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Treatment of food processing wastes for the production of medium chain fatty acids via chain elongation

Federico Battista *, Alessandro Zeni, Marco Andreolli, Elisa Salvetti, Fabio Rizzioli, Silvia Lampis, David Bolzonella

University of Verona, Department of Biotechnology, Strada Le Grazie 15, 37134 Verona, Italy

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ABSTRACT

The production of medium chain fatty acids (MCFAs) through reverse β-oxidation was investigated both on synthetic and real substrates. From preliminary batch tests emerged that caproic acid was maximized under an acetate/ethanol molar ratio of 5:1 at neutral pH. This ratio was then adopted in different semi-continuous tests operating with different amounts of the two reactants. It emerged that the MCFAs yield reached the maximum level of 6.7% when the total molar substrate amount was around 40–45 mmol/d, while the process was inhibited for values higher than 400 mmol/d. Semi-continuous tests using real waste as substrates, namely food waste condensate, cheese whey, and winery wastewater, confirmed the results obtained with the synthetic substrates. Better performances were obtained when an adequate molar ratio of the acetate and the electron-donor compound was naturally present. Therefore, a MCFAs yield of 25% and 10.5% was obtained for condensate of food waste and acidic cheese whey, respectively. Regarding MCFAs composition, caproic acid was the dominant form but small concentrations of octanoic acid were also found in the tests where ethanol was the electron donor (synthetic substrates and food waste condensate). Octanoic acid was not produced in test where lactic acid represented the electron donor molecules (cheese whey). Condensate and synthetic samples were dominated by *Pseudoclavibacter caeni* with an abundance of 38.19% and 33.38% respectively, while *Thomasclavelia* (24.13%) and *Caproiciproducens* (11.68%) was the most representative genus in acidic cheese whey sample.

1. Introduction

The most recent report by the European Biogas Association (EBA) confirmed Anaerobic Digestion (AD) as leading technology for the transition from a linear based to a circular economy in Europe (European Biogas Association, 2020). The new European policies, i.e. the EU Green Deal and the RePower Plan, aims to assure a further boosting of the biogas and biomethane sector in Europe in order to reduce the European dependency from fossil natural gas imported from other Countries (European Commission, 2022). Beyond biogas production, the AD process, if properly managed, is also suitable for producing high added organic compounds like Volatile Fatty Acids (VFAs) which are usually synthetized from fossil sources. In the last years, biological VFAs have being received an increasing interest becoming a trend topic in the biorefinery field (Battista et al., 2022) as they are considered the "building blocks" to produce multiple bio-based products, such as Medium-Chain Fatty Acids (MCFAs). This latter family of compounds has multiple application in food,

* Corresponding author.
E-mail address: federico.battista@univr.it (F. Battista).

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pharma, chemical and biofuel industry and, for this reason, can allow to increase the potential economic revenue of the biorefinery platforms (Menon and Lyng, 2020; Scarborough et al., 2018).

MCFAs are carboxylic fatty acids with a carbon chain ranging from 6 to 12 atoms. Currently, the 90% of the MCFAs market is produced from fossil-base sources (Agnihotri et al., 2022). As alternative, MCFAs are extracted from vegetable oils (palm oil, coconut oil or castor oil) but this technology presents two main drawbacks: i) it usually adopts no green solvents and ii) the oil recovery is low, representing the 5–15% of the starting substrates. For these reasons oil extraction cannot be classified as a sustainable technology (Venkateswar Reddy et al., 2020).

A promising biological process to synthetize MCFAs seems to be the so called "chain elongation". In this process VFAs coming from the acidogenic fermentation of organic substrates, together with electron donor-compounds, usually ethanol or lactic acid of biological origin, produce longer fatty acids: these two classes of compounds are used as substrates by microorganisms to perform cyclic reactions, known as reverse β-oxidations (Angenent et al., 2016; Carvajal-Arroyo et al., 2019). In this process the electron donor-compound provides an acetyl-CoA molecule, which is transferred to the VFAs carbon chain, elongating its length by two carbons at a time (Venkateswar Reddy et al., 2020).

Caproic acid is the MCFA whose economic revenue is estimated to grow up more in near future: its global market was of US$ 176.7 Million in the year 2020 and it is estimated to reach US$ 283.6 Million by 2027 (Research and Markets, 2022). There are not many works in scientific literature dealing with the conversion of organic wastes into MCFA. Moreover, the operational parameters, which allow to shift the biological pathways from the mere acidogenic fermentation to the chain elongation, differ in the previous works available in the scientific literature (Tang et al., 2022; Xie et al., 2021). The most influencing parameter is represented by the ratio between the electron-donor compounds and the acetic acid. Recently, Sarkar et al. (2021) adopted brewery spent grains in a double step for the sequential production of VFAs and MCFA. Acidogenic fermentation allowed for obtaining a total VFAs and acetic acid concentrations of 19.66 gCOD/L and 5.87 gCOD/L, respectively. The following conversion of the VFAs into caproic acid by reverse β-oxidation pathway was maximized when the mass ratio between ethanol and acetate was around 1.5:1, with a final caproic acid concentration of 9.10 gCOD/L. Tang et al. (Tang et al., 2022), who used synthetic lactate and acetate, found the optimal ratio in term of caproic acid yield was of 3:1 (w/w). Another optimal ratio value emerged from the work by Cavalcante et al. (2020), who worked with fermented sugarcane molasses as a substrate. Here, the optimal ethanol:acetate ratio (w/w) was 10:1 which allowed a final caproic acid concentration of 7.9 gCOD/L. All these works emphasized how large is the range of the optimal ethanol:acetate molar ratio. Another parameter affecting the chain elongation is the pH. Some previous works indicated the optimal pH range is 5.0–5.5, meaning slightly acid conditions (Candry et al., 2020a; Strik et al., 2022), but other ones obtained best performances at close neutral pH (6.0–7.0) (Liu et al., 2022).

