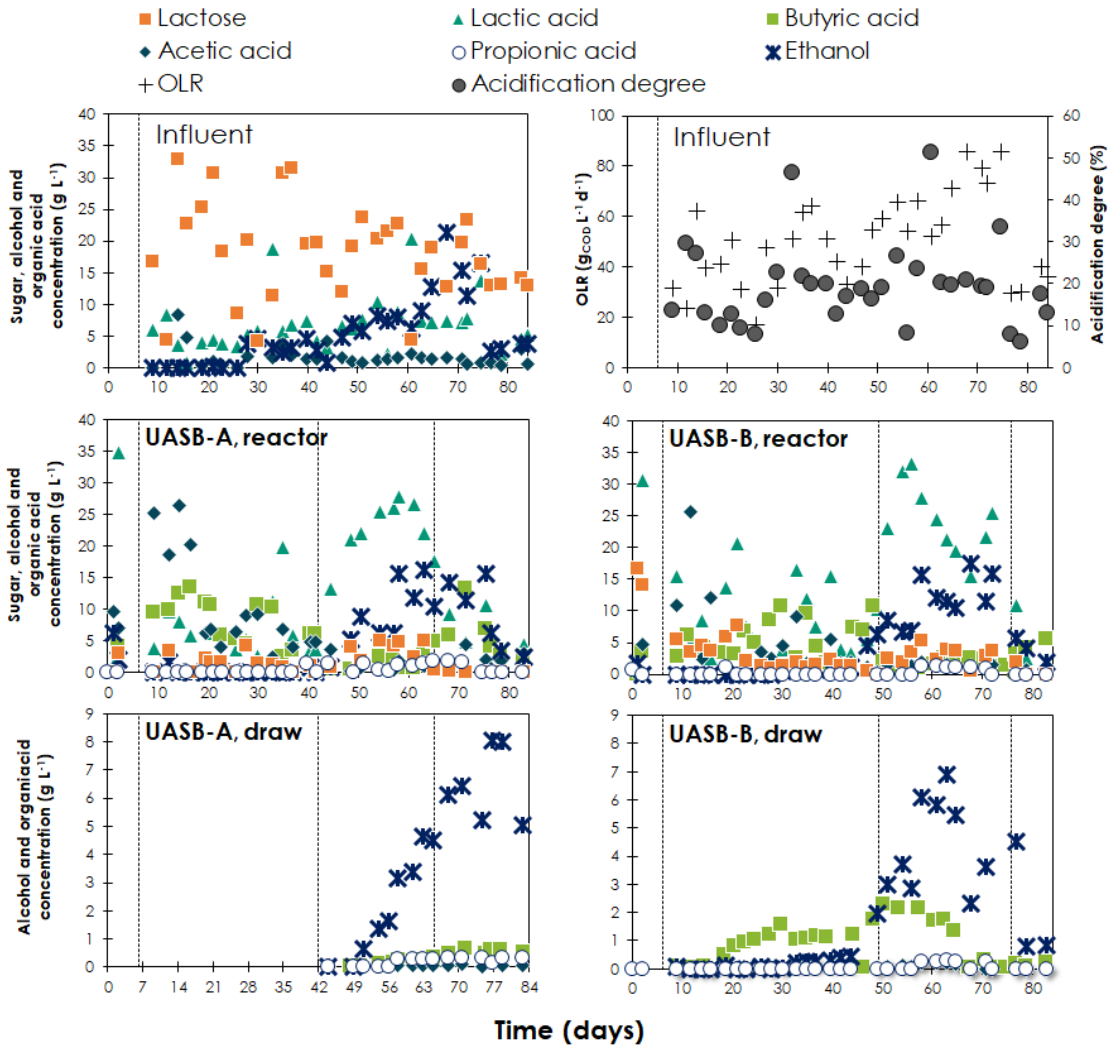


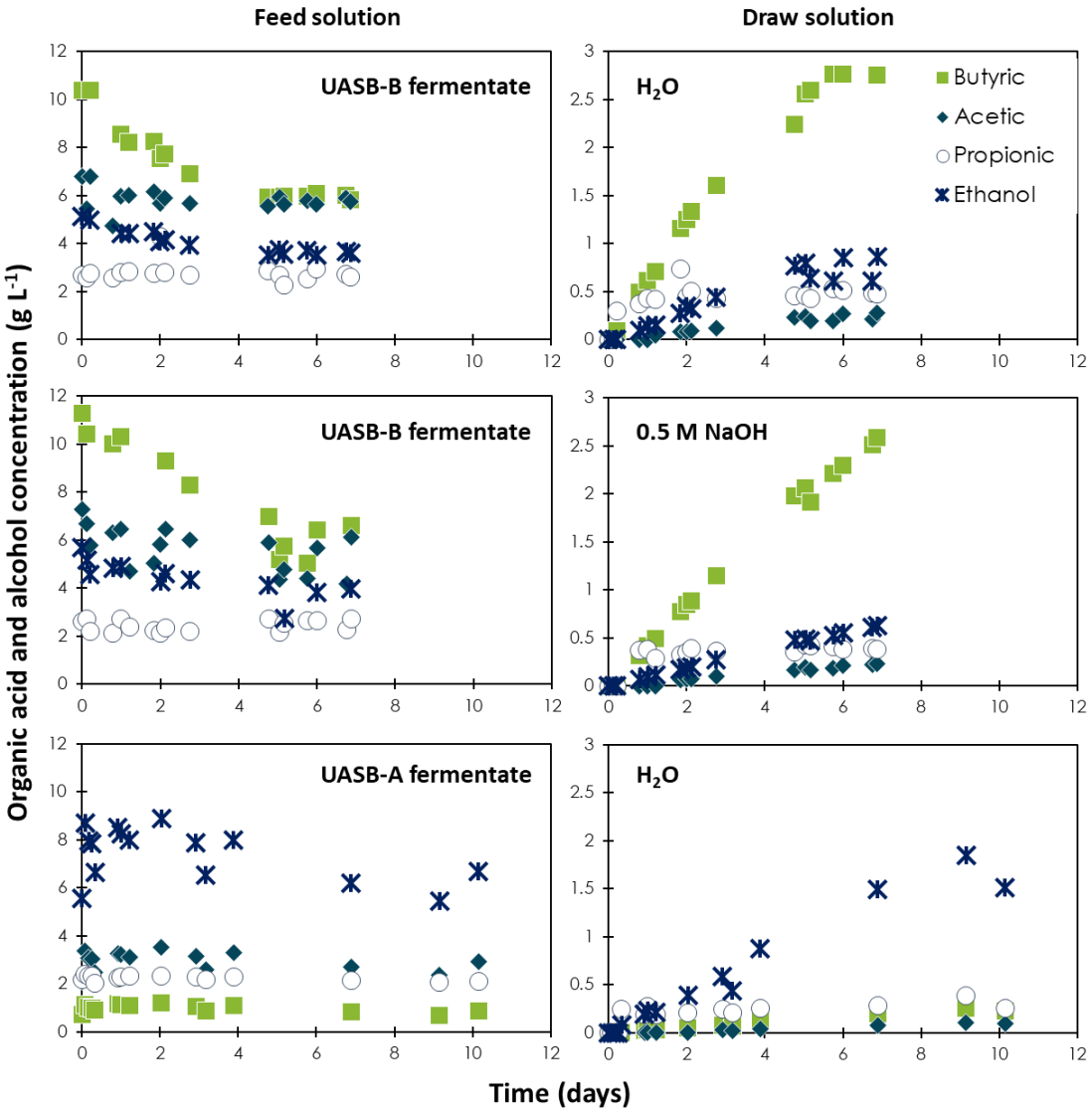
Figure 7. Sugar, alcohol, and carboxylic acid concentration in the influent, the two UASB reactor effluents and the respective draw solutions during the continuous experiment. The organic loading rate (OLR) and acidification degree of the influent are also reported. The experimental stages, separated by the vertical dotted lines, refer to Table 2.

3.3.4 Continuous VFA extraction through silicone membrane

In UASB-B, butyric acid was extracted at pH 5.0 up to a concentration of 1.0-1.5 g L^{-1} within 7 days, confirming the results obtained under batch conditions. The extraction of butyric acid benefited from the decrease of fermentation pH down to 4.5, which enabled to reach concentrations up to 2.2 g L^{-1} in the draw solution on days 54-58. However, starting from day 59, the butyric acid extraction was affected by the low production in UASB-B, which caused an inversion of the concentration gradient (Fig. 7). When the UASB reactors were operated at pH 4.5, ethanol was