Chemical Engineering Journal 497 (2024) 154539

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Membrane-based fermentation enables highly selective caproic acid production from wine lees

Paolo Dessì [a,b,1,*], Meritxell Romans-Casas [a,1], Elisabet Perona-Vico [c], Michele Tedesco [d], Hubertus V.M. Hamelers [e], Lluis Bañeras [c], M. Dolors Balaguer [a], Sebastià Puig [a]

[a] LEQUiA, Institute of the Environment, University of Girona. Campus Montilivi, Carrer Maria Aurèlia Capmany 69, E-17003 Girona, Spain
[b] Department of Agricultural Sciences, University of Naples Federico II, Piazza Carlo di Borbone 1, 80055 Portici, Italy
[c] Molecular Microbial Ecology Group, Institute of Aquatic Ecology, University of Girona, Maria Aurèlia Capmany 40, 17003 Girona, Spain
[d] TNO, Sustainable Process and Energy Systems, Lange Kleiweg 1D, 2288 GH Rijswijk, the Netherlands
[e] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911, MA, Leeuwarden, the Netherlands

ARTICLE INFO

Keywords:
Biorefinery
Chain elongation
Fermentation
Hexanoic acid
Pertraction
Product purification

ABSTRACT

Chain elongation is a green chemistry approach to convert organic waste or agro-industrial side-products into caproic acid. Nonetheless, such feedstocks may contain microorganisms and chemical pollutants that could deteriorate the product yield and purity. This study introduces a membrane-based fermentation process designed to isolate the caproic acid producing bacteria from these contaminants. A tubular polysiloxane (silicone) membrane submerged into a fermentor allowed the selective diffusion of ethanol and acetic acid while preventing the cross-over of bacteria and contaminants such as ions. Abiotic tests confirmed that membrane thickness, feedstock pH and flow velocity can be adjusted to independently control the diffusion of ethanol and acetic acid. Thereof, optimal ethanol:acetic acid ratios for chain elongation (6:1) were obtained from a feedstock solution with equimolar (1 M) concentrations of both. In the biotic tests, this resulted in a highly selective (>90 %) caproic acid production at a highest rate of 3.1 g/(L·d). Maintaining the pH above 6.8, thereby keeping most caproic acid in its dissociated form, prevented its back-diffusion through the membrane. Similar caproic acid productivity was achieved from diluted wine lees (1 M ethanol), amended with 1 M acetic acid. In contrast, unamended wine lees resulted in three times lower caproic acid production rate, although the product selectivity remained high (94 %). Downstream processing by acidification and phase separation yielded 6–13 mL/$L_{feedstock}$ of an oily product containing up to 784 g/L caproic acid (84.3 % purity). In conclusion, membrane-based fermentation enables highly selective caproic acid production from highly concentrated and unbalanced substrates.

1. Introduction

Population growth and prosperity increase in developing countries result in a high demand for chemical products. The energy- and carbon-intensive chemical industry is expected to become the first global oil consumer with a 25 % contribution (2.4 billion litres) by 2050 [1]. However, the emergence of a circular economy, as well as the evolution of carbon pricing, expected to exceed 100 €/ton by 2050, will make the business-as-usual model economically unsustainable [2]. Carbon-free vectors such as green electricity and H_2 will be increasingly adopted for energy generation. Instead, low-carbon feedstocks such as organic waste/by-products can replace the fossil fuel-based counterparts for chemical production, transforming the chemical industry into a biorefinery [3].

The agri-food sector is currently responsible for over one billion tons of organic waste, expected to further raise in response to population growth [4], representing a major societal and environmental concern. Rather than a waste, agri-food residues can be seen as a sustainable feedstock that matches the scale required by chemical industry and does not compete with food production. Several biorefineries models adopting agri-food waste such as potato peels [5], cassava processing residues [6], or mixed fruit and vegetable leftovers [7] have been proposed. In

* Corresponding author.
E-mail address: paolo.dessi@unina.it (P. Dessì).
[1] These two authors contributed equally to the manuscript.

https://doi.org/10.1016/j.cej.2024.154539
Received 26 March 2024; Received in revised form 8 July 2024; Accepted 2 August 2024
Available online 3 August 2024

most cases, the biorefinery model includes a fermentation step in which carbohydrates, proteins and/or lipids are biologically converted into simpler molecules such as short-chain carboxylic acids (i.e. lactic, acetic and butyric acids) and ethanol [8].

Depending on feedstock composition, microbial community structure, and operation conditions (pH, hydrogen partial pressure and temperature), a secondary fermentation may spontaneously occur, where ethanol or lactic acid act as electron donor to elongate short chain carboxylic acids into medium chain carboxylic acids. This mostly occurs through an enzymatic pathway called reverse β-oxidation (RBO). In RBO, the electron donor is first converted to Acetyl-CoA and enters a cyclic chain elongation reaction yielding a C_{n+2} from a C_n carboxylic acid. Thus, acetic (C_2) and butyric (C_4) acids are typically elongated to caproic acid (C_6) and even to caprylic acid (C_8) [9]. Ethanol is the most studied electron donor for chain elongation. Since its oxidation to Acetyl-CoA does not yield CO_2, it is superior to lactic acid in terms of conversion efficiency. Ethanol can be produced by alcoholic fermentation from carbohydrate-rich substrates [10] or from CO/CO_2 containing gas [11] and is found at high concentration in side streams from beer, wine and distillate production [9,12,13].

Caproic acid has a relatively high market value of 2.5 – 3.5 €/kg [14,15], and its extraction and concentration are technically simpler than for shorter chain acids. In fact, its low solubility in water (10.8 g/L under standard conditions) [16] facilitates downstream processing through physical/chemical techniques such as liquid–liquid extraction [17]. Caproic acid finds industrial application as a green pesticide and as an additive for food, paints, fragrances and pharmaceuticals [18]. Furthermore, it is a precursor for the synthesis of green fuels [19] and bioplastics [20], two products with steadily increasing markets in the € billion scale.

Caproic acid production rates of 50 g/(L·d), with 80 % selectivity, have been reported from a synthetic media containing acetic acid and ethanol in a continuously operated up-flow anaerobic filter bioreactor under optimal conditions (pH 7, yeast extract addition, and nitrogen sparging) [21]. However, the production rate and selectivity obtained from real substrates are significantly lower [22]. This can be attributed to (i) the presence of undesired microorganisms that diverge the carbon flow away from caproic acid production, and (ii) sub-optimal concentrations and electron donor:substrate ratios in the feedstock. Ethanol: acetic acid molar ratios of at least 3 are required for optimal chain elongation [23], while ratios in real waste are often lower. In such case, additional ethanol must be outsourced, negatively affecting operation costs and environmental sustainability. On the contrary, some potential substrates such as wine lees contain ethanol in excess (over 80 g/L), requiring dilution to prevent inhibition of bacteria [17]. This inevitably results in large fermentation volumes, with negative consequences in both capital and operational costs.

A bioreactor concept that avoids direct contact between contaminated, waste-derived substrates and chain elongating bacteria, while ensuring the availability of chain elongation precursors, would prevent some of the issues mentioned above. This can be achieved by using submerged membranes. To date, fermentors with integrated membrane modules have been mainly proposed to extract caproic acid from the fermentation broth [24]. In a previous study [25], a polyacrylonitrile hollow fibre membrane module was used to separate the butyric acid producing *C. tyrobutyricum* from the caproic acid producing *M. hexanoica*. With this method, the butyric acid rich medium produced by *C. tyrobutyricum* was transferred by filtration to *M. hexanoica* through the porous membrane, in a separate bioreactor, avoiding direct interactions between the two bacteria. However, besides requiring energy for filtration, this approach was only validated using pure substrates and is hardly applicable to waste-derived substrates due to the lack of selectivity and fouling propensity of the membrane.