The chain elongation process is also influenced by pretreatments, which are fundamental in the presence of very complex substrates. As emphasized by Kim et al. (2022), complex substrates are not suitable for the reverse β-oxidations. Alkaline, acidic, deacetylation and even thermal steps are often required in order to degrade the substrates in the VFAs and electron donor compounds. The effect of pretreatments can vary according to the substrates. For example, Ma et al. (2021a) tested different chemical, thermal and physical pretreatments on food wastes in attempt to increase the caproic acid production by reverse β-oxidations. While a combination of alkaline and thermal pretreatments inhibited the chain elongation, hydrothermal and ultrasound processes led to an increase of caproic acid of about 100%. On the contrary, a thermal alkaline pretreatment applied on bovine and ruminant animal manure improved of about 8.5 folder times the MCFAs production (Fu et al., 2023). Another consideration about the pretreatment is the need to consider their economic sustainability, so it is necessary to evaluate if the MCFAs increasing can cover the costs required for it.

The challenge of this work was the improvement of the knowledge on the operational parameters which lead to maximize the VFAs conversion into caproic acid production via the reverse β-oxidation mediated by Microbial Mixed Culture (MMC). To date, several previous works adopted pure cultures of *Clostridium* (*Cl. kluyveri; Cl. sporosphaeroides), Megasphaera (M. elsdenii), Bacillus, Ruminococcaceae, and Caproiciproducens (Ca. galactilolivorans*) to produce caproic acid through chain elongation (Jeon et al., 2017; Yuan et al., 2022; Zhu et al., 2017).

According to this scenario the present work considers in the first part where synthetic substrates were used to optimize the process conditions while in the second part real organic waste was used. In particular, the experimental campaign consisted of both batch and semi-continuous tests. Batch tests were carried out using synthetic substrates (acetate and ethanol) at different molar ratios and pH to determine the best conditions in terms of MCFAs and caproic acid production yields. Then, semi-continuous tests were performed in the best conditions to confirm observed evidence from the batch tests. In the last part of the study, different real substrates were treated: condensate from the Organic Fraction of Municipal Solid Waste (OFMSW) treatment (drying), acidic cheese whey and winery wastewater were treated in the semi-continuous mode. In particular, semi-continuous tests with synthetic substrates were performed with the best acetate-ethanol molar ratio and pH emerged from batch. Tests on different real substrates were conducted to observe the chain elongation process productivity with effective organic materials from municipal and agri-food wastes, whose composition can differ from the optimal conditions. Moreover, for semi-continuous tests a microbiology analysis was also performed to unravel the presence of caproic-acid-producing microorganisms and, consequently the shifting from acidogenic fermentation to chain elongation processes.

2. Materials and methods

2.1. Batch tests to determine the best operational parameters

The molar ratio between acetate and ethanol is one of the most important parameters influencing the chain elongation. The

stoichiometric acetate:ethanol molar ratio to produce caproic acid is 1:2 but higher ethanol concentrations are needed (Zhang et al., 2021). Batch tests were carried out to find two important operational parameters: i) the optimal acetate:ethanol molar ratio, and ii) the best pH values for the reverse β-oxidation. The tests adopted synthetic substrates (acetic acid and ethanol), which were fed in 0.5 L reactors with a working volume of about 250 mL under anaerobic conditions and at 37 ± 2 °C. The presence of the MMC and the macro and micronutrients were provided by an agricultural digestate (inoculum), having a Total Solids (TS) and Volatile Solids (VS) contents of 5.39% and 3.41% w/w, respectively. The inoculum:substrate ratio was of 2:1 (VS base), following the indication of a previous research work for caproic acid production from MMC (Liu et al., 2017). Three rounds of batch tests were performed at pH of 5.5, 7 and 9, respectively, according to the acetate:ethanol molar ratios reported in Table 1. The pH was set up by the addition of NaOH (2 M) and HCl (1 M) until the reaching of the desired value at the beginning of the test. All the tests were performed in triplicate.

The duration of the tests was of 10 days, which emerged in a previous study as the minimal time for starting the chain elongation reactions (Liu et al., 2017).

The concentrations of the VFAs and MCFAs (caproic, octanoic and decanoic acids) were measured at the end of the batch tests and were used to evaluate the yields of the process both in term of MCFAs and VFAs productions, according to the Equation 1 and Equation 2, respectively:

$$\text{MCFAs yield}(\text{YMCFAs}, \%\ \text{w/w}) = \frac{\text{amount of MCFAs(gCOD)}}{\text{intial COD amount from substrates(g)}} \times 100 \quad (1)$$

$$\text{VFAs Yield}(\text{YVFAs}, \%\ \text{w/w}) = \frac{\text{amount of VFAs(gCOD)}}{\text{intial COD amount from substrates(g)}} \times 100 \quad (2)$$

To evaluate the mass balance of the process, the biogas production was also monitored following the procedure by Holliger et al. (2016).

2.2. Semi-continuous tests

Semi-continuous trials were performed both on synthetic and on real substrates. The pH of all the trials was set up at 7, which emerged as the best condition from the previous batch tests, while the Hydraulic Retention Time (HRT) was set up at 25 d. The duration of each test was at least 2 HRTs. The operational temperature was set up at 37 °C for all the tests which were performed in anaerobic condition. The CSTR reactors, having a working volume of about 0.6 L, were inoculated with the same agricultural digestate, previously described, in order to assure a inoculum:substrates VS ratio of 2:1. The discharge and the feeding of the semi-continuous tests were manually provided. Specifically, the amount of volume corresponding to the dilution factor (1/HRT) was discharged and renovated every day. In that occasion, the pH value was checked and, eventually, adjusted.

The performances of the semi-continuous tests were evaluated considering the concentration of caproic and octanoic acids, according to Eq. 3.

$$\text{MCFAs Yield}(\text{MCFAY}, \%\ \text{w/w}) = \frac{\text{gCOD}_{MCFAs/\text{d}}}{\text{gCOD}_{inlet/\text{d}}} \times 100 \quad (3)$$

2.2.1. Semi-continuous tests on synthetic ethanol and acetate

Three different tests on synthetic substrates were carried out, according to the daily feeding reported in Table 2. The same volume of substrates (feeding) and effluent were daily fed in and discharged from reactors in order to keep the volume constant.

