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Efficient production of *n*-caproate from syngas by a co-culture of *Clostridium aceticum* and *Clostridium kluyveri*

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ABSTRACT

In recent years, the possibility of merging technologies for waste recovery such as those based on syngas fermentation and chain elongation has been studied for the production of medium chain fatty acids (MCFAs) and bioalcohols, in an attempt to integrate the concept of circular economy in the industry. Nevertheless, one of the main issues of this approach is the pH mismatch between acetogens and chain elongating microorganisms. This work reports, for the first time, the suitability of a co-culture of *C. aceticum* and *C. kluyveri* metabolizing syngas at near neutral pH in stirred tank bioreactors. For this purpose, bioreactor studies were carried out with continuous syngas supply. In the first experiment, maximum concentrations of *n*-butyrate and *n*-caproate of 7.0 and 8.2 g/L, respectively, were obtained. In the second experiment, considerable amounts of *n*-butanol were produced as a result of the reduction, by *C. aceticum,* of the carboxylates already formed in the broth. In both experiments, ethanol was used as an exogenous electron agent at some point. Finally, batch bottle assays were performed with a pure culture of *C. aceticum* grown on CO in presence of *n*-butyrate to assess and confirm its ability to produce *n*-butanol, reaching concentrations up to 951 mg/L, with a *n*-butyrate conversion efficiency of 96%, which had never been reported before in this species. Therefore, this work contributes to the state of the art, presenting a novel system for the bioproduction of MCFAs by combining syngas fermentation and chain elongation at near neutral pH, as opposed to the acidic pH range used in all previously reported literature.

1. Introduction

Nowadays, society is facing two major environmental issues, namely the need for sustainable manufacturing of chemicals and fuels from non-food sources and the need to mitigate greenhouse gas emissions. For instance, as part of the European Green Deal, the European Commission proposed, in September 2020, to achieve a reduction target for these emissions by 2030 of at least 55% compared to 1990, with the goal of making Europe the first climate-neutral continent (European Commission, 2021). Hence, there is a special interest in the development of production processes based on the use of renewable raw materials or even waste (e.g, gas emissions). A promising alternative is syngas fermentation, since it employs acetogenic bacteria as autotrophic biocatalysts that utilize CO and CO_2 through the Wood-Ljungdahl pathway for the efficient production of bioalcohols and biocommodities (e.g., acetate) (Bengelsdorf et al., 2013). Nevertheless, the distillation of these highly water soluble end products is rather expensive since it is an energy-intensive process (Grootscholten et al., 2013). An alternative is to convert the produced short chain acids from syngas into MCFAs, such as *n*-caproic acid (C_6) or *n*-caprylic acid (C_8), through a second fermentation step (Gildemyn et al., 2017). MCFAs are more hydrophobic, have a higher added-value and a higher energy density which makes them more suitable for the biofuels market (Steinbusch et al., 2011). Furthermore, the number of strains that can metabolize syngas to form *n*-caproic acid is very limited (i.e., *C. carboxidivorans* and *E. limosum* mainly), making it a rather unusual process (Lindley et al., 1987; Phillips et al., 2015). Likewise, this second bioconversion is performed through a biochemical process called chain elongation based on the reverse β-oxidation pathway. First, part of the ethanol is oxidized to acetate via substrate level phosphorylation to form adenosin triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). Secondly, the remaining ethanol is converted into acetyl-CoA. One molecule of acetyl-CoA is then added to an acetate molecule triggering the formation of *n*-butyrate, thus elongating its chain length by two carbons in each cycle. Lastly, similar to the previous step, another molecule of acetyl-CoA is used to elongate *n*-butyrate to *n*-caproate (Cavalcante et al., 2017;

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Spirito et al., 2014). An overview of the Wood-Ljungdahl and chain elongation pathways can be found in Fig. 1. Among all the chain elongating microorganisms, *Clostridium kluyveri* has been the most widely studied species.

MCFAs are typically obtained from vegetable oils (e.g., palm kernel and coconut oils) (Sarria et al., 2017). However, *n*-caproate and *n*-caprylate are found in these oils in percentages below 10% and this traditional method requires high pressures and temperatures, as well as the use of expensive metal oxides or mineral acids as catalysts (Gervajio et al., 2005). *n*-Caproate can also be produced in large quantities directly from synthetic substrates (e.g. ethanol, acetate) (Steinbusch et al., 2011), although the high cost of those substrates hinders the commercialization of this technology (Reddy et al., 2020). Bioelectrochemical systems are also an alternative to generate MCFAs from *in situ* generated H_2 or electricity that can use cheap waste as feedstock. However, it has significant drawbacks such as the need of expensive materials for reactor fabrication (e.g., membranes) and the large electricity expenses (Reddy et al., 2018b). Anaerobic digestion is another suitable technology for this purpose (Iglesias-Iglesias et al., 2021) due to its several advantages: a high level of purification, low nutrient requirements, reduced excess sludge and accumulation of valuable biogas for energy production, thus minimizing operating costs (Siles et al., 2007). On the other hand, this process is often carried out with mixed cultures and competing processes are possible (i.e., methanogenesis), being necessary sometimes the presence of suppressive agents (Steinbusch et al., 2011). On the other hand, the combination of syngas fermentation and chain elongation technologies for obtaining bioproducts is an attractive approach considered by several researchers due to its benefits compared to other thermochemical processes (e.g., Fischer-Tropsch process) (Kennes et al., 2016): low temperatures (30–37 °C) and near atmospheric pressures. Besides, the biocatalytic reactions are independent of the syngas (CO_2: CO:H_2) ratio, providing great flexibility regarding these proportions; and it withstands quite well the presence of impurities (e.g., sulfur compounds and chlorine) (Fernández-Naveira et al., 2017). Chain elongation can also take place in pure cultures or under non-sterile conditions with mixed cultures (Reddy et al., 2018a) and both readily biodegradable and non-biodegradable waste (e.g. plastic) can be used directly or indirectly as feedstock (Han et al., 2019). Besides, the use of syngas instead of dissolved sugars or liquid substrates is advantageous because the hydraulic retention time is uncoupled from the substrate supply in the bioreactor (Henstra et al., 2007).

Co-cultures performing both fermentations are interesting because they can be designed to have the advantageous interactions that exist in mixed cultures, with the absence of other microorganisms that could exert a negative effect on the performance of the process (Diender et al., 2021). However, to undertake this approach in a single reactor, one of the major challenges to be faced is the pH incompatibility between acetogens and chain elongating microorganisms. While acidic conditions promote solventogenesis (i.e., ethanol production) in acetogens, this is harmful to the survival of *C. kluyveri*, especially below pH 4.5 (Ganigué et al., 2016). Recently, various acetogens capable of yielding alcohols from gas fermentation, such as *C. autoethanogenum* at pH 6 and 4.75 (Abubackar et al., 2015) and *C. carboxidivorans* at pH 5.75 (Fernández-Naveira et al., 2016) have been reported in the literature.

The case of *C. aceticum* is quite noteworthy and will be object of study in this work. The first ethanol production by *C. aceticum* (0.6 g/L) was reported using CO as the sole source of carbon and electrons in batch studies (Mayer et al., 2018). A few month later, it w was shown that, in this strain, the solventogenic phase starts at a pH close to neutrality, reaching ethanol concentrations of up to 5.6 g/L at pH 6.9 (Arslan et al., 2019). It would therefore be interesting to combine this strain with *C. kluyveri*, taking advantage of the fact that ethanol production in *C. aceticum* takes place at a pH close to the optimum for *C. kluyveri*. So, the aim of the present work was to study the optimal operational conditions and the MCFAs and alcohols resulting from a formerly non-reported co-culture of *C. aceticum* and *C. kluyveri* fed syngas in bioreactors.