In previous studies [26,27], inexpensive, commercial polysiloxane (silicone) tubing was used to extract undissociated carboxylic acids and alcohols from fermentation broths. This occurs via liquid–liquid extraction (pertraction) where the compounds passively diffuse through the polysiloxane matrix driven by concentration gradient. More interestingly, it was observed that ethanol diffuses through the silicone membrane faster than acetic acid [28], and that microorganisms and soluble compounds such as ions, proteins and sugars are retained. Thus, differently from porous membranes, using non-porous, hydrophobic polysiloxane membranes for transferring waste-derived substrates not only allows to avoid contamination, but also to adjust the ethanol:acetic acid ratio by fine-tuning the diffusion rates. This would potentially increase caproic acid productivity and product purity.

This study proposed, for the first time, the use of a silicone membrane-based fermentor for selective caproic acid production from ethanol-containing substrates. Initially, abiotic tests were conducted to assess the diffusion rates of ethanol and acetic acid through the silicone membrane under different operational conditions (recirculation rates and substrate concentrations) and different types and thicknesses of silicone. Subsequently, biotic experiments were conducted to demonstrate the potential of membrane-based fermentors for caproic acid production from non-ideal feed solutions (i.e., low ethanol:acetic acid ratio and/or concentrations above toxicity levels). Finally, the technology was validated using a real substrate (wine lees) to further highlight its potential for recycling carbon from highly concentrated streams into caproic acid.

2. Materials and methods

2.1. Reactor set-up

The experiments were conducted in glass fermentors of 2 L total volume and 1.4 L working volume (Fig. 1). The fermentors were equipped with a water jacket for temperature control at 35 ± 1 °C through a recirculating bath (Digit-Cool, Selecta, Spain), and were placed on the top of a magnetic stirrer (Stuart SB161, Cole-Parmer, UK) for mixing. A membrane module consisting of a silicone tubing coil (Altec, UK) was installed into a purposely made plastic structure obtained by 3D printing (Fig. S1 in the Supporting Information) and placed in the middle of the fermentor. The temperature probe was installed inside the fermentor, near the membrane coil. Two recirculation lines were constructed using Marprene tubing and peristaltic pumps (Watson Marlow, UK). The first one was used to recirculate a feed solution (either a synthetic solution or wine lees) from a 1 L bottle to the silicone tubing coil, to allow ethanol and carboxylic acids to diffuse to the fermentor (Fig. 1). The second one was used to recirculate the fermentor medium for homogenization (internal recirculation). The fermentor was equipped with a pH control system consisting of a pH probe (model 5303, Crison, Spain) connected to a peristaltic pump (101U/R, Watson Marlow, UK) through a relay controller (MultiMeter 44, Crison, Spain). The pump dosed a NaOH (3 M) solution to the fermentor through a T connection in the recirculation line when the pH was below 6.8. The NaOH solution was stored in a 0.1 L bottle connected to a N_2-containing gas bag to avoid under-pressure and consequent oxygen intrusion. Both recirculation lines included ports for liquid samples. Ports for gas samples were also installed on the top of the fermentor and feed bottle.

2.2. Abiotic tests

Abiotic tests were conducted to evaluate the effect of silicone tubing thickness and material (general purpose or high strength material, with different characteristics, as summarized in Table S1 in the Supporting Information), and operation parameters (flow velocity and feed concentration) on the ethanol and acetic acid diffusion rate. For all tests, both the fermentor and feed solutions contained 1.95 g/L 2-(N-morpholino)-ethanesulfonic acid (MES buffer) to avoid sharp pH changes. In addition, the feed solution contained ethanol and acetic acid at the required concentrations. A 10 M NaOH solution was used to adjust the initial pH of the fermentor to 7, optimal for chain elongation. Instead,

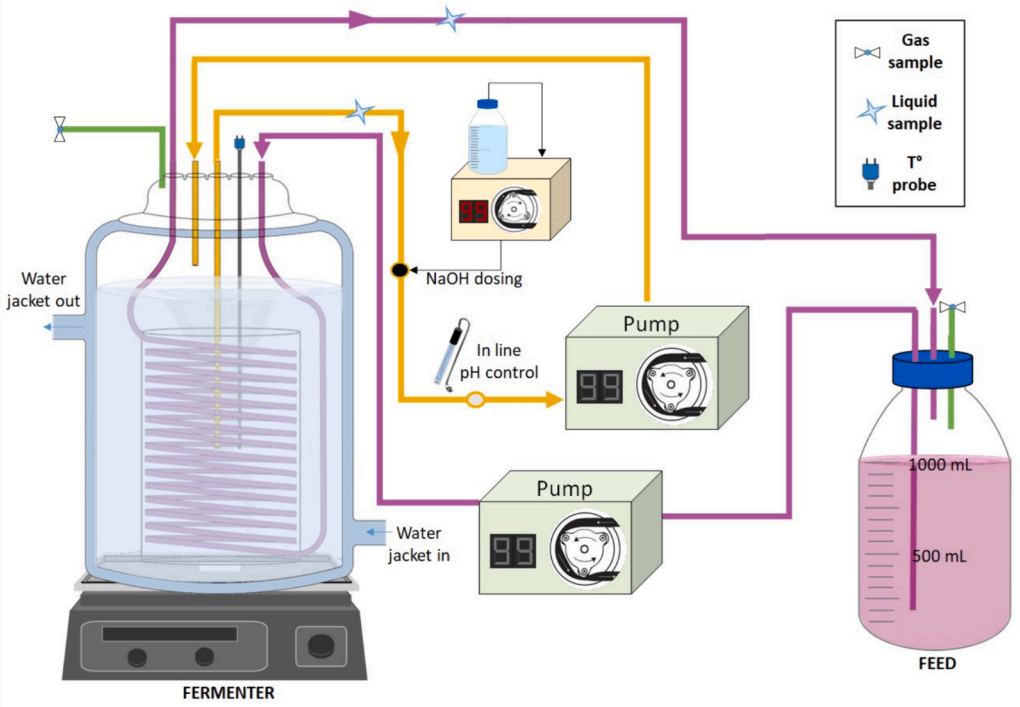

Fig. 1. Schematic overview of the experimental set-up.

Table 1
Summary of the abiotic tests. For each parameter tested, the constants are presented in grey, while variables are presented in white.

Parameter tested	Feed flow velocity (m/h)	Ethanol and acetic acid concentrations (M)	Tubing wall[a] (mm)	Silicone type[b]
Feed flow velocity	200	0.1	1.0	General purpose
	400			
	2000			
	4000			
Feed concentration	400	0.1		
		0.5		
		1.0		
Tubing thickness		0.1	0.25	
			0.50	
			1.00	
Silicone material			0.5	General purpose
				High-strength

[a] Tubing with 0.50 or 1.00 wall thickness had an internal diameter of 2 mm, whereas tubing with 0.25 mm wall thickness had an internal diameter of 0.5 mm; [b] Two different silicone materials were tested. Their properties are summarized in Table S1 in the Supporting Information.

the pH of the feed solution was adjusted to 4.5 to simulate a typical fermentation effluent. The internal recirculation rate of the fermentor was set at 170 mL/min, and a pH >6.8 was maintained by automatic control. The other operation parameters were specific of the test conducted and summarized in Table 1.

2.3. Biotic tests with synthetic medium

The biotic tests (Table 2) were performed in fed-batch mode using the same reactor configuration described for the abiotic test, except for a control experiment (Test 2) where the feed recirculation line and the silicone membrane were omitted. The fermentor medium had the following composition (g/L): KH_2PO_4 (0.1), NaCl (0.8), NH_4Cl (1.0), $MgCl{\cdot}6H_2O$ (0.2), KCl (0.1), $CaCl_2{\cdot}2H_2O$ (0.02), and MES buffer (1.95). A Wolfe's trace metal solution and a vitamin solution (1 mL/L each) were also added. The synthetic feed solution had the same composition of the fermentor medium, with the addition of ethanol and acetic acid (1 M each, Test 1) or only ethanol (1 M, Test 3).