2.2.2. Semi-continuous tests on real substrates

Condensate of food waste (COND), Acid Cheese Whey (ACW), Cheese Whey (CW) and Winery Wastewater (WW) were used as real substrates for caproic acid production. The amount of the different substrates daily fed to the semi-continuous reactor was 25 g. The main physical and chemical characteristics of substrates are summarized in Table 3.

COND was obtained as the by-product of OFMSW dehydration by a rotary drum dryer for 17 h at 105 °C (Salimi et al., 2021) and was supplied from the National Technical University of Athens (Greece) to our laboratory. It contained low concentrations of volatile organic compounds, i.e. ethanol and acetic acid (Table 3). ACW was the main by-product from soft cheese and yogurt production, while with the term CW was referred to the by-product derived from hard cheese, as Parmesan. ACW and CW were tested as they have a high environmental impact, being rich in organic matter, and are very abundant in the European Union and worldwide (Gottardo et al., 2022). Finally, WW was represented by an expired red wine.

Table 1
Set-up of the batch tests both at pH 5.5, 7 and 9.

Acetate: Ethanol Molar ratio	Inoculum (g)	Acetate (g)	Ethanol (g)
1:2	240	1.56	2.4
1:3	240	0.48	0.92
1:5	240	0.84	3.16
1:10	240	0.84	6.24

Table 2
Daily feeding of the semi-continuous tests with synthetic substrates.

	Ethanol (g/d)	Acetic Acid (g/d)	Water (g/d)	Molar amount of substrates daily fed in the system (mmol/d)
SYN1	20.0	5.0	0.0	442
SYN2	2.0	0.5	20.5	44.2

Table 3
Main chemical characteristics of the real substrates used in the experimentation.

	COND	ACW	CW	WW
TS (% w/w)	0.02 ± 0.01	6.67 ± 0.13	6.62 ± 0.05	2.52 ± 0.03
VS/TS (%)	99.98 ± 0.01	98.60 ± 0.02	99.50 ± 0.01	97.35 ± 0.06
COD (g/L)	23.46 ± 0.41	71.43 ± 0.89	67.31 ± 1.56	27.60 ± 0.54
Acetic acid (gCOD/L)	1.98 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.08 ± 0.01
Butyric Acid (gCOD/L)	0.00 ± 0.00	4.18 ± 0.51	1.34 ± 0.26	1.34 ± 0.28
Ethanol (gCOD/L)	8.36 ± 0.51	0.00 ± 0.00	0.00 ± 0.00	12.75 ± 0.19
Lactic acid (gCOD/L)	0.00 ± 0.00	21.58 ± 0.85	12.32 ± 0.02	0.00 ± 0.00

2.3. Sample collection, 16S rRNA gene metabarcoding and bioinformatic analysis

Samples of the inoculum (INO), the synthetic substrate (SYN2) and the real substrates Condensate (COND) and Acid Cheese Whey (ACW) were collected after reaching the steady state conditions (25 days from the beginning of the first HRT) and conserved at −20 °C until the following analysis.

The DNA extraction and metabarcoding analysis were performed at BMR Genomics S.r.l. (Padua, Italy). In detail, DNeasy 96 PowerSoil Pro QIAcube HT kit was employed for DNA extraction (Qiagen, Hilden, Germany); secondly, V3-V4 regions of 16 S rRNA gene were amplified with the primers, followed by the enzymatic purification with a Thermolabile Exonuclease I (Euroclone spa) before carrying out a second amplification, necessary for adapter ligation and dual index library preparation. The 16 S rRNA gene amplicons paired-end sequencing was performed with the MiSeq Illumina platform (dual-indexing approach, 2 ×300 bp) (Illumina, San Diego, CA, USA). A mock community was included as control. The raw files were obtained in FASTQ format; the reads were submitted to SRA archive and are available under the bioproject n. PRJNA935712. Metabarcoding reads were then analysed using Qiime2 (v. 2021.4.0.). At first, adapters were trimmed using Cutadapt then DADA2 was used to denoise the sequences; this step included read quality filtering and trimming, error rate estimation, dereplication, read merging and chimera detection. Operational Taxonomic Units (OTUs) were retrieved using trained sequences (OTUs at 99%) from Silva database (v. 138).

2.4. Analytical methods

The main parameters for the chemical characterization of the substrates and the reaction medium, i.e., Total Solids (TS), Volatile Solids (VS), Chemical Oxygen Demand (COD), have been determined according to Standard Methods (APHA-AWWA-WPCF, 2005). A portable probe (Eutech pH 700) was used to measure the pH. The VFAs contents of the different tests were obtained through a liquid ionic chromatography method (Dionex ICS 1100 with AS23 column), while MCFAs (octanoic and decanoic acids) were measured by an external laboratory. Ethanol and lactate concentrations were determined by Megazyme kits (Megazyme, 2018, 2020). All the VFAs and MCFAs values are expressed as COD. The amount and the composition of biogas production was measured once a day through water displacement method and a portable biogas analyzer, respectively (BIOGAS5000, Geotech, United Kingdom).

3. Results and discussions

3.1. Batch tests for the determination of the best operational parameters using synthetic substrates

Batch tests were carried out to define the best molar ratio for VFAs and electron donors as well the best operational pH. Regarding the pH, it emerged that all the biological processes were totally inhibited at pH 9, while acidogenic fermentation, the chain elongation and the biogas production performed well both at slightly acidic (pH=5.5 ± 0.2) and neutral pH conditions (pH=7 ± 0.2).