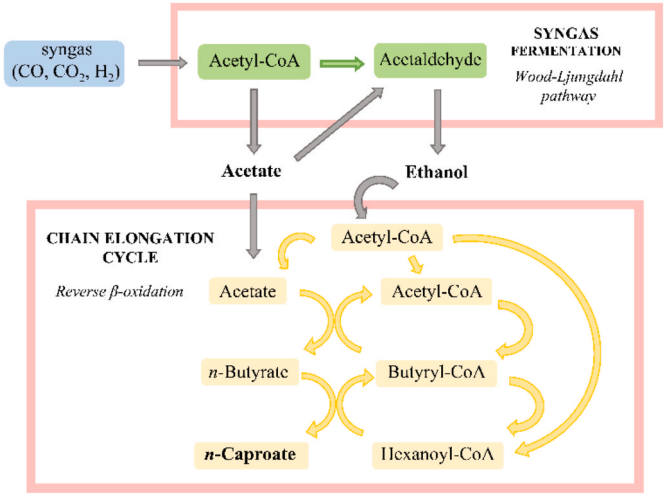

Fig. 1. Metabolic pathways occurring during the course of bioreactor experiments: Wood-Ljungdahl pathway (upper part) used by acetogenic bacteria, and reverse β-oxidation cycle (bottom part) used by chain elongating organisms.

2. Material and methods

2.1. Microbial strains and culture media

C. aceticum DSM 1496 and *C. kluyveri* DSM 555 were obtained in freeze-dried pellet form from the Deutsche Sammlung von Mikroorganismen und Zellkulturen MnbH (Braunschweig, Germany). After rehydration, all incubations were carried out under shaking conditions at 150 rpm and 33 °C.

The basal medium in serum bottles used for *C. aceticum* growth contained (per liter distilled water): 0.20 g NH_4Cl, 3 g yeast extract, 1.76 g KH_2PO_4, 8.44 g K_2HPO_4, 0.33 g $MgSO_4{\cdot}7H_2O$, 10 g $NaHCO_3$, 1 mL rezasurin (0.1% w/v), 2 mL trace metals solution, 0.30 g L-cysteine-HCl and 0.92 g $Na_2S{\cdot}9H_2O$.

The composition of the trace metal solution was (per liter distilled water): 15 g nitrilotriacetic acid, 30 g $MgSO_4{\cdot}7H_2O$, 5 g $MnSO_4{\cdot}H_2O$, 10 g NaCl, 1 g $FeSO_4{\cdot}7H_2O$, 1.8 g $CoSO_4{\cdot}7H_2O$, 1 g $CaCl_2{\cdot}2H_2O$, 1.8 g $ZnSO_4{\cdot}7H_2O$, 0.1 g $CuSO_4{\cdot}5H_2O$, 0.2 g $KAl(SO_4)_2{\cdot}12H_2O$, 0.1 g H_3BO_3, 0.1 g $Na_2MoO_4{\cdot}2H_2O$, 0.25 g $NiCl_2{\cdot}6H_2O$, 3 mg $Na_2SeO_3{\cdot}5H_2O$ and 4 mg $Na_2WO_4{\cdot}2H_2O$

The composition of the vitamin solution was (per liter distilled water): 25 mg D-biotin, 25 mg folic acid, 50 mg pyridoxine-HCl, 50 mg thiamine-HCl, 50 mg riboflavin, 50 mg nicotinic acid, 50 mg D-Ca-pantothenate, 25 mg vitamin B_{12}, 50 mg p-aminobenzoic acid and 25 mg lipoic acid.

After dissolving all the chemicals in 40 mL distilled water in serum bottles, the medium was purged with pure N_2 for a few minutes and then with pure CO. The pH was adjusted to 7.5 either with HCl or NaOH 2 M, while flushing CO through the medium. The bottles were sealed with butyl rubber septa and aluminium crimps and then autoclaved at 121 °C for 20 min.

The composition of the liquid medium for anaerobic cultures and growth of *C. kluyveri* in serum bottles, as well as the composition of the trace metal solution and the vitamins stock solution, were the same as reported in some of our previous studies (San-Valero et al., 2020).

Once all the components had been dissolved in 40 mL distilled water, the serum bottles were flushed for a few minutes with pure N_2, while adjusting the pH to 6.8 with either HCl or NaOH 2 M. Afterwards, 0.83 mL ethanol (96%, v/v) was added just before sealing the bottles with butyl rubber septa and aluminium crimps, and then autoclaving at

121 °C for 20 min.

2.2. Bioreactor experiments with continuous syngas feeding

The experiments were performed in 2 L Eppendorf BIOFLO 120 bioreactors, with a working volume of 1.2 L, and at a constant temperature of 30 °C (Eppendorf AG, Hamburg, Germany). Before inoculation of *C. kluyveri*, the protocol followed was similar to that of Arslan et al. (2019) with slight modifications. In all the experiments, the previously described *C. aceticum* medium was introduced in the vessel, except for $MgSO_4 \cdot 7H_2O$, L-cysteine-HCl, $Na_2S \cdot 9H_2O$ and vitamins, and subsequently autoclaved for 20 min at 121 °C. After cooling down to room temperature (20 °C), the reactor medium was flushed with pure N_2 through a microsparger, for 1 h and a half, while stirring at 250 rpm. Thereafter, the N_2 supply was stopped, and syngas feed was started using a constant flow rate of 10 mL/min, regulated by means of a mass flow controller (Aalborg GFC 17, Müllheim, Germany). The $CO:CO_2:H_2:N_2$ ratio in the syngas mixture was 30:5:15:50. Next, the pH was adjusted to 7.5 and the autoclaved solutions of $MgSO_4 \cdot 7H_2O$, L-cysteine-HCl and $Na_2S \cdot 9H_2O$ were added through one of the reactor's ports. A seed inoculum of *C. aceticum* (10% v/v, 120 mL) in early exponential growth phase and the corresponding vitamins were also introduced in the reactor. The pH was monitored on-line and adjusted, when needed, through the addition of HCl or NaOH 1 M solutions connected to peristaltic pumps. In order to avoid overpressure inside the system, a gas outlet port was used, from which the gaseous samples were also taken. The experimental set-up used is shown in Fig. 2.

In all the experiments, once the desired ethanol concentration was reached after the solventogenic stage, 160 mL of *C. kluyveri* inoculum, with the corresponding vitamins, were added to the fermentation broth. On a daily basis, one or two liquid samples were withdrawn from the bioreactors with a sterile syringe for subsequent optical density measurements at $\lambda = 600$ nm (OD_{600nm}) and analysis of metabolites. Occasionally, 1 mL gas samples were taken from the outlet of the bioreactors for H_2, CO and CO_2 determinations. These studies were carried out to study the behavior of *C. aceticum* and *C. kluyveri* co-cultures in a single bioreactor, combining syngas fermentation and chain elongation processes.