The inoculum was collected from a bioelectrochemical system producing butyric acid from CO_2 [29]. It was placed in a serum bottle and

Table 2
Summary of the biotic tests conducted either with synthetic feed (Tests 1–3) or with wine lees (Tests 4–6).

Test	Feedstock specifications	Initial ethanol concentration (M)	Initial acetic acid concentration (M)	Number of replicates
1	Synthetic – Equimolar	1.0	1.0	3
2	Synthetic – Equimolar – Control[a]	1.0	1.0	1
3	Synthetic – Only ethanol	1.0	0	1
4	Wine less (WL)	2.3	<0.1	2
5	Diluted WL	1.0	<0.1	1
6	Diluted WL with acetic acid addition	1.0	1.0	2

[a] No silicone membrane. Ethanol and acetic acid were directly added to the fermentor broth.

routinely maintained by sparging H_2:CO_2 (80 %:20 %) twice per week. The inoculum (70 mL) was centrifuged at 6000 rpm for 10 min, the supernatant was discarded, and the pellet was resuspended in the same volume of fresh medium sparged with N_2 to minimize the exposure to O_2. The inoculum was then added to the fermentor containing 1330 mL of medium pre-sparged with N_2, resulting in a 5 % ratio. Based on the results obtained in the abiotic tests, the biotic tests were conducted using a general purpose silicone membrane with an internal diameter of 2 mm and a wall thickness of 0.5 mm. The superficial velocity inside the silicone tubing was set to 400 m/h (feed recirculation rate of 21 mL/min), the temperature was 35 ℃, the internal recirculation was set at 170 mL/min and the pH was automatically maintained above 6.8.

2.4. Biotic tests with wine lees

Wine lees (WL), consisting of the settled fraction after alcoholic fermentation, were collected from a local wine factory (La Vinyeta, Masarac, Catalunya, Spain) and thoroughly characterized (Table 3). Experiments were performed similarly to those with synthetic solutions (Table 2), which was replaced by WL, either undiluted (2.3 M ethanol, Test 4) or diluted (1 M, Test 5). In one test (Test 6), the diluted WL was amended with acetic acid (1 M) to allow comparison with the tests performed with synthetic feed. No pretreatment was applied, since the low concentration of solids allowed to recirculate the WL inside the silicone tubing without any issue.

2.5. Product separation

Caproic acid-containing fermentate obtained either from synthetic feed (Test 1) or WL (Test 6) was acidified to pH 3 with 5 M HCl and settled overnight. The resulting oily supernatant was recovered and centrifuged (Hettich EBA 21, Germany) for 5 min at 14,000 rpm to obtain a caproic acid rich oil. The remaining solution was neutralized with 5 M NaOH to assess the chemical dosing necessary for the whole hypothetic downstream processing.

2.6. Sampling and chemical analysis

Liquid (2 mL) and gas (5 mL) samples were collected from the respective sampling ports by using a plastic syringe and a gas tight glass syringe (Hamilton, US), respectively. The optical density (OD, 600 nm) of liquid samples was measured using a spectrophotometer (Hach DR3900, Germany). The OD values were converted to cell dry weight (CDW) using the procedure described in the Supporting Information. A Basic 30+ EC-meter and a Basic 20+ pH meter (Crison Instruments, Spain), were used to assess electric conductivity and pH, respectively. Gas pressure in both the fermentor and the feed solution bottle was assessed by digital pressure sensor (Testo 512, Spain). The composition of liquid samples (carboxylic acids and alcohols) and gas samples (CO_2, CH_4, CO, H_2, O_2 and N_2) was analysed by gas chromatography using, respectively, a GC 7890 A (Agilent Technologies, USA) equipped with a DB-FFAP column and a flame ionization detector (FID), and a Micro GC 490 (Agilent Technologies, USA) equipped with both a CP-Molesieve 5A and a CP-Poraplot U column. Details on the analytical methods have been reported by Romans-Casas et al. [30]. Total solids (TS) and volatile solids (VS), as well as total and soluble COD, were measured according to the standard APHA Standard Methods for the Examination of Water and Wastewater [31]. Total organic carbon (TOC) was analysed by a TOC-V CSH analyser (Shimadzu, Japan). Ions were analysed by ion chromatography (Dionex ICS-5000, ThermoFisher, USA) as described in the Supporting Information.

Table 3
Wine lees characterization.

Parameter	Unit	Values
pH	–	3.56
Conductivity	mS/cm	2.09
Total Solids (TS)	g/L	28.08 ± 2.72
Volatile Solids (VS)		23.98 ± 2.11
COD		284.0 ± 5.2
COD_{sol}		274.5 ± 3.3
TOC_{sol}	g/L	98.16 ± 1.03 (8.18 ± 0.09)
Acetic acid	(M)[a]	1.99 ± 0.10 (0.03 ± 0.00)
Ethanol		105.70 ± 8.14 (2.29 ± 0.17)
Anions (Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-})	mg/L	24.78 ± 0.53, 0.23 ± 0.01, 0.09 ± 0.00, 3.29 ± 0.04, 193.12 ± 0.92
Cations (Ca^{2+}, K^+, Na^+, NH_4^+)	mg/L	66.69 ± 0.83, 946.05 ± 13.73, 20.97 ± 0.42, 20.48 ± 0.23

[a] Values in mol/L in brackets.

2.7. Scanning electron microscopy

A piece of the silicone tubing was cut and prepared for scanning electron microscope (SEM) analysis at the end of a replicate tests with synthetic medium (Test 1). It was fixed for four hours in a 0.1 M cacodylate buffer solution with 2.5 % (w/v) glutaraldehyde (pH 7.4), dried with graded ethanol and critical point drier (model K-850, Emitech, Germany) and sputter-coated with a carbon evaporator (Turbo Evaporator K950, Emitech). Micrographs of the coated sample were obtained by SEM (FESEM Hitachi S4100) at 10 μA and acceleration voltages ranging from 1 to 20 kV. Images were acquired with Quartz PCI. Sample preparation and analysis were performed by the *Serveis Tècnics de Recerca* (STR) at the University of Girona.

2.8. Microbial community analysis

Samples for microbial community analysis were collected both at the beginning and at the end of each biotic experiment with synthetic media. Suspended cells were harvested at the start and end of the experiment, attached cells (biofilm) were collected only at the end of the experiment. Biofilms were detached from three sections of the silicone tube by swabbing two times the whole surface in a longitudinal section of 30 mm using a sterile cotton swab. The swabs were then submerged in sterile distilled water and vortexed for 10 s at maximum speed. Detached cells, as well as inoculum and bulk suspended cells, were collected by centrifugation (4,400 rpm, 15 min, 4 °C). Pelleted cells were stored at −20 °C until DNA was extracted using the FastDNA SPIN kit for soils (MP Biomedicals, USA) following the manufacturer's instructions. The extracts were distributed in aliquots and stored at −20 °C. DNA concentration was measured using a Nanodrop 1,000 spectrophotometer (Thermo Fisher Scientific, USA).

The composition of microbiomes present in the bioreactor was determined by barcoded amplicon-based Illumina sequencing of the 16S rRNA gene V4 region using the 515–806 primer pair [32]. Illumina MiSeq flow cell (V2) sequencing was conducted by the RTSF Core facilities at the Michigan State University USA (https://rtsf.atsci.msu.edu/). Raw sequencing data were quality filtered, trimmed, dereplicated, merged and, after a process of chimera removal, were clustered into amplicon sequence variants (ASVs) using the DADA2 Pipeline [33]. Taxonomic assignations were done using the Silva 138.1 database as a reference (www.arb-silva.de) at a minimum bootstrap level of 80 %. The relative abundance of ASVs per sample was calculated using the phyloseq package in R [34]. When needed, taxonomic assignations were refined by BLASTn searches using the refseq_rna database as a reference and excluding environmental isolates (blast.ncbi.nlm.nih.gov). Additional bioinformatic methodology is included in the Supporting Information. All sequences obtained in this study have been submitted to the GenBank database under the SRA accession number PRJNA1061376.