San-Valero et al. (2020), who worked with synthetic VFAs in pure culture composed by *Clostridium kluyveri*, achieved the best caproic acid production in the pH range of 7.0–7.5. In particular, they observed that under pH values of 6.5, the VFAs start to be present also in the dissociated form, which can pass through the microbial membrane, inhibiting their metabolism. However, another work by Candry et al. (Candry et al., 2020b) emphasized how the optimal pH depends on the microorganism choice, especially when working in pure culture conditions. Values of pH below 6 resulted in caproic acid production by *Caproiciproducens galactitolivorans* (Kim et al., 2015), while pH above 6 was better for propionic acid formation by *Veillonella* and *Aminobacterium* spp. Basic pH conditions were also studied previously. Fernando-Foncillas and Varrone (2021) investigated the effect of pH on the fermentation of a mixture of sewage sludge and food wastes. They demonstrated that pH 9 was able to assure a good yield of VFAs production as VFAs are in the undissociated form, but this condition was not advantageous for MCFAs synthesis, which was optimized at slight acidic-neutral pH values. A

similar result was achieved by Sarkar et al. (2021): they observed caproic acid concentrations around 9 g_{COD}/L for pH values in the range 6.5–7.0, while lower concentrations were observed for higher pH. Roghair et al. (2018b) explained that basic pH was responsible to favor the ethanol-oxidizing microorganisms which do not perform chain elongation. It led the Excessive Ethanol Oxidation (EEO) phenomenon, which reduces the amount of acetyl-CoA available for VFA chain elongation. Furthermore, acetic acid derived from EEO led to the medium acidification, with the further inhibition of the system (Grootscholten et al., 2013).

Consequently, a pH value of 7 was selected for the following semi-continuous tests.

The influence of different acetate:ethanol molar ratios was shown in Fig. 1. The performances were referred to pH 7 but the values are very similar also for pH 5.5.

The MCFAs production yields at pH 5.5 were slightly lower, even if the trend was similar. They accounted for the 0.23%, 3.14% and 4.59% w/w for the acetate:ethanol molar ratio of 1:2, 1:3 and 1:5, respectively. While, no MCFAs were detected at pH 9 in all the three acetate:ethanol molar ratios.

The results demonstrated that acetate:ethanol molar ratio is a very important parameter to shift the biological pathway from acidogenic fermentation towards the reverse β-oxidation reactions.

Theoretically, the molar ratio 1:2 allows acetic acid elongation through ethanol use. The results of the batch tests demonstrated this ratio led to a very low MCFAs production, with a yield of 0.48% w/w. This is consistent with some previous research works: according to Zhang et al. (2021) ethanol is first converted into acetyl-CoA, but 1/6 of it is used to supply energy in ATP form. The remaining 5/6 of the acetyl-CoA enter the cyclic chain elongation pathway to increase the carbon chain of the VFAs through sequential enzymatic reactions, which clearly have not a full efficiency (Angenent et al., 2016). Another very recent work by Wang et al. (2023a) adopted real substrates (food wastes) to produce ethanol by yeast fermentation and VFAs by acidogenic fermentation. The two effluents were mixed at different ratios. It emerged that the best yeast fermentation: Acidogenic fermentation ratio was 2:1. Even if the authors did not calculate the acetate:ethanol ratio, their work confirmed that a higher electron donor compound is fundamental for the starting of the chain elongation process. On the other hand, very high ethanol concentrations are not equally suitable for β-oxidation. It can be easily explainable as under this condition, the acetic acid concentration is too low to complete the cyclic pathway. Moreover, an ethanol excess can cause the inhibition of the microorganisms too, inhibiting the process, as observed by Ma et al. (2022). This trend was confirmed by the present work when operating at 1:10 acetate:ethanol molar ratio, which led to the complete failure of the process because of the excess of ethanol presence. On the contrary, the best performance was achieved working with an acetate:ethanol molar ratio of 1:5, which led to a MCFAs yield of about 6% w/w. Regarding the composition of the produced MCFAs, caproic acid was the main acid with about 95% w/w, while octanoic and decanoic acids were present in low concentrations: 3.5% and 1.5% w/w, respectively. This test also showed that a good VFAs production yield was around 27% w/w, with butyric acid representing 85–90% w/w of the total VFAs. It confirmed that reverse β-oxidation reactions completed just the first step of chain elongation with the conversion of the acetic acid into butyric acids, while the second cycle had just started. For this reason, the following continuous process had a longer duration, with an HRT of 25 days, similarly to some previous research works (Liu et al., 2017; Zhang et al., 2021).

Considering the COD conversion into VFAs (27%) and MCFAs (6%), it is fundamental to remark that the largest part of COD was converted into biogas, whose specific production was of about 580 mLbiogas/g_{COD}. This means that batch mode was not the best configuration for a process having the scope to produce MCFAs by reverse β oxidation as it promoted an immediate consumption of the substrates for biogas production by AD. On the contrary, it is important to favor the selection of the biomass by a gradual and continuous addition of the substrates. Consequently, the continuous mode could represent a more appropriate feeding mode to assure the shifting from acidogenic fermentation pathway, for VFAs and biogas productions, to reverse β-oxidation.

3.2. Semi continuous tests

The influence of the molar ratio between the acetic or butyric acids and the electron-donor compounds were studied along semi-continuous tests both on synthetic and on real substrates. The main results were correlated with the main operational parameters in Table 4.

3.2.1. Semi continuous tests on synthetic substrates

Semi continuous tests on synthetic substrates were carried out according to the best acetate:ethanol molar ratio of 1:5 and neutral pH. These were the best conditions observed during the experimental campaign on batch trials. Along this work three different

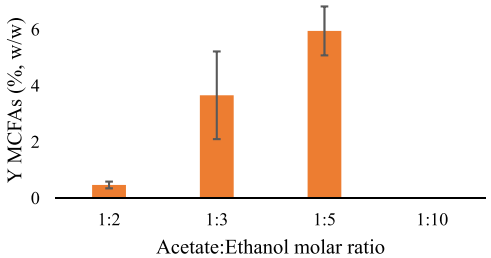

Fig. 1. MCFAs production yields at the different acetate: ethanol molar ratios at pH of 7.

conditions were tested (Table 2).