2.3. Bottle batch experiments with C. aceticum

In order to analyze the ability of *C. aceticum* to reduce *n*-butyrate to *n*-butanol, a batch bottle study was carried out with a pure culture of *C. aceticum*. To the previously described medium for this strain, 0.1 mL of *n*-butyric acid (99%) was added and the pH was subsequently adjusted to 7.5 with either HCl or NaOH 2 M. Next, the bottles were flushed first with N_2 and then with CO in the liquid and headspace for 6 min with each gas. Finally, the bottles were sealed with butyl rubber septa and aluminium crimps and autoclaved for 20 min at 121 °C. Once the bottles had cooled down to room temperature, 5 mL seed inoculum in the early exponential growth phase were transferred to each serum bottle (40 mL), and the corresponding vitamins were added. All bottles were incubated on an orbital shaker at 150 rpm and 33 °C. A daily liquid sample of 1 mL was withdrawn from each bottle for OD_{600nm} measurements and quantification of metabolites.

2.4. Analytical methods

OD_{600nm} measurements, used to estimate growth, were done with a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The concentrations of acids and alcohols were determined by High Performance Liquid Chromatography (HPLC) (HP1100, Agilent Co., USA), with an Agilent Hi-Plex Column (300 × 7.7 mm) and both a diode array detector (DAD) and a refractive index detector (RID) maintained at 50 °C. A 0.005 M H_2SO_4 solution was used as mobile phase, with a flow rate of 0.80 mL/min and a column temperature of 45 °C. All liquid samples were centrifuged at 7000 rpm during 5 min and filtered through 0.22 μm PTFE syringe filters.

H_2 and CO concentrations were analyzed by gas chromatography (GC, Agilent Technologies, Madrid, Spain). The GC consisted of a thermal conductivity detector (TCD) and a 15-m HP-PLOT Molecular Sieve 5 A column (0.53 mm ID, 50 μm film thickness). The temperature program was the same as reported in Arslan et al. (2019).

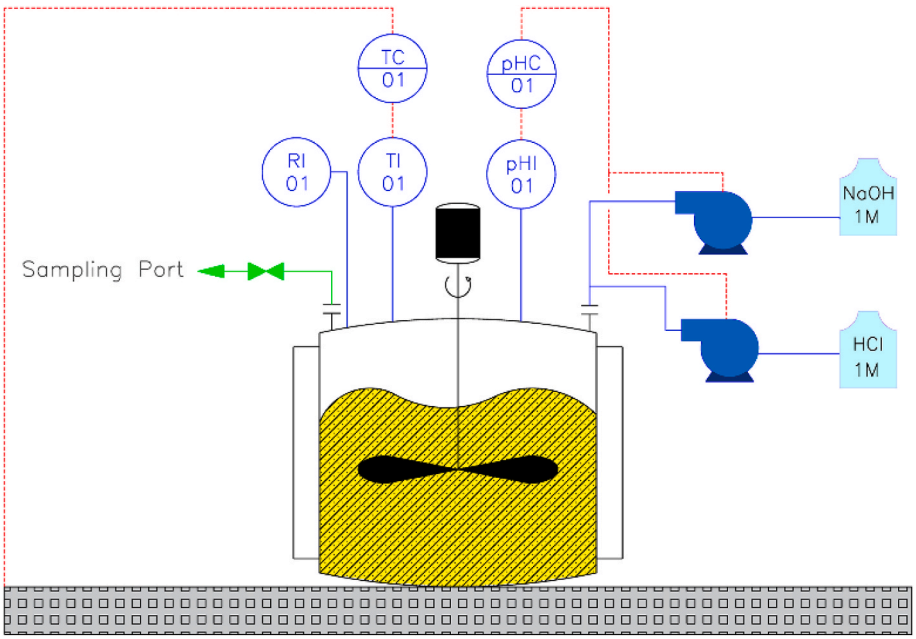

Fig. 2. Experimental set-up used in studies with stirred tank bioreactors continuously fed syngas.

3. Results and discussion

3.1. Bioreactor experiments with continuous syngas feed

3.1.1. MCFAs production by the co-culture of C. aceticum and C. kluyveri

Solvent-producing acetogenic bacteria, fermenting C_1 gases, will generally first produce volatile fatty acids (VFAs), which will then further be converted into solvents (Fernández-Naveira et al., 2019). Besides, higher pH values, close to the optimal for growth, have been found to be beneficial both for growth and acid production in acetogens (Gildemyn et al., 2017). Therefore, the pH was set at 7.5 on starting the bioreactor experiment with *C. aceticum* and it was maintained constant during the lag phase (62 h).

Then, once exponential growth and acetate production were observed, pH regulation was stopped in order to allow acidification and stimulate solventogenesis. The OD_{600nm} and acetate concentration reached then 0.87 and 1 g/L, respectively (Figs. 3 and 4). Thereafter, OD_{600nm} increased exponentially up to a maximum value of 2.1 at pH 7.14 (t = 91 h). In the following days, the pH dropped to 6.58 (t = 134 h), with an acetate concentration of 4.6 g/L at that pH. In order to avoid any possible inhibitory effect, and since the lowest pH supported by *C. aceticum* is not reported in the literature, the pH was adjusted to 6.9 at this point. This new pH value was maintained for 4 days, during which OD_{600nm} slowly declined from 1.97 to 1.70, and the acetate concentration continued to increase steadily up to 9 g/L after 230 h. Since no ethanol production was observed, the pH was decreased to 6.8 and an increase in ethanol concentration was noticed, up to 329 mg/L, at t = 305 h (Figs. 3 and 4). The pH was then further lowered by 0.05 unit intervals in search of a value at which higher ethanol productivity could occur without compromising the biomass concentration. The ethanol concentration reached 3.2 g/L at t = 542 h and pH 6.5; however, the OD_{600nm} dropped significantly from 1.6 to 1.3, so the pH was slightly increased again, to 6.6, to circumvent further biomass decay. Though OD_{600nm} had been gradually increasing again, reaching a maximum of 2.6, at t = 407 h; from this point onwards, it followed a declining trend, a behavior previously reported with this strain at low pH (Arslan et al., 2019). On the other hand, acetate, which had reached a maximum concentration of 15.9 g/L, decreased for the first time to 15.4 g/L, at t = 496 h, while an increase of 0.9 g/L in ethanol concentration was observed at the same time. This indicated that acetate was being converted to ethanol at a faster rate than its *de-novo* synthesis.

Next, at t = 593 h, 160 mL of a *C. kluyveri* culture in early exponential phase was inoculated in the reactor, when the acetate and ethanol

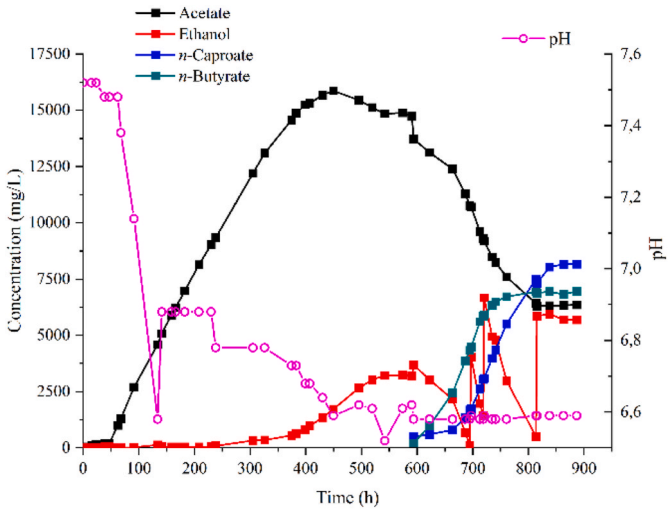

Fig. 3. Metabolites concentrations in the culture broth with the co-culture (*C. aceticum* + *C. kluyveri*) (left axis) and the working pH (right axis) versus time (h) during the 1st bioreactor experiment.