produced in both UASB reactors and extracted up to a concentration of 7 g L^{-1} (Fig. 7), suggesting that non-porous silicone membranes can be used for alcohol extraction as well. Unlike carboxylic acids, alcohols do not dissociate in water, and the extraction is therefore not affected by the pH.

3.4 Off-line VFAs and alcohol extraction from cheese whey fermentate

The effluents of UASB-A and UASB-B were collected on day 71, while the reactors were operated at pH 4.5 and 5.0, respectively, and tested for off-line VFAs and alcohol extraction upon acidification (pH 3.0). The fermentate of UASB-B mainly consisted of butyric acid (10.8 ± 0.4 g L^{-1}), with lower concentrations of ethanol (5.4 ± 0.3 g L^{-1}) and acetic (7.0 ± 0.2 g L^{-1}), propionic (2.7 ± 0.0 g L^{-1}) and lactic (2.9 ± 0.1 g L^{-1}) acid, whereas the fermentate of UASB-A contained mainly lactic acid (19.4 ± 1.1 g L^{-1}) and ethanol (5.9 ± 0.4 g L^{-1}) with lower concentrations of acetic (2.2 ± 0.1 g L^{-1}), propionic (2.2 ± 0.0 g L^{-1}) and butyric (0.8 ± 0.0 g L^{-1}) acid.