2.9. Calculations

Diffusion fluxes were calculated as the moles of either ethanol or

acetic acid transported per membrane surface unit in the time unit. Reynolds numbers were calculated using the following equation:

$$Re = \frac{uD}{\upsilon} \quad (1)$$

Where u is the superficial velocity of the fluid (m/h), D is the internal diameter of the tubing (m), and υ is the kinematic viscosity of water at 35 °C (2.61×10^{-3} m^2/h).

Production rates were calculated as the moles of product synthesized per fermentor volume unit in the time unit. Carbon conversion efficiencies were calculated as the ratio between the carbon detected in the target product and the carbon supplied as ethanol and/or acetic acid. Product selectivity was calculated as the ratio between the carbon detected in the target product and in the sum of all fermentation products.

For the extraction tests, the recoverable product was calculated as the total caproic acid that naturally separates from water forming an oily layer due to oversaturation (exceeding the solubility value of 10.8 g/L). The recovered product was defined as the kg of caproic acid recovered as oil per kg of feed solution. Product purity was determined as the caproic acid concentration in the final product divided by that of the pure product (930 g/L).

3. Results and discussion

3.1. Ethanol and acetic acid diffusion through silicone membrane

Abiotic tests confirmed that both ethanol and acetic acid can diffuse through the silicone tubing by concentration driven pertraction, and that ethanol diffuses at a higher rate than acetic acid, as previously reported [28]. At an initial concentration of 0.1 M, ethanol and acetic acid diffused with a flux of 1.94 and 0.25 mmol/(m^2·h), respectively, resulting in an ethanol:acetic acid ratio of 7.9 (Fig. 2A). Increasing the feedstock concentration to 1 M caused a 14-fold and 11-fold increase of the ethanol and acetic acid flux, respectively, resulting in an ethanol: acetic acid ratio of 10.0. Such a difference was attributed to the higher vapor pressure of ethanol in comparison to acetic acid (13.3 *vs* 3.6 kPa at 35 °C). Silicone is a hydrophobic material that discourages the permeation of soluble compounds. However, the flexibility of the silicon-oxygen chains generates void spaces that favour the diffusion of volatile compounds through the matrix in the presence of a concentration gradient [35]. Thus, alcohols and undissociated carboxylic acids will diffuse through the silicone matrix proportionally to their hydrophobicity and volatility, while the diffusion of hydrophilic, dissociated carboxylic acid is negligible.

Interestingly, increasing the superficial velocity of the feed solution inside the silicone tubing from 200 to 4000 m/h had a positive effect on the ethanol diffusion, but a negative effect on the acetic acid diffusion, resulting in an increase of the ethanol:acetic acid ratio from 7.6 to 26.5 (Fig. 2B). This can be attributed to the increased inertial forces (Reynolds value increased from 154 to 3077) inside the tubing, which favoured ethanol extraction, as previously reported for medium chain carboxylic acids extraction through hydrophobic membranes [17]. Oppositely, the shear forces caused by the high superficial velocity might discourage the initial sorption of the hydrophilic acetic acid molecules into the hydrophobic silicone material, resulting in a lower flux. Decreasing the membrane wall thickness from 1.00 mm to 0.25 mm resulted in an increase in both the ethanol and acetic acid flux, but also caused a decrease of the selectivity, with an ethanol:acetic acid ratio decreasing from 14.8 to 6.1 (Fig. 2C). These results suggested that, besides controlling acid dissociation by adjusting pH, both superficial velocity and tube wall thickness can be tweaked to achieve the desired ethanol:acetic acid molar ratio for chain elongation. Based on the results, a tubing thickness of 0.5 mm and a flow velocity of 400 m/h were selected to obtain optimal ethanol:acetic acid ratios for chain elongation in the biotic experiment.

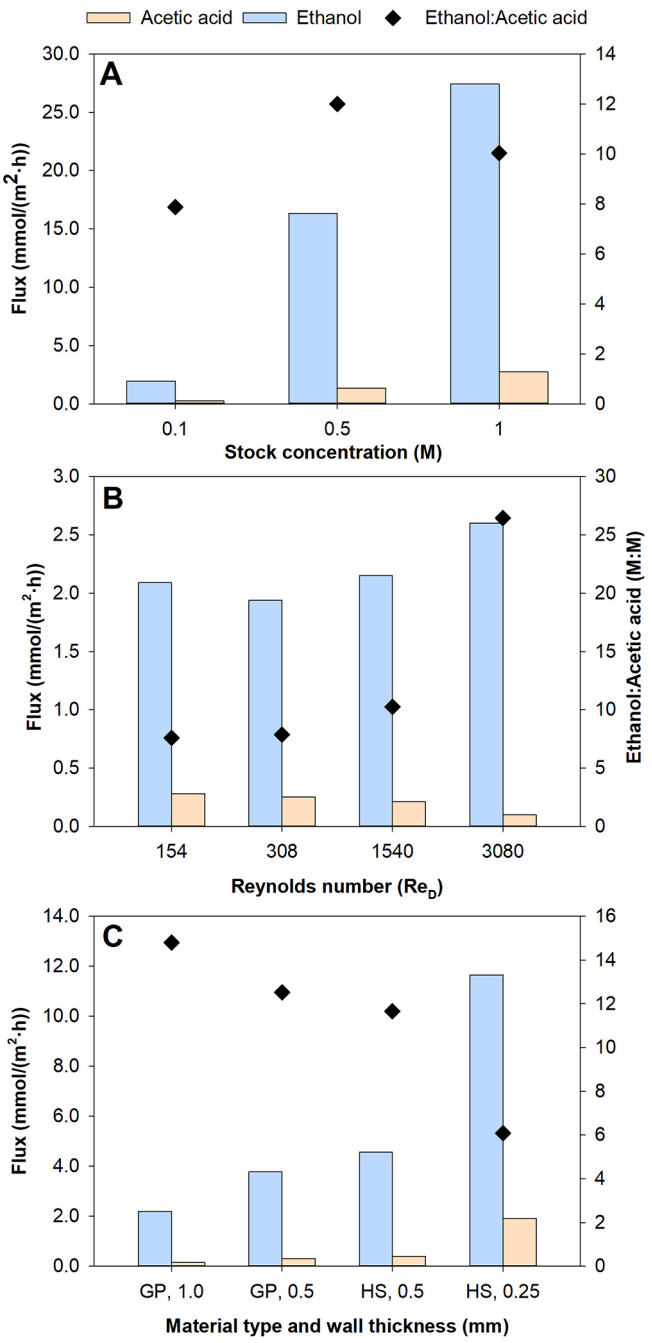

Fig. 2. Effects of feed concentration (A), superficial velocity (B) and silicone type and wall thickness (C) on the ethanol and acetic acid flux through the silicone membrane. Tests A and B were performed with used membrane, while Test C was conducted with new membrane, which slightly affected the acetic acid (but not the ethanol) flux.