Since the first days of the experimentation, SYN1 test failed, so data are not shown in Fig. 2. The analysis of the daily effluent from the reactor evidenced an accumulation of the two substrates: acetic acid and ethanol as they did not undergo conversion by reverse β-oxidation. After 14 days the acetic acid concentration in the reaction medium was detected at about 70 g/L, demonstrating the inhibition of the system. Consequently, the SYN1 test was dismantled after twenty days. The failure of SYN1 test demonstrated that acetate: ethanol molar ratio is not the only important parameter to have a successful chain elongation process. The total substrate molar concentration is equally important. Recently, Zhang et al. (2021) found that the optimal total substrates concentration for caproic acid production was in the range of 650–850 mmol/L. Over this value they observed a decline in the caproic acid productivity. Another previous study also reported an inhibition of the reverse β-oxidation when the ethanol concentration was higher than 700 mmol/L (Yin et al., 2017; Wang et al. 2023c). On the contrary, this work showed different results: the failure of the synthetic tests was observed for high amount of the substrates, such as in SYN1 test, whose feeding was constituted by a total substrate amount of about 440 mmol/d. This high daily substrate amount was not properly assimilated and converted by the microbial consortium and consequently started to accumulate in the system, inhibiting it in few days. On the contrary, in tests SYN2, which worked at daily feeding amount of substrate of about 40–45 mmol/d (Table 2), was observed a chain elongation reaction. It represented an important achievement as higher substrate concentration means the possibility to operate with a lower dilution and, consequently, with a lower reactor volume with a positive effect on the economic performance of the process.

Fig. 2 illustrates the trends for acetic, butyric, caproic and octanoic acids over time of these tests.

The 10-fold lower molar concentration of the total substrates, daily fed to the system, assured the good achievement of the two tests. SYN2 test was very effective both in terms of reaction kinetics and of VFAs and MCFAs productions: after 5 days since the beginning of the tests the butyric acid started to appear, simultaneously to a first decline of acetic acid concentration (Fig. 2). It demonstrated the instauration of the first cycle of reverse β-oxidation which resulted in acetic acid chain elongation to butyric acid. Around day 10 of the experimentation of the SYN2 tests, the further butyric acid chain elongation led to the appearance of caproic acid, which increased rapidly to about 20–25 g_{COD}/L. The caproic acid concentration remained stable at this value for about a complete HRT. Then, the caproic acid concentration declined to 15 g_{COD}/L (days 30–50 days, Fig. 2) and octanoic acid appeared reaching a stable concentration of 1.50–1.55 g_{COD}/L. However, it is important to remark that octanoic acid appearance justified only partially the caproic acid decline. Its appearance occurred simultaneously with the raising of acetic and butyric acids concentrations in the effluent, demonstrating that the daily feed of acetic acid was not completely assimilated and adopted by the microbial consortia, remaining partially accumulated in the reaction medium. This phenomenon was strictly correlated to the high caproic acid content, too, which comported a decreasing of the kinetic of the reverse β-oxidation reactions, according to the Le Châtelier chemical principle. The simultaneous production and recovery of caproic acid from the reaction medium can help to keep the performance of the system high. This solution could be also economically convenient considering its applications in the synthesis of a large portfolio of bio-based products (Rizzioli et al., 2021).

The concentrations of the acids remained almost constant along the last HRT of the SYN2 test, as effect of the reaching of a steady state condition of caproic and octanoic acids values of 14–17 g_{COD}/L and 1.50–1.55 g_{COD}/L, respectively. The final MCFAY for SYN2 was 6.69%, but it reached 9–12% in correspondence of the highest caproic acid concentration along the first HRT of the test.

Table 4
Correlation of the MCFAs production with the operational parameters.

	MCFAs yield (% w/w)	acetic or butyric acid / Electron donor compound (molar ratio)	MCFAs/ VFAs (production rate)	Operational conditions
COND (this study)	25.17 ± 1.65	1: 5.50	0.93 ± 0.04	Semi-continuous test, HRT 25, 37 °C, pH 7.0
ACW (this study)	10.45 ± 0.72	1: 5.04	0.64 ± 0.21	Semi-continuous test, HRT 25, 37 °C, pH 7.0
CW (this study)	5.41 ± 2.39	1: 8.99	0.36 ± 0. 13	Semi-continuous test, HRT 25, 37 °C, pH 7.0
WW (this study)	failed test	1: 208	failed test	Semi-continuous test, HRT 25, 37 °C
Opuntia Ficus Indica (Rizzioli et al., 2024)	about 30%	1: 4.16	about 0.30	Semi-continuous test, HRT 5, 37 °C, pH 7.0
Sugarcane molasses (Cavalcante et al., 2017)	about 40%	1: 8.00	about 0.65	Batch test, 35 °C, pH 5.5
Synthetic substrates (Tang et al., 2022)	about 35%	1: 3.00	about 0.45	Batch test, 37 °C, pH 5.5
Food wastes (Wang et al., 2023b)	18.5%	1: 2.00	about 0.30	Separate yeast fermentation and food fermentation for ethanol and VFAs production, respectively. Batch test for caproic production at 55 °C and pH 5
Food wastes and sewage sludge (Gottardo et al., 2023)	about 10%	Not available	about 0.15–0.20	Semi-continuous tests, HRT 4–6, 55 °C, uncontrolled pH, food waste- sewage sludge volumetric ratio 1:1
Food wastes (Crognale et al., 2023)	5.5%	2: 0.85	about 0.30	Semi-continuous tests, HRT 4, 37 °C, pH 7

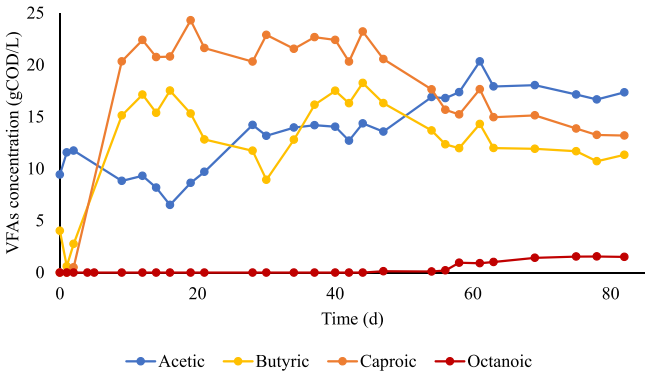

Fig. 2. VFAs and caproic acid trends for SYN2.