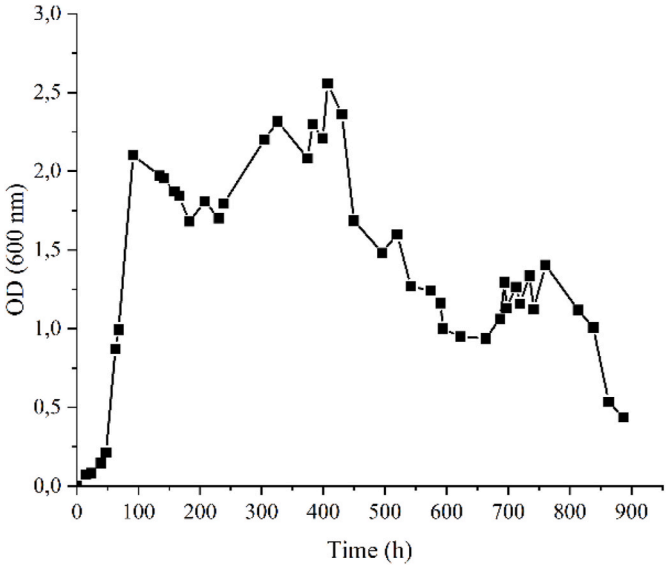

Fig. 4. Optical density profile (λ = 600 nm) versus time (h) in the 1st bioreactor experiment.

concentrations were 14.7 and 3.2 g/L, respectively. It has been shown that *C. kluyveri* is not inhibited at acetate concentrations below 15 g/L (Candry et al., 2018). However, the chain elongation process requires an excess of ethanol over carboxylates and a high ethanol:acetate ratio can promote the production of longer chain fatty acids, such as *n*-caprylate versus *n*-caproate (Kucek et al., 2016). As a consequence of the inoculation of *C. kluyveri*, the ethanol concentration rose to 3.7 g/L, due to its presence in that inoculum, and OD_{600nm} decreased from 1.2 to 1, because of the dilution effect, but then increased to 1.3, at t = 695 h, proving that *C. kluyveri* was efficiently growing. The initial *n*-caproate concentration of 490 mg/L, carried over from the inoculum, rapidly increased to 1.7 g/L, between t = 594 h and 695 h. Simultaneously, the concentration of *n*-butyrate, another product of *C. kluyveri*, increased from 220 mg/L to 4.3 g/L. These bioconversions result from various reactions. On the one hand, acetogens convert C_1 gases from syngas into acetate and ethanol according to **Reactions 1–7** (Diender et al., 2016; Fernández-Naveira et al., 2017).

$$4CO + 2H_2O \rightarrow CH_3COO^- + H^+ + 2CO_2 \quad (1)$$

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O \quad (2)$$

$$2CO + 2H_2 \rightarrow CH_3COO^- + H^+ \quad (3)$$

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \quad (4)$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \quad (5)$$

$$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O \quad (6)$$

$$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2 \quad (7)$$

On the other hand, *C. kluyveri* uses ethanol as the electron donor and acetate as the electron acceptor to produce *n*-butyrate, which can further be elongated to *n*-caproate. The overall reactions, whose stoichiometry depend on the available substrate concentrations, are shown in **Reactions 8** and **9** (Diender et al., 2016).

$$6C_2H_5OH + 4CH_3COO^- \rightarrow 5C_3H_7COO^- + H^+ + 3H_2O + 2H_2 \quad (8)$$

$$6C_2H_5OH + 5C_3H_7COO^- \rightarrow 5C_5H_{11}COO^- + CH_3COO^- + H^+ + 3H_2O + 2H_2 \quad (9)$$

It is therefore difficult to estimate exactly how much ethanol and

acetate are produced by *C. aceticum*. Nevertheless, it can be deduced that the consumption rates of acetate and ethanol by *C. kluyveri* are higher than their production rates by *C. aceticum*. It was even necessary, at t = 695 h, to supply exogenous ethanol (9 mL) upon its depletion. This addition increased the ethanol concentration up to 4 g/L. Owing to the renewed availability of the electron donor, *n*-butyrate and *n*-caproate continued to accumulate via chain elongation, reaching 5.9 and 3 g/L, respectively, at t = 719 h, before adding 12 mL ethanol again. This resulted in an ethanol concentration of 6.7 g/L, which was basically consumed in the following 100 h; and the *n*-caproate concentration (7.5 g/L) was for the first time higher than for *n*-butyrate (6.9 g/L). This means that, for a large part of the experiment, **Reaction 8** was favored over **Reaction 9**, leading to *n*-butyrate accumulation. According to the stoichiometry presented in **Reactions 8** and **9**, the Gibbs free energies (ΔG^0) for these reactions were calculated using the Gibbs free energies of formation of products and reactants (Chase, 1998; Thauer et al., 1977). The ΔG^0 for **Reaction 8** is 51.87 kJ/mol, while the ΔG^0 for **Reaction 9** is −345 kJ/mol, so thermodynamically, **Reaction 9** would be favored. Therefore, the accumulation of *n*-butyrate over *n*-caproate, before t = 760 h, could be explained by kinetic rather than thermodynamic aspects. Also, it is known that increasing the ethanol:acetate molar ratio improves the accumulation of longer chain fatty acids, i.e. *n*-caproate over *n*-butyrate (Kucek et al., 2016), so the lack of an adequate ethanol:acetate ratio could be another reason that explains this fact. Even so, it sounds reasonable to believe that a certain amount of *n*-butyrate has to be formed first, since it is the precursor for the formation of *n*-caproate. Fig. 3 shows that *n*-butyrate accumulation started to slow down when the acetate concentration was around 8 g/L. Above this value, **Reaction 9** was hindered somehow. Finally, 12 mL ethanol (5.8 g/L) was supplied again, though it was not completely exhausted as OD_{600nm} started to drop dramatically to 0.4 in a few hours. The final *n*-caproate, *n*-butyrate, acetate and ethanol concentrations were 8.2, 7.0, 6.4 and 5.7 g/L, respectively. It is worth mentioning that, with the inoculation of *C. kluyveri*, H_2 production was continuously detected, at trace levels, until the end of the experiment. Part of the hydrogen provided by *C. kluyveri* can be used by *C. aceticum*. Also, CO was never detected in any of the gas samples taken from the bioreactor outlet, suggesting that CO was fully utilized by *C. aceticum*.

The behavior of *C. aceticum*, prior to the inoculation of *C. kluyveri*, differs somewhat from other works. For example, Arslan et al. (2019) achieved a maximum ethanol concentration of 5.7 g/L by applying a cyclic pH shifting approach. In that study, the initial growth pH was 8 instead of 7.5 and the highest production of alcohols occurred at pH 6.8–6.9, after natural acidification (Arslan et al., 2019). In contrast, in the present work, although the first appearance of ethanol occurred at pH 6.8, its production rate was slow, while reducing the pH to 6.6 increased this rate. In any case, biomass decline is expectedat these pH levels, when ethanol is produced with basically no ATP generation nor energy conservation (Schuchmann and Müller, 2014).