3.2. Biotic experiments with synthetic medium

3.2.1. Caproic acid production from equimolar ethanol and acetic acid

In the first days, ethanol and acetic acid progressively diffused from the feedstock bottle to the fermentor broth through the silicone membrane at a controlled molar ratio of 6:1 (Fig. 3A). The biomass concentration in the fermentors slowly increased in the first 4 days (Figure S2 in the Supporting Information), although no products were detected. On day 4, when ethanol concentration reached 174 mM (8 g/L), caproic

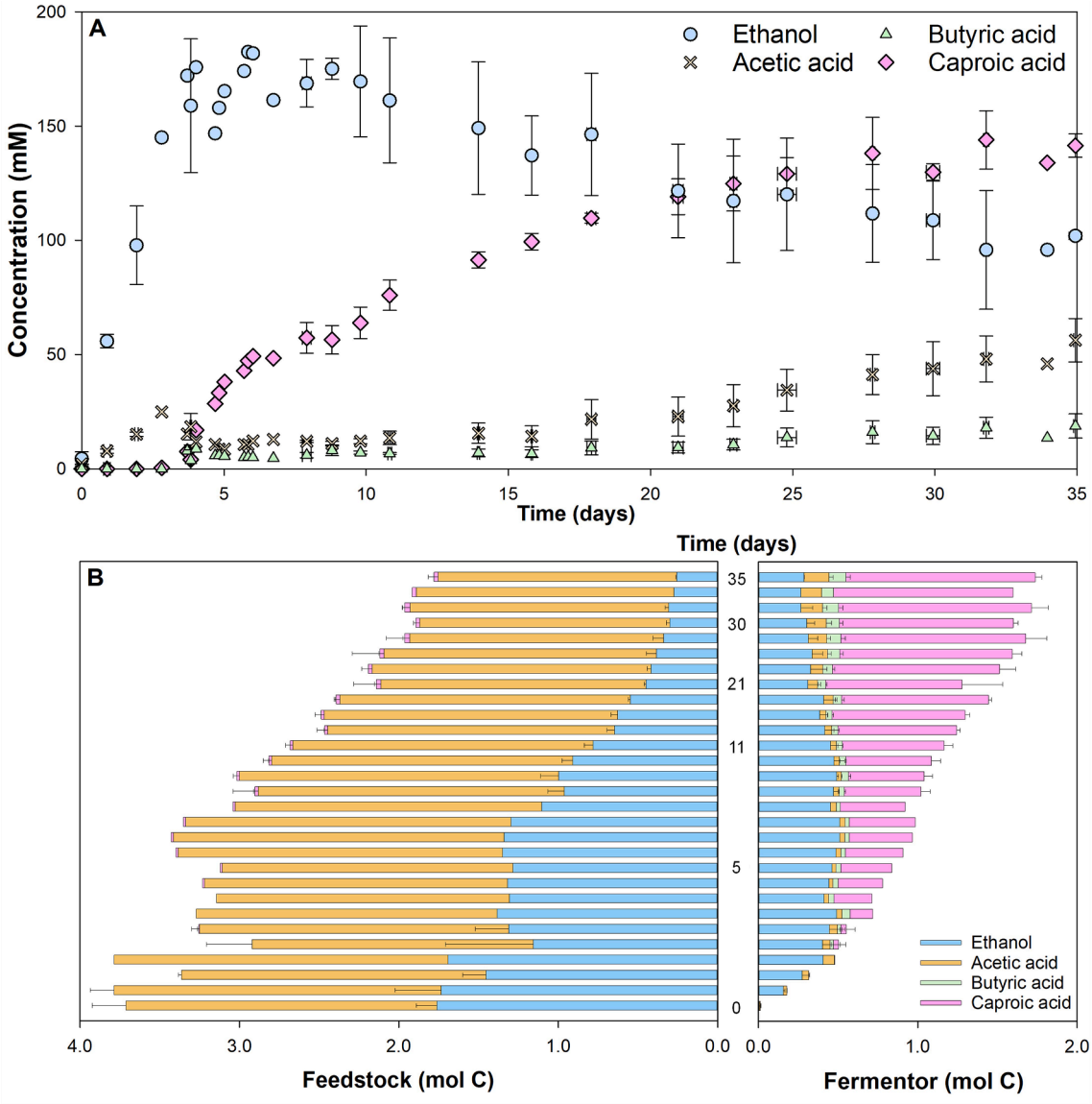

Fig. 3. Ethanol and carboxylic acid concentration profiles in the fermentor during operation (A); carbon balance feedstock-fermentor over time (B). Bars and whiskers show mean values and standard deviations (n = 3).

acid production was detected, ethanol concentration plateaued, and acetic acid concentration decreased confirming the chain elongation onset [36]. Ethanol concentrations between approximately 100 and 200 mM (4.6 and 9.2 g/L) are indeed known to induce chain elongation in mixed culture fermentation [37]. The onset of chain elongation pathways (days 5–9) coincided with a sharp increase of biomass, which reached 2.95 ± 0.54 g_{CDW}/L (Figure S2 in the Supporting Information). Caproic acid production remained negligible in the control fermentor without the membrane module (Figure S3 in the Supporting Information). This confirmed that the membrane-based fermentor, where ethanol and acetic acid are dosed by diffusion through the silicone membrane, enables caproic acid production from concentrated (1 M) feed streams that would otherwise inhibit the chain elongating microorganisms.

Caproic acid was initially produced at a rate of 26.7 mM/d, equal to 3.1 g/(L·d) and accumulated in the fermentors (Fig. 3A). However, its production rate declined over time due to product inhibition. Once caproic acid concentration exceeded 40 mM, its average production rate dropped to 5.2 mM/d on days 6–21, and then its concentration plateaued at 148.1 ± 8.6 mM (17.2 ± 1.0 g/L) by day 35. In total, 84 ± 3 % of the ethanol initially present in the feed bottle diffused towards the fermentor, confirming that the ethanol-consuming chain elongation reaction can be used to maintain a concentration gradient between feedstock and fermentor. However, acetic acid diffusion was limited to 24 ± 7 % due to its lower diffusion rate. Caproic acid concentration in the feed solution remained negligible, confirming that dissociated acids (>99 % at pH 7) do not counter-diffuse through the silicone membrane, allowing the product to accumulate in the fermentor. Overall, around 52 % of the carbon initially introduced in the system as ethanol or acetic acid (2.0 mol C) were transferred from the feed bottle to the fermentor within the 35 days operation, 62 % of which was converted into caproic acid (Fig. 3B), resulting in a conversion efficiency of 32 %. The remaining carbon was detected as residual ethanol (13 %), acetic acid (7 %) and butyric acid (6 %). Since the carbon detected in gas phase (CO_2 and CH_4) was negligible (in the order of μmol C), the remaining carbon (12 %) was likely consumed for microbial growth.

The production profile and final caproic acid concentration were in line with previous batch experiments with *C. kluyveri* [38]. However, the production selectivity (on a carbon basis) was higher than in previous studies [39,40]. In the period of maximum production rate (day 4–6),

caproic acid was essentially the only product synthesised, while butyric and acetic acid concentrations remained constant in the range of 10 mM. This can be attributed to the dosing effect of the silicone membrane, which constantly provided an optimal ethanol:acetic acid ratio to the microbial community, avoiding acetate accumulation in the fermentor. The thickness and the total surface of the membrane, along with operation parameters such as pH and flow velocity, can be tailored to achieve optimal diffusion ratios from any ethanol and carboxylic acid containing feedstock, regardless of their absolute and relative concentrations.

On average, 3.0 ± 0.3 mol ethanol and 1.1 ± 0.3 mol acetic acid were consumed to produce 1 mol caproic acid, and 1.7 ± 0.2 mol NaOH per mol caproic acid were consumed for pH control. The ethanol and NaOH consumption values were relatively close (within a 15–20 % range) to the stoichiometric values required for chain elongation through the RBO pathway (Eq. (2)), while acetic acid consumption was 40 % higher than required. This suggested that, while a share of the acetic acid was inevitably used for microbial growth [41], competing reactions, such as excessive ethanol oxidation and methanogenesis, were limited.

$$3\,CH_3COO^- + 12\,CH_3CH_2OH \rightarrow 5\,CH_3(CH_2)_4COO^- + 8\,H_2O + 2\,H^+ + 4\,H_2 \quad (2)$$

NaOH dosing resulted in an increase of the Na^+ concentration from the initial 0.6 ± 0.0 g/L to the final 6.2 ± 0.1 g/L (Figure S4 in the Supporting information). The Na^+ accumulation should be monitored, particularly when NaOH is used for pH control, since concentrations above 8.6 g/L have been reported to inhibit chain elongation [42]. The concentration of other macronutrients, including NH_4^+, K^+ Mg^{2+}, Ca^{2+} and PO_3^{2-}, decreased over time but none of them was depleted (Figure S4 in the Supporting Information), confirming that the production plateau was caused by caproic acid accumulation. This agrees with previous studies on chain elongation by mixed cultures at neutral pH, where the final caproic acid concentration rarely exceeded 150–180 mM [22].