3.2.2. *Semi continuous tests on real substrates*

3.2.2.1. Chain elongation from condensate of the organic fraction of municipal solid waste. Fig. 3 shows the VFAs and MCFAs concentrations over time for COND test.

The trend observed in this experimental trial were similar to those observed in SYN2 test. OFMSW condensate seemed to be an ideal substrate for the reverse β-oxidation reaction, as it contained both acetic acid and ethanol in concentrations close to the optimal ratio of 1:5. Consequently, the butyric and caproic acids production started very fast, around the fifth day since the beginning of the tests, demonstrating the instauration of the two cycles of the chain elongation. In the first 10 days, the caproic acid concentration went up at around 12 g_{COD}/L, remaining stable until the end of the first HRT. However, towards the end of the HRT, a gradual caproic acid decrease was detected until a new stable condition at lower values of about 7 g_{COD}/L was observed. At the same time, octanoic acid appeared reaching a stable concentration in the range of 1.20–1.25 g_{COD}/L. Unlike SYN2 tests, COND reactor did not accumulate acetic acid, whose concentration was always lower than 2 g_{COD}/L over all the test duration. Instead, the butyric acid increasing to 6–8 g_{COD}/L seemed to indicate the chain elongation processes were not inhibited. Moreover, the simultaneous appearance of octanoic acid demonstrated that a further reverse β-oxidation cycle occurred for the elongation of caproic acid to octanoic acid. The reactor kept constant fatty acids concentrations for more than 20 days. This is a very important achievement as the reaching of the steady state is fundamental in the process scale-up optic. MCFAY was good for COND test with a 25.17% in steady state condition and a peak of about 42.5% in correspondence of a caproic acid concentration of 12 g_{COD}/L.

3.2.2.2. Chain elongation from Acid Cheese Whey (ACW) and Cheese Whey (CW). ACW and CW were two interesting substrates for reverse β-oxidation as they represented an alternative to the conversion of acetic acid and ethanol. Table 3 shows these substrates are rich in butyric acid and lactic acid. This latter worked as electron donor compound instead of ethanol..

Being rich in these organic acids, both the reactors quickly accumulate butyric acid in the first 15–20 days of the tests (Fig. 4). The phenomenon was more evident in ACW test (Fig. 4A), substrate richer in butyric and lactic acids than CW (Table 3), but can also be observed in CW (Fig. 4B). Consequently, the maximum butyric acid concentration was higher (45–50 g_{COD}/L) in ACW than in CW test (30–35 g_{COD}/L). The caproic acid did not appear in the first part of the trial as occurred both for the tests on synthetic substrates and condensate. Caproic acid started to be synthetized and to increase its concentration in the reaction medium with the starting of the decline of the butyric acid and the simultaneous increasing of the acetic acid (15–25 days). This was a very interesting result as it suggested that caproic acid elongation did not occur as effect of the direct elongation of butyric acid, but after the degradation of lactic acid into acetic one. In fact, in ACW the butyric concentration moved from 40 to 50 g_{COD}/L to 15–25 g_{COD}/L, with the simultaneous

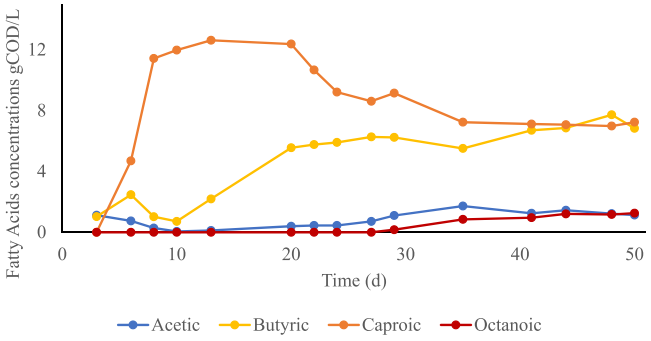

Fig. 3. VFAs and caproic acid trends for COND test.

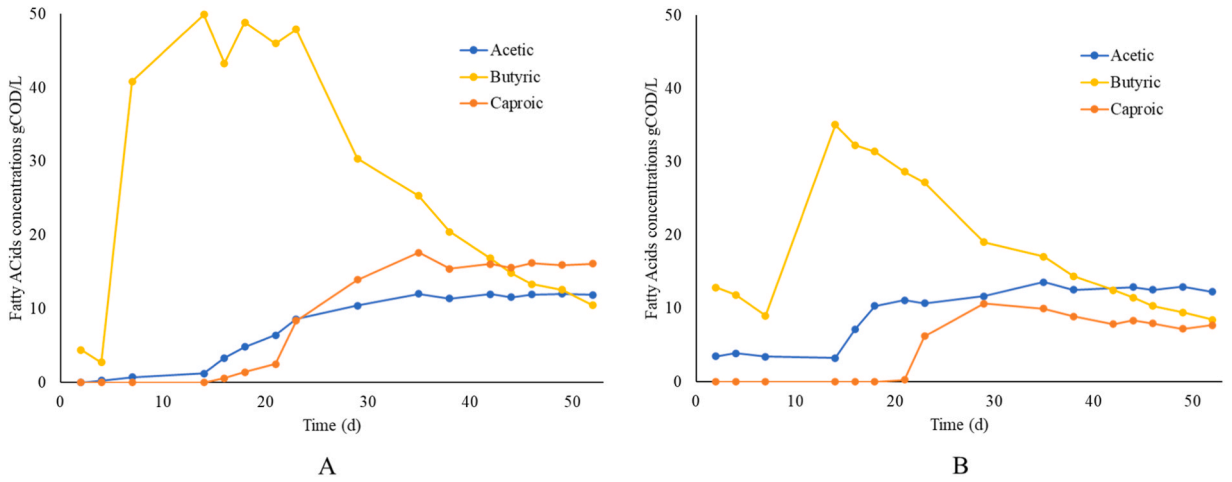

Fig. 4. VFAs and caproic acid trends for ACW (A) and CW (B), respectively.