3.1.2. *n*-Butanol production by the co-culture of *C. aceticum* and *C. kluyveri*

The previously described bioreactor experiment demonstrates that a co-culture of *C. aceticum* and *C. kluyveri* is able to produce *n*-caproate from syngas, at near neutral pH. Furthermore, it shows that solventogenesis in *C. aceticum* took place at high rate at pH close to 6.6. Based on this, a second bioreactor was started up in which the pH was maintained at 6.6 immediately after natural acidification. This was expected to favor earlier ethanol production and avoid excessive accumulation of acetate observed in the previous assay. Therefore, the goal of the present experiment was to study MCFAs accumulation and the behavior of the co-culture adopting this new pH strategy.

Initially, as in the previous experiment, the pH was adjusted to 7.5, before inoculation of *C. aceticum*. After a lag phase of about 2 days, and once OD_{600nm} reached 0.5, pH regulation was stopped allowing natural acidification and accumulation of 563 mg/L acetic acid at that time (Figs. 5 and 6). Consequently, the pH gradually decreased and OD_{600nm} reached its maximum value of 2.6 after 118 h, when the pH was 6.93. The pH continued to drop naturally to 6.62 and then it was maintained at 6.6 after t = 136 h, when the acetate and ethanol concentrations were 3.8 g/L and 242 mg/L, respectively. However, in the previous experiment the pH was adjusted to 6.6 when the acetate and ethanol concentrations had already increased up to 15.7 and 1.3 g/L, respectively. The pH value of 6.6 was first maintained, for the next 89 h, but was then increased again to 6.7 because of the observed biomass decay and OD_{600nm} dropping to 1.3. Furthermore, at this stage, the acetate concentration started to decrease, and ethanol concentration increased. Just before inoculating 160 mL of *C. kluyveri* seed culture into the bioreactor, the concentrations of acetate and ethanol, at t = 310 h, were 3.9 g/L and 1.5 g/L, respectively, corresponding to an ethanol:acetate molar ratio of 0.5, which was indeed higher than the ratio of 0.28 of the previous described experiment, as successfully planned with this new pH-strategy. Nonetheless, at the same time, this led to lower net total concentrations of the electron donor and the electron acceptor than in the previous experiment, due to the earlier pH drop. The inoculation of *C. kluyveri* led to a dilution effect of previously accumulated metabolites and the presence of *n*-butyrate and *n*-caproate, whose concentrations were respectively 235 mg/L and 719 mg/L. At this point, OD_{600nm} started dropping dramatically, attributed to the decay of *C. aceticum*. Several attempts were made in order to try to reactivate the strain by raising the pH again. Although the optimum pH for solventogenesis in *C. aceticum* is slightly below neutral; optimal growth conditions require a more alkaline pH. Conversely, *C. kluyveri*'s optimal activity and growth pH is closer to neutrality. Therefore, at t = 520 h, and only two days after raising the pH from 6.7 to 7.5, the biomass exhibited a sudden growth, with OD_{600nm} increasing from 0.4 to 1.5. At that pH, it was hypothesized that mainly *C. aceticum* had grown, since increases of OD_{600nm} values do not allow to distinguish which organism is growing most. On the other hand, according to the consumption and production profiles, the activity of *C. kluyveri* started 7 days after its inoculation (at t = 480 h), with an increase in *n*-butyrate concentration and ethanol consumption. From this point onwards, the concentration of *n*-butyrate remained higher than *n*-caproate, reaching maximum values of 1.96 g/L and 1.14 g/L, respectively. This behavior was also observed in the previous experiment, especially in the first days after inoculation of *C. kluyveri*, when the ethanol:acetate ratio was lower, though, finally, more *n*-caproate than *n*-butyrate did accumulate, but only at the end of the study, when the ethanol:acetate ratio was higher.

Finally, unexpectedly, considerable amounts of *n*-butanol were first

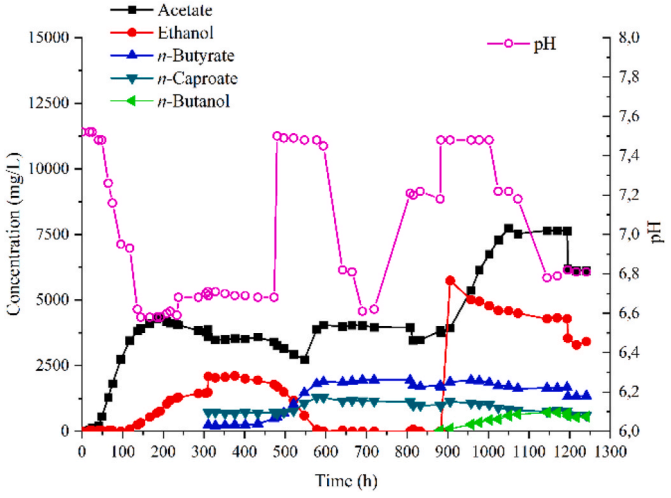

Fig. 5. Metabolites concentrations in the culture broth with the co-culture (*C. aceticum* + *C. kluyveri*) (left axis) and the working pH (right axis) versus time (h) during the 2nd bioreactor experiment.

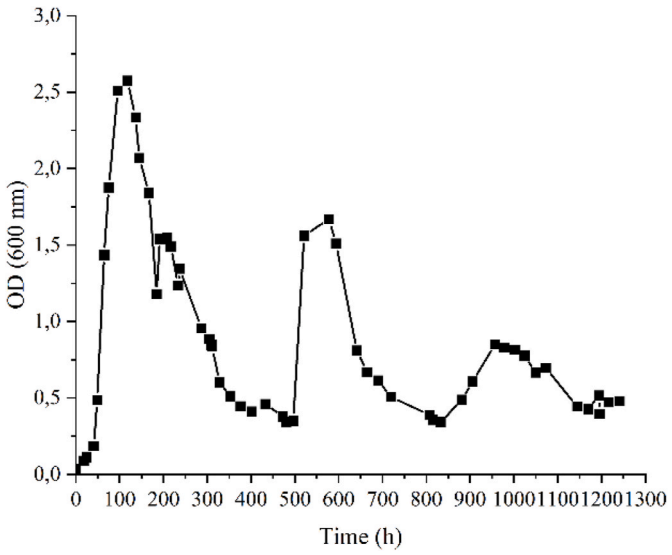

Fig. 6. Optical density profile (λ = 600 nm) versus time (h) in the 2nd bioreactor experiment.

found at t = 900 h, reaching a maximum concentration of 719 mg/L. In the first bioreactor experiment, *n*-butanol production had also been observed after inoculation of *C. kluyveri*, but at concentrations below 200 mg/L. No *n*-hexanol production was found during any of the experiments. To date, *C. aceticum* has never been reported to produce *n*-butanol in pure culture. A slight decrease in the concentration of *n*-butyrate could be observed, concomitant with the appearance of *n*-butanol, indicating that this should be a reduction process from acid towards alcohol using syngas as a source of energy and electrons (reductive power).

This bioconversion of acids to the corresponding alcohols, not only for *n*-butanol, but also for *n*-hexanol and *n*-octanol, has occasionally been reported with other co-cultures as well as with mixed cultures (Diender et al., 2016; Ganigué et al., 2016; Richter et al., 2016). Normally, this process would be favored at lower pH, since the percentage of undissociated *n*-butyric acid, which is the substrate for the reduction, is higher. However, at pH < 7, *C. aceticum* loses part of its activity. Furthermore, the pK_a of the VFAs and MCFAs is in the range of 4.7–4.8 (Lonkar et al., 2016), which means that at pH close to 7, most of the acid will be in the carboxylate form, and *n*-butanol production is not expected to be high under these conditions.