3.2.2. Caproic acid production in absence of exogeneous acetic acid

In the RBO pathway, in absence of organic substrates, ethanol is oxidized to acetic acid to provide ATP and NADH for chain elongation. Stoichiometrically, one ethanol molecule every six is oxidized, while the remaining five molecules are used for chain elongation [43]. The RBO pathway can proceed even in absence of exogeneous acetic acid, since it is produced via ethanol oxidation. To confirm this, also considering that some wastewaters (e.g., from wineries or distilleries) mostly contain ethanol, a test without acetic acid in the feed was performed. Caproic acid production occurred from day 6 onwards, and the lack of exogeneous acetic acid had no negative effect on biomass growth (Figure S2 and S5 in the Supporting Information). This suggests that ethanol oxidation to acetic acid, yielding ATP via substrate-level phosphorylation, occurred similarly in presence or absence of exogeneous acetic acid, resulting in a similar microbial growth. However, the acetic acid produced by ethanol oxidation was likely not sufficient to sustain high rate caproic acid production, resulting in three times lower production rates (8.4 mM/d) in comparison to the tests with exogeneous acetic acid. This confirms the results obtained by Allaart et al. [44] who reported that, in absence of short chain carboxylates, two molecules of ethanol (instead of six) were used for the RBO pathway for each molecule oxidized to acetic acid. Interestingly, the same authors reported a shift towards butyric acid and acetic acid production after reaching caproic acid concentrations above 10–15 mM, which was attributed to a reversal of the Rnf complex. This was not the case in this study, where caproic acid was essentially the only product synthesised (Figure S5 in the Supporting Information) until reaching a final concentration of 55 mM.

3.2.3. Microbial community analysis

Chain elongating bacteria including *Clostridiaceae* and *Ruminococcaceae* were found at < 2 % relative abundance in the original inoculum (Fig. 4A). Despite this, caproic acid production was observed after a relatively short lag phase of four days (Fig. 3A), suggesting the ability of chain elongating microorganisms to thrive when the environmental conditions become favourable. By the end of the experiments, in fact, chain elongating microorganisms dominated the microbial community (Fig. 4A). Scanning electron microscope analysis confirmed the formation of a biofilm on the silicone membrane (Figure S6 in the Supporting Information). This was composed by rod-shaped, cocci and filamentous microorganisms, as previously reported for chain elongating granules [45]. Principal coordinate analysis (PCoA) revealed that the silicone attached microbial community clustered separately from the bulk community (Fig. 4B), suggesting significant differences among the respective microbial community structures.

The attached community was more diverse than the bulk community, including 200 exclusive taxa (i.e., present only on silicone biofilm samples) (Figure S7 in the Supporting Information). Most represented taxa were *Clostridiaceae* (40 ± 8 %) and *Oscillospiraceae* (28 ± 8 %). *Clostridiaceae* were mostly classified as *Clostridium sensu stricto 12* (Figure S8 in the Supporting Information) with > 99 % similitude with *C. kluyveri*. *C. kluyveri* strains have been intensively studied as chain elongating microorganisms [36,38]. *Oscillospiraceae* could not be classified at species level but the most abundant ASVs were tentatively (similarity over 97 %) identified as *Oscillibacter* sp., a bacterium previously detected in chain elongation reactors using acetic acid and ethanol as substrates [46,47]. Instead, suspended cells were largely dominated (75 ± 17 %) by *Clostridium sensu stricto 12* (i.e. *Clostridium kluyveri*). Interestingly, the relative abundance of *Oscillospiraceae* in the bulk was < 2 %, suggesting the propensity of these microorganisms to attach to the silicone surface.

3.3. Biotic experiments with wine lees

Replacing the synthetic feedstock with wine lees, containing 2.3 M of ethanol (Test 4 in Table 2), resulted in a high ethanol diffusion rate of 125.5 ± 3.5 mM/d through the silicone membrane with 0.5 mm wall thickness. This resulted in a fast accumulation of ethanol in the fermentor, whose concentration exceeded 430 mM causing inhibition of the microbial community, and in a caproic acid concentration of only 11.7 ± 3.0 mM after 18 days of operation (Fig. 5A). Although this issue can be easily solved by using a thicker membrane or reducing the membrane surface in contact with the liquid, in this study, the wine lees was diluted to 1 M ethanol (Test 5) to allow a better comparison with the results obtained with the synthetic medium. Diluting the wine lees resulted in a lower ethanol diffusion rate of 46.5 mM/d, and caproic acid was produced at a highest production rate of 8.4 mM/d up to a concentration of 63.5 mM (Fig. 5B). Such production rate was similar to that obtained with the synthetic medium containing 1 M ethanol (Test 3, Figure S5 in the Supporting Information). An average caproic acid production selectivity of 94 % was achieved from diluted wine lees, with peaks of > 99 % during the period of high production rate. Such selectivity is substantially higher than those achieved in comparable studies using real substrates as ethanol source (Table 4).

Supplementing the diluted wine lees with 1 M acetic acid (Test 6) boosted the reaction kinetics [48,52], resulting in threefold caproic acid production rates and over twofold final concentrations (Fig. 5C), comparable to those achieved with the synthetic medium (Fig. 3A). Since high production rates are essential for process scale-up, acetic acid addition is required for substrates poor in carboxylic acids, although this may slightly worsen the final product purity (Figure S9 in the Supporting Information). Rather than outsourced, acetic acid can be naturally and sustainably produced from wine lees by ethanol oxidation.

3.4. Downstream processing

A simple downstream processing was performed to separate the caproic acid produced from both the synthetic (Test 1) and real (Test 6)