increasing of acetic acid until 10–15 g_{COD}/L (Fig. 4A). Similarly, the butyric acid passed from 30 to 35 g_{COD}/L to about 10 g_{COD}/L, during the appearance of the acetic acid which reached a concentration in the range of 10–15 g_{COD}/L (Fig. 4B). More details on the lactic acid pathway were provided by Asunis et al. (2020), who explained that lactose is first hydrolyzed into glucose and galactose, which were then converted into lactic acid. Then, lactic acid followed two different metabolic routes: a fraction is transformed into acetic acid and butyric acid, which explained the appearance of acetic acid in both the reactors. Another route led to the lactic acid conversion into acetyl-CoA, which acted as an electron donor compound allowing the elongation of butyric acid into caproic acid (Cavalcante et al., 2017). Clearly, all these reactions were more evident in ACW tests, having a higher initial butyric concentration than CW. Even the process kinetics seemed to be higher in ACW than in CW.

Both the tests reached the steady state condition in the second HRT with a final caproic acid concentration of 15–16 g_{COD}/L (corresponding to a MCFAY of 10.45%) for ACW test and 7–8 g_{COD}/L (corresponding to a MCFAY of 5.41%) for CW test, respectively. It is interesting to observe that caproic acid remained stable until the end of the test (Fig. 4): no decrease in its concentration was observed as happened in synthetic and COND tests. At the same time, octanoic acid was never detected in the reaction medium. This suggests that different electron donor-compounds can influence the kinetic and the final composition of the MCFAs production.

The good performances of the chain elongation from ACW were confirmed by some works in scientific literature. For instance, Wang et al. (2023b) classified different organic substrates according to their ability to produce MCFAs, putting ACW at the second place, after fruits and vegetable wastes. More interesting is the work by Duber et al. (2018), who achieved very similar results to the performance of present research: they obtained a caproic acid production of about 18 g/L, reaching the stabilization of its production after 37 days since the starting of the test. Instead, a slightly lower MCFAs production of about 11 g/L was obtained by Chwialkowska et al. (2019). The work is equally interesting as the authors used both ethanol and lactate as electron donor compounds, observing that both substrates promoted the chain elongation process, but lactate resulted in the most efficient.

3.2.2.3. Chain elongation from WW. Winery wastewater (WW) was also tested for reverse β-oxidation but the test failed completely as caproic acid was never produced. The chemical composition of the substrate can explain the negative result. As evident from Table 3, WW is very rich in ethanol. The acetic acid- ethanol molar ratio was of about 1:12, too far from the optimal one causing the inhibition of the chain elongation by the EEO, previously described (Roghair et al., 2018a). The ethanol excess led to the instauration of the Excessive Ethanol Oxidation (EEO), which is able to reduce the amount of acetyl-CoA available for VFAs chain elongation Furthermore, acetic acid derived from EEO led to the medium acidification, with the further inhibition of the system (Grootscholten et al., 2013). This result confirmed the importance of finding real substrates having a balanced ratio of the acid to be elongated and the compound acting as electron donor for the instauration of the reverse β-oxidation reactions. Thus, the failure of the chain elongation along this work by the adoption of an expired red wine, which was still rich in ethanol concentration. Instead, the usage of a byproduct from wine production seemed to make possible the MCFAs synthesis. Hernández-Correa and Buitrón (2023) achieved a maximum MCFAs production of 11% (on TS based). In particular, they used a by-product from wine production with a sugars content of about 25 g/L, which were then converted in ethanol and acetic and butyric acids allowing the instauration of the chain elongation.

3.3. Microbiology analysis

The bacterial diversity analysis was performed through Simpson and Shannon indexes (Table 5). The results showed a significant decrease in bacterial richness between the inoculum (INO) (Shannon 2.97; Simpson 0.9173) compared to ACW (Shannon 2.253; Simpson 0.8599), SYN2 (Shannon 2.308; Simpson 0.8339) and COND (Shannon 2.065; Simpson 0.8068) samples. These results suggested a specific bacterial speciation due both the available substrates as well as the chemical/physical parameters fixed during the chain-elongation process.

Focusing on the bacterial relative abundance in INO, COND, ACW and SYN2 (Fig. 5), it has been observed that INO include a high variability of Operational Taxonomic Units, mainly belonging to orders *Caldicoprobacterales, Bacteroidales e Pseudomonadales* (see Supplementary Materials), while COND and SYN2 were dominated by members of order *Micrococcales* wherein *Pseudoclavibacter caeni* represented the unique species – with an abundance of 38.19% and 33.38% respectively.

On the other hand, only 9.85% of *P. caeni* was detected in ACW substrate. This species was first isolated from sludge of a sewage disposal plant and evidenced the capability to produce acids from different sugars – including lactose - and showed a weakly positive reaction in assimilation of acetate and caproate. However, the use of lactate was not observed. Since this species was already detected in chain-elongation fermenters (Coma et al., 2016; Duber et al., 2022) it was suggested that *P. caeni* may be indirectly or directly involved in the chain-elongation process in mixed cultures. Alternatively, due to its strictly aerobic metabolism, it can be an indication of some possible oxygen contamination in the batch trial (Duber et al., 2022). *Erysipelotrichales* was the most abundant order in ACW (24.16%), mainly represented by *Thomasclavelia* genus (Lawson et al., 2023) (24.13%) which was absent in COND and with a very low relative abundance (0.5%) in SYN2. It is an anaerobic fermentative organism that can convert multiple substrates into short-chain carboxylate and lactate. *Thomasclavelia* (which was proposed as a new genus to encompass former genus *Erysipelatoclostridium)* was recently observed during caproate production suggesting a role both in acidogenic phase fermentation and lactate-based chain-elongation (Palomo-Briones et al., 2022).