Richter et al. (2016) found considerable amounts of long chain bioalcohols in the condensate of their system using a *C. ljungdahlii* + *C. kluyeri* co-culture. It occurred presumably as a consequence of the reduction, by *C. ljungdahlii*, of carboxylates already formed. Concentrations of *n*-butanol and *n*-hexanol of, respectively, 4.21 g/L (56.8 mM) and 4.73 g/L (46.3 mM), were obtained. Non negligible amounts of *n*-octanol were also found, reaching 0.78 g/L (6 mM). However, the authors state that even if a co-culture in which the strains are compatible in terms of pH is used, and an extraction system is set up to keep the concentration of alcohols low, thus avoiding inhibition, the specificities of long chain bioalcohols would never be high anyway. This is owing to the two competitive processes, i.e., chain elongation and carboxylate reduction.

3.1.3. Strengths and limitations of the system and comparison with other co-cultures

It was deduced, from both experiments, that the two critical parameters for achieving a better stability of the co-culture of *C. aceticum* and *C. kluyveri* and high production of longer chain metabolites are: (a) achieving an adequate ethanol:acetate ratio without the need to add exogenous ethanol; and (b) identifying the best pH that does not compromise the activity of any of the strains.

The challenging task of improving the rate of ethanol production by acetogenic bacteria was recently investigated by Benito-Vaquerizo et al. (2020). In their work, a metabolic model was developed to optimize the production of MCFAs in a syngas-fermenting co-culture of *C. autoethanogenum* and *C. kluyveri*. They found that ethanol production in *C. autoethanogenum* could be increased by blocking acetaldehyde dehydrogenase, formate dehydrogenase (ferredoxin) or the formate transport reaction via proton symport. This would allow an increase in ethanol production of up to 150% when H_2 is used as a second electron source compared to wild-type *C. autoethanogenum* without any genetic modification. Also, it was found that the presence of *C. kluyveri* in co-culture with *C. autoethanogenum* triggers solventogenesis in the acetogen, presumably due to continuous ethanol elimination by *C. kluyveri* (Diender et al., 2019). Seemingly, *C. kluyveri* is considered to produce a stress response in *C. autoethanogenum* and the result is a promotion of the flow of electrons from CO to ethanol, a shift that is apparently controlled by thermodynamics and not by gene expression. In that co-culture, an excess of H_2 supply (120 mmol·L^{-1}·d^{-1}) led to an abrupt shift towards solventogenic activity of *C. autoethanogenum*, finding 45% of the total electrons in the products in the formation of ethanol, in contrast with 20–30% when solely CO is used as substrate.

On the other hand, the pH issue has been previously reported by Ganigué et al. (2016), who concluded that pH was a critical parameter for successfully combining syngas fermentation and chain elongation processes. In this case, the authors suggested a minimum pH range of 4.7–4.8 to allow solventogenesis and growth of *C. kluyveri* at the same time in the mixed culture studied. A similar problem was faced by Diender et al. (2016), who studied a synthetic co-culture consisting of *C. autoethanogenum* and *C. kluyveri* in a single vessel capable of metabolizing CO and syngas. The authors chose an initial fermentation pH of 6, although the functional pH range of such co-culture was 5.5–6.5. Since their system had no pH control, the fact that the pH reached values above 6.5 during the experiment, as a result of solventogenic reactions, led to inhibition of *C. autoethanogenum*. Similarly, Richter et al. (2016) also employed a co-culture of *C. ljungdahlii* and *C. kluyveri* in a continuous syngas fermentation system with in-line product extraction. In this case, the co-culture operated in a rather narrow pH range of 5.7–6.4, obtaining the highest average concentrations of *n*-butyrate and *n*-caproate at pH 6.0. In the system presented in the present study, a similar issue needs to be dealt with. On the one hand, the applied pH range (6.6–7.5) corresponds to the operational pH of *C. kluyveri* (6.0–7.5), however, the limitation is imposed by the minimum pH supported by *C. aceticum* without abruptly losing its activity. A possible alternative to deal with this challenge would be the implementation of a cell recycling unit to maintain the OD_{600nm} at values above 1 (Abubackar et al., 2018). Even so, one of the major benefits of the system presented in this study is precisely the more alkaline pH range used, since the concentration of undissociated acids is lower and therefore the toxicity exerted on the bacteria is also lower. Although the inhibitory effect of *n*-caproate has not been studied in detail in this work, Fig. 3 shows how from approximately 4 g/L (34.4 mM), at t = 740 h, the chain elongation activity starts to cease. Candry et al. (2018) stated that *n*-caproate linearly inhibits the activity of *C. kluyveri*, with an upper limit of 91.3 ± 10.8 mM for a pH range of 7.4–8.2, whereas the toxicity limit observed by Diender et al. (2016) for a *C. kluyveri* + *C. autoethanogenum* co-culture is 12 mM at pH 6. So, this seems reasonable since the pH, when the activity of *C. kluyveri* started to decline, was 6.6 in the first bioreactor experiment.

Table 1 shows the maximum concentrations of MCFAs and bioalcohols reached by co-cultures formed by some acetogen and *C. kluyveri,* reported in the literature. It can be seen that the concentrations of *n*-butyrate and *n*-caproate reported in the present work are the highest. This was also possible thanks to the external, additional, supply of ethanol whenever ethanol produced by *C. aceticum* got exhausted. The data reported by Diender et al. (2016) are concentrations estimated from the Gompertz equation and not the observed ones, since

Table 1
Maximum metabolite concentrations reached and pH ranges used in the literature with pure co-cultures of acetogens and chain elongators (*C. kluyveri*).

Microorganisms	Substrate	Operation Mode	pH range	Fatty acids (g/L)		Alcohols (g/L)			Reference
				C_4	C_6	C_4	C_6	C_8	
C. aceticum + *C. kluyveri*	syngas + ethanol	STBRs	6.6–7.5	7.0	8.2	0.72	–	–	This study
C. autoethanogenum + *C. kluyveri*	syngas/CO	bottle study	6.0–7.2	2.27 ± 0.02	1.16 ± 0.03	0.43 ± 0.01	0.41 ± 0.02	–	(Diender et al., 2016)[a]
C. autoethanogenum + *C. kluyveri*	syngas	CSTRs	6.2	0.63	1.63	–	–	–	(Diender et al., 2019)[b]
C. ljungdahlii + *C. kluyveri*	syngas	continuous system + in-line product extraction	5.7–6.4	2.7	1.3	4.2	4.7	0.78	(Richter et al., 2016)[c]

Abbreviations: STBRs: stirred-tank bioreactors; CSTRs: continuous stirred-tank reactors.
[a,b,c] Calculations for the conversion of units from mM to g/L were done using the following molecular weights (g/mol): 88.11 *n*-butyrate, 116.16 *n*-caproate, 74.12 *n*-butanol, 102.16 *n*-hexanol, and 130.23 *n*-octanol.

both *n*-butyrate and *n*-caproate are involved in other reactions (i.e. formation of *n*-caproate from *n*-butyrate and conversion to bioalcohols).