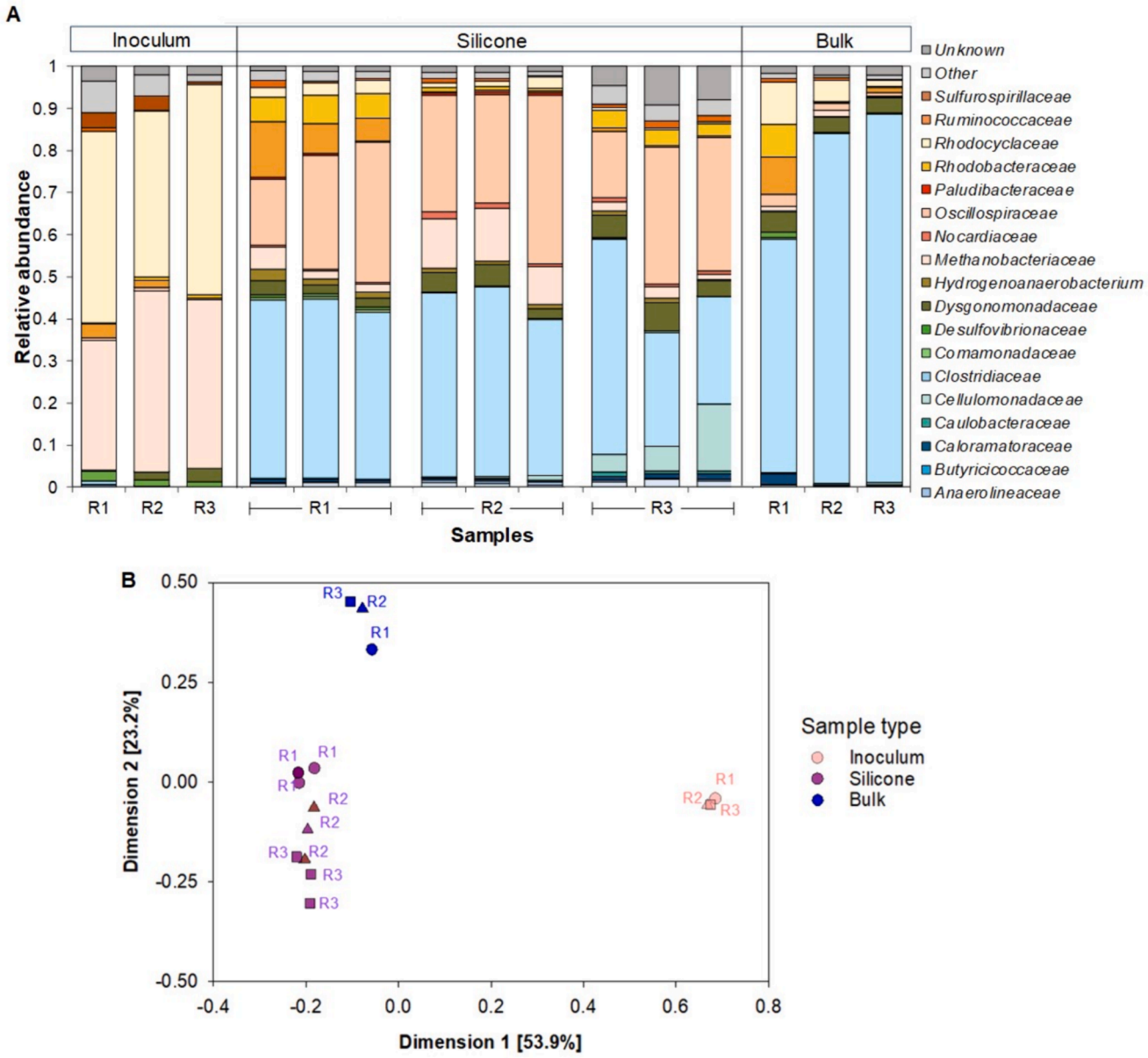

Fig. 4. Family-level community structures of inoculum, silicone biofilm and bulk samples from biotic Test 1 (A). ASVs found with < 1 % relative abundance are grouped as "Others"; Principal Coordinate analyses (PCoA) plot of microbial community structures based on Bray-Curtis' dissimilarity (B). R1, R2 and R3 refer to the three replicate assays conducted. Three silicone biofilm samples were analysed for each replicate assay.

wine lees fermentate (containing 0.185 and 0.151 M caproic acid, respectively). Acidification to pH 3 (to obtain undissociated caproic acid) followed by phase separation resulted in the formation of a white, oily emulsion (Figure S10 in the Supporting Information). Follow-up centrifugation allowed to recover 6.3 mL/L_{feed} (from the real wine lees fermentate) and 13.0 mL/L_{feed} (from the synthetic fermentate) of 6.41–6.75 M (80–84 %) pure caproic acid, accounting for 64.4 and 52.5 % of the recoverable product (Table 5). Such a difference was due to the different initial caproic acid concentration in the two fermentates, suggesting that achieving high caproic acid concentrations already in the fermentation stage facilitates product purification. The main contaminants detected were butyric acid (0.09–0.13 M) and hexanol (0.01–0.02 M), while no ions were present, as suggested by the negligible conductivity of 0.1 – 0.2 μS/cm. Such a result is similar to that obtained by Martinez et al. [12], who recovered around 13 mL/L of 87 % pure caproic acid starting from a fermentate containing 189 mM caproic acid. A further purification step, e.g. by combining adsorption/desorption processes with membrane-based separation, may allow to obtain purities above 95 % [53].

Dosage of chemicals, both during chain elongation (NaOH dosing for pH control) and downstream processing (acidification with HCl to pH 3 for oil separation followed by neutralization of the subnatant with NaOH) clearly limits the economic and environmental feasibility of the proposed approach. Substantially higher amounts of chemicals per unit of caproic acid extracted were required for the real than for the synthetic fermentate, due to the lower initial caproic acid concentration and purity (Table 5). This further highlights the importance of selectively producing caproic acid to decrease the costs associated to downstream processing.

3.5. Practical implications

Besides avoiding microbial contamination, the key advantage of silicone membrane-based fermentation is the possibility to control

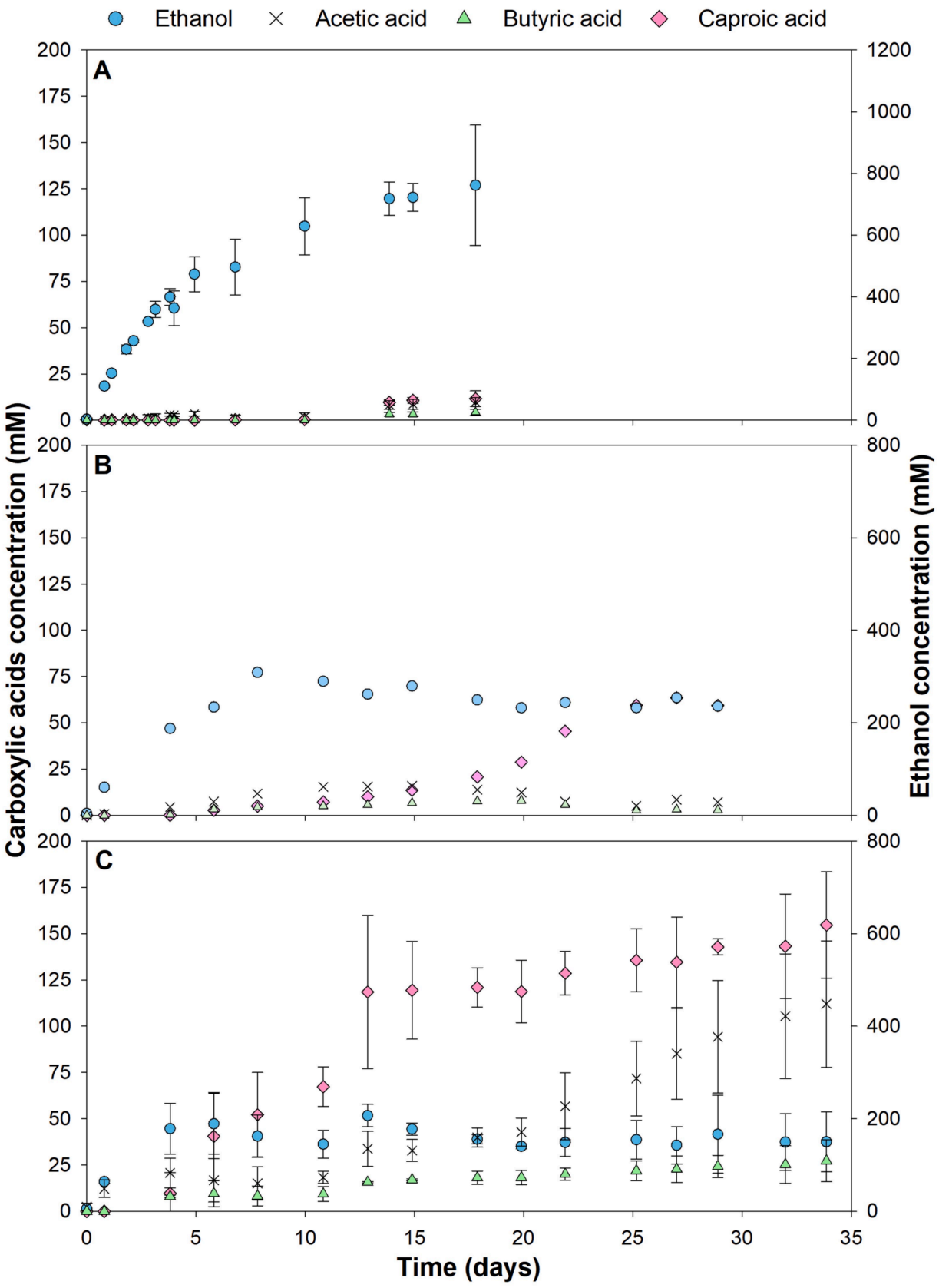

Fig. 5. Carboxylates and ethanol concentration profiles in the fermentor during operation when using raw wine lees (A), diluted wine lees (B), and diluted wine lees amended with 1 M acetic acid (C). Figure A and C refer to average and standard error values of replicate fermentors (n = 2).