Members of order *Tissierellales* were found in COND, SYN2 and ACW samples with a percentage of 7.14%, 12.86%, and 6.47%, respectively. The main genera identified were *Sporanaerobacter* (*S. acetigenes*) in either ACW (5.2%) or COND (6.22%), and *Keratinibaculum* (*K. parault*unense) (7.15%) in SYN2 samples. Although *S. acetigenes* is not considered to be a caproate producer, it is reported that this genus can produce H_2, acetic acid, isobutyric and isovaleric acids from different sugars (Hernandez-Eugenio et al., 2002). Moreover, this genus evidenced a possible correlation with caproate production using acetate and ethanol as sources of carbon end energy (Yu et al., 2019). Therefore, this genus may indirectly affect the caproate production during the chain-elongation process. *Keratinibaculum* is an anaerobic, moderately thermophilic and alkaliphilic genus with proteolytic activity and able to growth on some saccharides (Zagrodnik et al., 2020). *K. parault*unense was able to produce mainly acetic, propionic, isobutyric, butyric, and isovaleric acids in peptone medium. Low amounts of methanol, ethanol, isobutanol, and caproic acid were produced when chicken feather or peptone were used as substrates. On the other hand, to the best of the authors' knowledge, this is the first time that *K. parault*unense is detected during chain-elongation process.

Bacillales were found in all the batch tests with a percentage of 11.47% in COND, 7.8% in SYN2 and, 1.22% in ACW. *Bacillus* represented the main genus in COND, and SYN2 samples, whereas *Rummeliibacillus* was the most abundant genus in the ACW fermenter. *Bacillus* was found in chain elongation process for caproic acid production from waste-derived lactic acid and butyric acid (Nzeteu et al., 2022), while *Rummeliibacillus* spp. are dominant during the acclimation process of anaerobic fermentation microbiome with acetate and ethanol for chain elongation (Li et al., 2023).

Clostridiales accounted for 8.59, 1,76%, and 11.87% in COND, SYN2 and ACW samples respectively. Among this order, *Clostridium kluyveri* was the dominant species in COND (8.32%), and SYN2 (1.41%). On the other hand, *Clostridium luticellarii* (7.52%) was the most representative species in ACW fermenter. *Clostridium kluyveri* is a well-known species performing chain-elongation, ubiquitously found in anaerobic fermentation batch. Moreover, although this organism is not capable of utilizing sugars such as glucose or lactose, it is proved that *C. kluyveri* can efficiently convert ethanol and acetate to butyrate and caproate. *C. luticellarii* evidenced high taxonomic similarity with *C. kluyveri* (Wang et al., 2015). It can produce butyrate (Wang et al., 2015) and frequently identified in methanol-based chain-elongation process (de Leeuw et al., 2020). Members of *Eubacteriales* were particularly identified in COND (9.06%) and ACW (19.27%) fermenters. Unlike, only 0.24% of this order was found in SYN2 sample. Whereas in the COND a dominant genus was not observed, *Caproiciproducens* sp. (11.68%) was the most representative genus in ACW sample. It is a strictly anaerobic organism able to produce lactate, acetate, butyrate and caproate by using different sugars (Esquivel-Elizondo et al., 2021; Kim et al., 2015). In fact, this genus was often identified during the process for bioproduction of MCFA such as caproic acid (Ma et al., 2021b).

[Lachnospirales] order accounted for 11.08% in ACW sample, and it was significantly positively associated with butyric acid production (Zhao et al., 2022).

4. Conclusions

The reverse β-oxidation was studied to find the best operational conditions to increase caproic acid production. Batch tests, adopting synthetic substrates allowed to identify neutral pH values and a molar ratio of ethanol:acetic acid of 5:1 as the optimal conditions. Semi-continuous tests allowed to show the importance of the total substrates amount too. The best MCFAs yield with synthetic substrates of about 6.7% w/w was achieved in correspondence of the total substrates amount of around 40–45 mmol/d. It started to decrease when the substrates amount overcame the 400 mmol/d. Finally, the results indicated a specific bacterial speciation during the chain-elongation process toward bacterial population that can be indirectly or directly involved in the chain-elongation process such as *Pseudoclavibacter caeni, Thomasclavelia* and *Caproiciproducens.*

Table 5
Statistical indices (Simpson and Shannon) of bacterial diversity at order level.

Index	INO	COND	SYN2	ACW
Simpson	0.9173	0.8068	0.8339	0.8599
Shannon	2.97	2.065	2.308	2.253

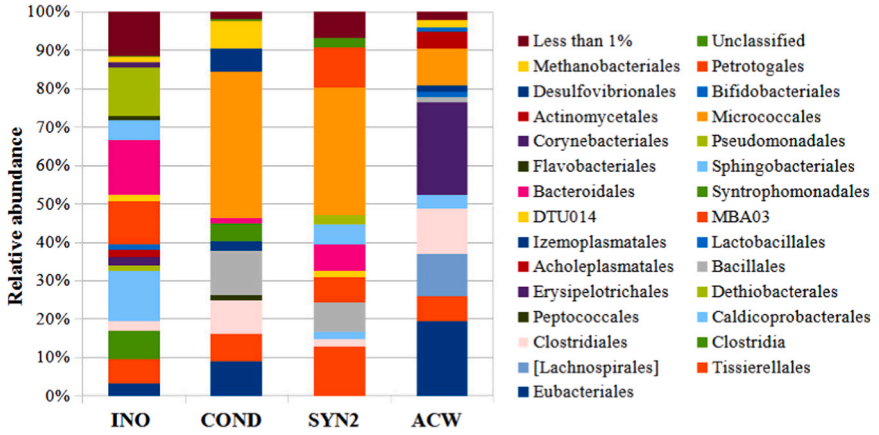

Fig. 5. Bacterial community composition at order level revealed in INO, COND, SYN2 and ACW samples. Order names have been reported following Oren and Garrity (2021).

CRediT authorship contribution statement

Federico Battista: Conception, Supervision, Redaction of the paper. **Alessandro Zeni:** Conduction of the tests. **Marco Andreolli, Elisa Salvetti, Silvia Lampis:** microbiology analysis, Redaction of the paper. **Fabio Rizzioli:** Supervision and Conduction of the test, Redaction of the paper. **David Bolzonella:** Funding and general supervision.

Declaration of Competing Interest

The authors declare that have not conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work.

Data availability

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2023.103453.

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