3.2. Evidence of n-butyrate reduction to n-butanol in C. aceticum

Although only few studies have reported on the fermentation of CO and syngas to MCFAs by co-cultures of acetogenic and chain elongating organisms, it is not unusual to end-up detecting higher alcohols besides MCFAs in such assays, as described before. The potential of some carboxydotrophic bacteria to convert different fatty acids into their corresponding alcohols in presence of syngas has been demonstrated (Perez et al., 2013), so it is reasonable to consider that in these co-cultures the acetogenic strain is responsible for such bioconversion. A recent study reported that genes responsible for the production of *n*-butanol from acetyl-CoA were detected in *C. aceticum*, though the production of *n*-butanol in pure cultures of that species has never been reported so far (Song et al., 2015). These experiments provide clear experimental evidence that the acetogen *C. aceticum* can produce *n*-butanol from *n*-butyrate in pure culture. The data are summarized and plotted, respectively, in Table 2 and Fig. 7. After inoculation of the strain, no biomass growth was observed for almost two weeks, resulting in a lag phase of 332 h (data not shown). This suggests that perhaps *n*-butyrate contained in the medium may have had some inhibitory effect on the strain. Nevertheless, the acetate concentration had been slowly increasing from its original concentration of 187 mg/L to 405 mg/L, within the first 6 days. Then, once significant bacterial growth started, a sudden increase in acetate concentration was observed, reaching its maximal concentration of 1.41 g/L, after 403 h, and with a concomitant fast pH decrease. The maximum *n*-butanol concentration reached was 951 mg/L. According to a mass balance calculation, the amount *n*-butyrate metabolized by *C. aceticum* and reduced to *n*-butanol was 96%, clearly demonstrating that all that *n*-butyrate must have been transformed and reduced to the corresponding alcohol. On the other hand, the highest *n*-butanol production rate occurred when the pH was close to 7; however, since it was not possible to control the pH inside the bottles, the effect of pH on *n*-butanol production could not be thoroughly investigated in this study. Furthermore, a final ethanol concentration of 234 mg/L (t = 403 h) was also detected, which resulted from solventogenesis and conversion of CO to ethanol, as such reaction was recently proven to be possible in *C. aceticum* at a pH close to 7 (Arslan et al., 2019).

Therefore, in the above-described co-culture fermentation, it can be ascertained that *C. kluyveri* is in charge of elongating acetic acid, produced by *C. aceticum*, into *n*-butyric acid, which is later converted to *n*-butanol by the acetogenic strain. In the co-culture of this work, the biological reduction of *n*-butyrate by *C. aceticum* did not exceed 49%, which was possibly due to the lower concentration of undissociated *n*-butyric acid at the working pH range (6.99–7.77) in comparison with more acidic conditions, as explained by Richter et al. (2016) for *C. ljungdahlii*. Thus, although those bacteria seem not to have the enzymatic machinery to convert C_1 gases to *n*-butyrate, they are able to convert that acid into *n*-butanol.

4. Conclusions

This research demonstrated the suitability to combine syngas fermentation and chain elongation technologies in one single bioreactor to produce MCFAs and bioalcohols. For this purpose, a co-culture consisting of *C. aceticum* (acetogen) and *C. kluyveri* (chain elongating microorganism) was successfully used in a pH range of 6.6–7.5. This work used *C. aceticum* for the first time as a new acetogenic bacterium able to co-exist with *C. kluyveri* at pH values close to neutrality, which had never been tested before, thus contributing to progress beyond the state of the art. All previous co-cultures of this type, with other acetogens, reported in the literature, operated at lower pH ranges. The present system is more advantageous in that respect, as the proportion of

Table 2
Experimental conditions of the bottle assay with a pure culture of *C. aceticum* grown on CO and in the presence of *n*-butyrate.

$[n\text{-Butyrate}]_{in}$	$[n\text{-Butyrate}]_{end}$	$[n\text{-BuOH}]_{max}$	$[\text{Acetate}]_{max}$	pH_{in}	pH_{end}
mg/L	mg/L	mg/L	mg/L		
2319	1135	951	1417	7.68	6.99

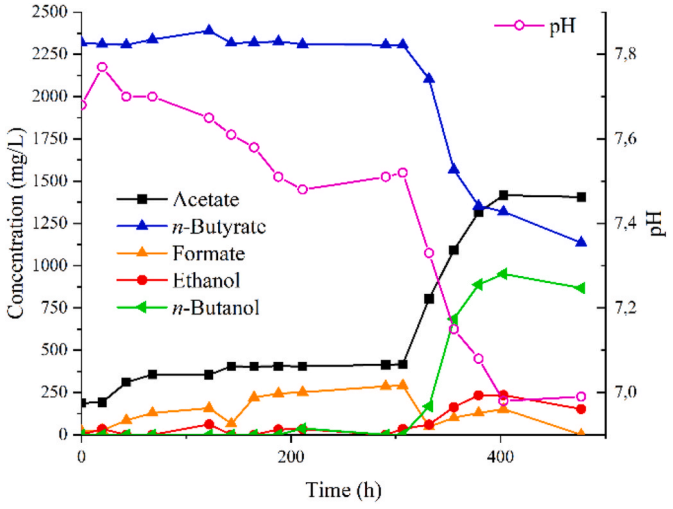

Fig. 7. Metabolites production by *C. aceticum* grown on CO and in presence of *n*-butyrate.

carboxylates is higher, while they are more soluble and exert a lower inhibitory effect on the microorganisms, thus improving the productivity of MCFAs. However, two main limitations exist, i.e., the ethanol: acetate ratio achieved before inoculation of *C. kluyveri*, which was lower than 1; and the drop in *C. aceticum* activity at pH levels close to the optimum growth pH of *C. kluyveri*. Therefore, these would be the key factors to be optimized in future research. The implementation of a cell recycling unit to help maintain stable OD_{600nm} values, changing the pH strategy or genetic engineering are some aspects that could be considered as well. Besides, other aspects that would be worth being further studied are the implementation of an *n*-caproate removal system, which avoids inhibition and stimulates the production of MCFAs; the elucidation of the optimal conditions for *n*-butanol production; and testing additional experimental configurations that improve mass transfer.

In the co-culture bioreactor experiments, the maximum concentration of *n*-caproate and *n*-butyrate were, respectively, 8.2 and 7.0 g/L, using exogenous ethanol as an additional source of electrons at some point, besides the naturally produced alcohol. Also, *n*-butanol was detected in the culture broth, at concentrations up to 719 mg/L. On the other hand, the potential of *C. aceticum* to reduce *n*-butyrate to *n*-butanol, in pure culture, using CO was demonstrated on an experimental basis for the first time, producing up to 951 mg/L *n*-butanol in a batch bottle assay. These facts suggest that in this type of co-culture, the acetogen takes care of the metabolic reduction of the carboxylates, previously formed by *C. kluyveri*, to the corresponding alcohols.

To sum up, this approach is a sustainable alternative for the bioproduction of commercially interesting chemicals and biofuels from gaseous emissions from diverse industries (e.g., steel industries) at ambient temperature and atmospheric pressure, and with flexible ratios of the gaseous compounds.

Credit author statement

Carla Fernández-Blanco: Data curation; Experimental work; Writing and revising the manuscript. María C. Veiga: Resources; Supervision; Revising the manuscript; Funding acquisition. Christian Kennes: Conceptualization; Validation; Supervision; Collaboration in writing and revising the manuscript; 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.