569

570 **Figure 8.** Off-line VFAs and alcohol extraction from the UASB reactor effluents,
571 acidified to pH 3.0, via silicone membrane pertraction at 20°C. The UASB-A and
572 UASB-B fermentate was collected while the reactors were operated at pH 4.5 and 5.0,
573 respectively. Lactic acid data are omitted since it was not detected in the draw solutions.

574

575 Acidifying the fermentate to pH 3.0, and, in turn, increasing the share of undissociated
576 VFAs, fostered their diffusion rate through the silicone membrane. Butyric acid was
577 extracted with a maximum flux of 0.53 g m^{-2} h^{-1}, exceeding the concentration of 2 g L^{-1}

578 in the draw solution within 5 days (Fig. 8). Indeed, at pH 3.0, over 99% of the butyric
579 acid is in undissociated form, as compared to the 40% undissociated at pH 5.0. On the
580 other hand, by decreasing the pH the diffusion of acetic and propionic acid is favoured
581 as well, leading to a lower purity of butyric acid in the draw solution (65.4% and 69.2%
582 on carbon content basis, using water and 0.5 M NaOH, respectively, as draw solution).
583 In contrast, an over 90% purity was observed in the experiments performed at pH 5.0
584 and 4.5 (Fig. 3).
585
586 Using NaOH as the draw solution resulted in a slower VFAs migration (maximum flux
587 of 0.26 g d^{-1} h^{-1}), but a concentration gradient was maintained due to the dissociation of
588 the extracted VFAs, and the subsequent formation of sodium salts. In contrast, a plateau
589 was reached when pure water was used as the draw solution (Fig. 8). The overall mass
590 transfer coefficient (K_{OV}) of butyric acid from the UASB-B fermentate was 0.109 and
591 0.101 µm s^{-1} using, respectively, water and NaOH as the draw solution. Those values
592 are comparable to the K_{OV} of 0.157 µm s^{-1} previously obtained for butyric acid
593 extraction from acidified fish fermentate using silicone membrane [26].
594
595 Despite the high lactic acid concentration (19.4 g L^{-1}) in the UASB-A fermentate, 85%
596 of which in undissociated form at pH 3, lactic acid did not migrate through the
597 membrane, resulting in concentrations below the detection limit in the draw solution.
598 This is due to its low volatility (Henry constant 9.6×10^{-9} atm m^{-3} mol^{-1}) and solubility in
599 water, whereas the more volatile ethanol (Henry constant 5.0×10^{-6} atm m^{-3} mol^{-1})
600 migrated with a Kov of 0.083-0.096 µm s^{-1}.
601

3.5 Future research directions

Despite the promising results obtained in this study, more research efforts are required to advance the technology readiness level of the integrated process. Further studies should focus on membrane characteristics (material, length, and thickness) and process operating parameters (pH, temperature, and recirculation flow). A second process to be downstream implemented, e.g. electrodialysis-based technologies [42], is required to concentrate the VFAs extracted and, at the same time, to avoid their accumulation in the draw solution, keeping a sufficient concentration gradient between the fermentation broth and the draw solution to allow VFA migration. Enhancing in-line VFA extraction further reduces their toxic effect on the microorganisms, and thus positively affects H_2 yields [42], resulting in further economic benefits.

4. Conclusions

This study proposes a novel approach for cheese whey valorisation that can also be applied to other waste streams, where dark fermentation is combined to a relatively low-cost extraction process to obtain H_2 and high-purity butyric acid. This study showed that:

- HPR up to 2.0 L L^{-1} d^{-1} can be obtained by fermenting cheese whey at pH 5.0;
- Non-porous silicone membranes favours extraction of longer chain fatty acids over short chain acids, which is a unique advantage for processes generating VFA mixtures;
- Up to 3 g L^{-1} of high purity (>90% on carbon content basis) butyric acid can be in-line extracted without affecting the steady-state H_2 production, decreasing the

NaOH requirement and saving the energy which is otherwise required for extraction;

- Ethanol can be extracted using silicone membranes, whereas sugars and nutrients are retained;
- Low pH values increase VFAs extraction rate in silicone membrane pertraction, but drastically decrease the HPR and can negatively affect the selectivity of the extraction process.

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Conflict of interest

The authors declare no conflict of interest.

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652 (IWWG), https://www.tuhh.de/iue/iwwg/task-groups/waste-biorefinery.html.
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