Table 4
Summary of batch experiments on chain elongation of real substrates using ethanol as the main electron donor.

Reactor type	Inoculum	Substrate	Electron donor	T (°C)	pH	Caproic acid production rate (mM/d)	Caproic acid concentration (mM)	Selectivity, %[a]	Reference
Flasks	Enriched biomass	Fermented sugarcane molasses + acetic acid	Ethanol (indigenous)	35	7	n.a.	31.0	n.a.	[48]
Serum bottles	Pit mud and activated sludge	Liquor making wastewater	Ethanol and lactic acid (indigenous)	35	6.5	n.a.	105.9	61	[49]
Plastic drum	Enriched biomass	Grape pomace	Ethanol (indigenous)	37	7	53.4	189.4	67	[12]
Upflow with in-line product extraction	Natural microbiomes	Yeast-fermentation beer	Ethanol (indigenous)	30	5.5	18.1	n.a.	79	[50]
Two-stage fermentor	Anaerobic sludge	Fermented fruit and vegetable waste	Ethanol (indigenous and added)	30	7.5	n.a.	128.3	81	[51]
Fermentor with submerged membrane	Enriched biomass	Wine lees + acetic acid	Ethanol (indigenous)	35	7	25.8	155.0	84	This study
		Wine lees				8.4	64.6	94	

[a] Percentage of caproic acid in comparison to all fermentation products (molC/molC) at the point the highest caproic acid concentration was reached. When not available, the information was extrapolated from the published graphs; n.a., information not available.

Table 5
Caproic acid recovery from synthetic and wine lees fermentates after downstream processing.

		Synthetic	Wine lees
Fermentate characterization	pH	6.96	7.30
	Conductivity (mS/cm)	15.3	18.4
	Ethanol (M)	0.078	0.149
	Acetic acid (M)	0.044	0.111
	Butyric acid (M)	0.023	0.026
	Caproic acid (M)	0.185	0.151
Product characterization	Recoverable[a] (mol/L_{feed})	0.129	0.081
	Recovered (mol/L_{feed})	0.084	0.042
	Recovered (%)	64.4	52.5
	pH	3.1	3.1
	Conductivity (μS/cm)	0.11	0.17
	Hexanol (M)	0.02	0.01
	Butyric acid (M)	0.13	0.09
	Caproic acid (M)	6.41	6.75
	TOC_{sol} (M)	42.8	45.1
	Purity (%)[b]	80.1	84.3
Chemical dosing (mol/mol_{HCa})	NaOH, pH control	1.51	1.80
	HCl, acidification	4.08	7.49
	NaOH, neutralization	2.41	7.00

[a] Considering a caproic acid solubility of 0.093 M and fermentor volume of 1.4 L. [b] Commercial product: ~ 8 M concentration, 48 M TOC, 99.5 % purity.

ethanol and carboxylic acid diffusion rates. This potentially allows to achieve optimum substrate:electron donor ratios for chain elongation from any ethanol and (optionally) short chain carboxylic acid containing feedstock by tailoring membrane thickness, flow velocity and pH. Exogeneous ethanol addition can be thereby avoided even in case of feedstocks with low ethanol:carboxylic acid ratios, with positive economic and environmental impacts. This technology can be implemented to *retro*-fit existing fermentors by addition of a membrane module either inside or outside (e.g. in a recirculation line). An external module is a practical solution that allows to access the membrane without emptying the fermentor in case of malfunctioning, but results in a higher plant footprint. The low cost of silicone tubing (as low as 0.10 €/m) allows to counteract the relatively low diffusion rates of the feedstocks by increasing the installed membrane surface. As an alternative, membranes with a thinner wall can be produced and installed to increase fluxes. Being diffusion through the membrane based on a concentration gradient, no energy is required, other than for circulating the feedstock inside the membrane, resulting in contained operation costs.

Despite the promise, several limitations must be considered, and some bottlenecks must be solved, to bring this technology towards commercialization. The first key challenge is to translate the process from batch to continuous. In fact, despite the remarkable selectivity achieved in batch mode, the production rates were significantly lower than those obtained in continuous processes. Wu et al. [13] achieved a caproic acid production rate of 121.4 mM/d from diluted liquor making wastewater in a continuously operated expanded granular sludge bed (EGSB) reactor, although over one year start-up time was necessary to achieve such a high productivity. Long-term experiments in continuous mode are required to determine whether such production rates can be achieved and maintained over time with the membrane-based fermentor. These experiments will also reveal whether, and to what extent, biofilm formation on the silicone membrane affects the process, either positively (help biomass retention) or negatively (compromise substrate diffusion).

The second big challenge is to develop an effective product extraction unit, and install it on-line on the fermentor (e.g., on recirculation line). Kucek et al. [17] proposed a membrane contactor unit to continuously extract undissociated medium chain carboxylic acids from an upflow fermentor fed with wine lees and operated at pH 5.2. Online extraction allowed to mitigate product inhibition and promoted the further elongation of caproic acid to valuable caprylic acid (C_8). However, in this study, the caproic acid was mostly in its dissociated form (pH 7), which makes such extraction method ineffective. Developing a sustainable alternative, e.g., integrating in-line liquid–liquid extraction and membrane electrolysis to control pH avoiding chemical dosing [54], is crucial to achieve economic and environmental sustainability.

4. Conclusions

A novel membrane-based fermentation process provided a cost-effective solution to maintain optimal condition for chain elongation, while preventing contamination, when dealing with complex, highly concentrated, ethanol rich organic waste streams. The diffusion of ethanol and carboxylic acids through the membrane can be tweaked by optimizing the membrane thickness and parameters such as pH and flow velocity. This allowed to obtain optimal electron donor:substrate ratios that resulted in highly selective caproic acid production even when starting from suboptimal organic feeds. Downstream processing, consisting of acidification and physical separation, resulted in an oil containing 84.1 % pure caproic acid that can be potentially used as green pesticide or additive for animal feed. Developing high-rate bioreactor design for continuous operation, and a sustainable alternative to chemical dosing for both pH control and downstream processing, is essential to advance the technology, achieve economic sustainability

and reduce the carbon footprint.

CRediT authorship contribution statement

Paolo Dessì: Writing – original draft, Investigation, Conceptualization. **Meritxell Romans-Casas:** Writing – original draft, Visualization, Investigation, Data curation. **Elisabet Perona-Vico:** Writing – review & editing, Methodology, Formal analysis. **Michele Tedesco:** Writing – review & editing. **Hubertus V.M. Hamelers:** Writing – review & editing. **Lluis Bañeras:** Writing – review & editing, Resources, Formal analysis. **M. Dolors Balaguer:** Writing – review & editing, Supervision. **Sebastià Puig:** Writing – review & editing, Supervision, Funding acquisition.

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

Data will be made available on request.

Acknowledgements

This research was carried out in the project "PANGEA" funded by the Spanish Ministry of Innovation and Science (ref. PID2021-126240OB-I00). PD acknowledges the support of the European Union's Framework Programme for Research and Innovation Horizon 2020 (2014-2020) under the Marie Skłodowska-Curie Grant Agreement No. 101029266, and the project RESTART, funded through the "Dipartimenti di Eccellenza 2023-2027" initiative of the Italian Ministry of University and Research. M.R-C is grateful for the support of Spanish Government (FPU20/01362). E.P-V is supported by INVESTIGO 2022 (ref. INV-1 00050, Catalan Government). S.P. is a Serra Hunter Fellow (UdG-AG-575) and acknowledges the funding from the ICREA Academia award. LEQUIA and EcoAqua have been recognized by the Catalan Government (Ref 2021 SGR01352 and 2021 SGR01142).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.154539.

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