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References

Abubackar, H.N., Veiga, M.C., Kennes, C., 2015. Carbon monoxide fermentation to ethanol by *Clostridium autoethanogenum* in a bioreactor with no accumulation of acetic acid. Bioresour. Technol. 186, 122–127. https://doi.org/10.1016/j.biortech.2015.02.113.

Abubackar, H.N., Veiga, M.C., Kennes, C., 2018. Production of acids and alcohols from syngas in a two-stage continuous fermentation process. Bioresour. Technol. 253, 227–234. https://doi.org/10.1016/j.biortech.2018.01.026.

Arslan, K., Abubackar, H.N., Veiga, M.C., Kennes, C., 2019. Solventogenesis in *Clostridium aceticum* producing high concentrations of ethanol from syngas. Bioresour. Technol. 292, 121941 https://doi.org/10.1016/j.biortech.2019.121941.

Bengelsdorf, F.R., Straub, M., Dürre, P., 2013. Bacterial synthesis gas (syngas) fermentation. Environ. Technol. 34, 1639–1651. https://doi.org/10.1080/09593330.2013.827747.

Benito-Vaquerizo, S., Diender, M., Parera Olm, I., Martins dos Santos, V.A.P., Schaap, P. J., Sousa, D.Z., Suarez-Diez, M., 2020. Modeling a co-culture of *Clostridium autoethanogenum* and *Clostridium kluyveri* to increase syngas conversion to medium-chain fatty-acids. Comput. Struct. Biotechnol. J. 18, 3255–3266. https://doi.org/10.1016/j.csbj.2020.10.003.

Candry, P., Van Daele, T., Denis, K., Amerlinck, Y., Andersen, S.J., Ganigué, R., Arends, J. B.A., Nopens, I., Rabaey, K., 2018. A novel high-throughput method for kinetic characterisation of anaerobic bioproduction strains, applied to *Clostridium kluyveri*. Sci. Rep. 8, 9724. https://doi.org/10.1038/s41598-018-27594-9.

Cavalcante, W. de A., Leitão, R.C., Gehring, T.A., Angenent, L.T., Santaella, S.T., 2017. Anaerobic fermentation for *n*-caproic acid production: a review. Process Biochem. 54, 106–119. https://doi.org/10.1016/j.procbio.2016.12.024.

Chase Jr., M.W., 1998. NIST-JANAF thermochemical tables. J. Phys. Chem. Ref. Data 9, 1–1951 fourth ed.

Diender, M., Parera Olm, I., Gelderloos, M., Koehorst, J.J., Schaap, P.J., Stams, A.J.M., Sousa, D.Z., 2019. Metabolic shift induced by synthetic co-cultivation promotes high yield of chain elongated acids from syngas. Sci. Rep. 9, 82. https://doi.org/10.1038/s41598-019-54445-y.

Diender, M., Parera Olm, I., Sousa, D.Z., 2021. Synthetic co-cultures: novel avenues for bio-based processes. Curr. Opin. Biotechnol. 67, 72–79. https://doi.org/10.1016/j.copbio.2021.01.006.

Diender, M., Stams, A.J.M., Sousa, D.Z., 2016. Production of medium-chain fatty acids and higher alcohols by a synthetic co-culture grown on carbon monoxide or syngas. Biotechnol. Biofuels 9, 1–11. https://doi.org/10.1186/s13068-016-0495-0.

European Commision, 2021. EU Reference Scenario 2020: Energy, Transport and GHG Emissions: Trends to 2050. https://doi.org/10.2833/35750.

Fernández-Naveira, Á., Abubackar, H.N., Veiga, M.C., Kennes, C., 2016. Efficient butanol-ethanol (B-E) production from carbon monoxide fermentation by *Clostridium carboxidivorans*. Appl. Microbiol. Biotechnol. 100, 3361–3370. https://doi.org/10.1007/s00253-015-7238-1.

Fernández-Naveira, Á., Veiga, M.C., Kennes, C., 2017. H-B-E (hexanol-butanol-ethanol) fermentation for the production of higher alcohols from syngas/waste gas. J. Chem. Technol. Biotechnol. 92, 712–731. https://doi.org/10.1002/jctb.5194.

Fernández-Naveira, Á., Veiga, M.C., Kennes, C., 2019. Selective anaerobic fermentation of syngas into either C_2-C_6 organic acids or ethanol and higher alcohols. Bioresour. Technol. 280, 387–395. https://doi.org/10.1016/j.biortech.2019.02.018.

Ganigué, R., Sánchez-Paredes, P., Bañeras, L., Colprim, J., 2016. Low fermentation pH is a trigger to alcohol production, but a killer to chain elongation. Front. Microbiol. 7, 702. https://doi.org/10.3389/fmicb.2016.00702.

Gervajio, G.C., 2005. Bailey's industrial oil and fat products. In: Shahidi, F. (Ed.), Fatty Acids and Derivatives from Coconut Oil. J. Wiley & Sons, Inc., pp. 1–55. https://doi.org/10.1002/047167849x.bio039.pub2

Gildemyn, S., Molitor, B., Usack, J.G., Nguyen, M., Rabaey, K., Angenent, L.T., 2017. Upgrading syngas fermentation effluent using *Clostridium kluyveri* in a continuous

fermentation. Biotechnol. Biofuels 10, 1–15. https://doi.org/10.1186/s13068-017-0764-6.

Grootscholten, T.I.M., Steinbusch, K.J.J., Hamelers, H.V.M., Buisman, C.J.N., 2013. Chain elongation of acetate and ethanol in an upflow anaerobic filter for high rate MCFA production. Bioresour. Technol. 135, 440–445. https://doi.org/10.1016/j.biortech.2012.10.165.

Han, W., He, P., Shao, L., Lü, F., 2019. Road to full bioconversion of biowaste to biochemicals centering on chain elongation: a mini review. J. Environ. Sci. 86, 50–64. https://doi.org/10.1016/j.jes.2019.05.018.

Henstra, A.M., Sipma, J., Rinzema, A., Stams, A.J., 2007. Microbiology of synthesis gas fermentation for biofuel production. Curr. Opin. Biotechnol. 18, 200–206. https://doi.org/10.1016/j.copbio.2007.03.008.

Iglesias-Iglesias, R., Portela-Grandío, A., Treu, L., Campanaro, S., Kennes, C., Veiga, M.C., 2021. Co-digestion of cheese whey with sewage sludge for caproic acid production: role of microbiome and polyhydroxyalkanoates potential production. Bioresour. Technol. 337, 125388. https://doi.org/10.1016/j.biortech.2021.125388.

Kennes, D., Abubackar, H.N., Diaz, M., Veiga, M.C., Kennes, C., 2016. Bioethanol production from biomass: carbohydrate vs syngas fermentation. J. Chem. Technol. Biotechnol. 91, 304–317. https://doi.org/10.1002/jctb.4842.

Kucek, L.A., Spirito, C.M., Angenent, L.T., 2016. High *n*-caprylate productivities and specificities from dilute ethanol and acetate: chain elongation with microbiomes to upgrade products from syngas fermentation. Energy Environ. Sci. 9, 3482–3494. https://doi.org/10.1039/c6ee01487a.

Lindley, N.D., Loubiere, P., Pacaud, S., Mariotto, C., Goma, G., 1987. Novel products of the acidogenic fermentation of methanol during growth of *Eubacterium limosum* in the presence of high concentrations of organic acids. J. Gen. Microbiol. 133, 3557–3563. https://doi.org/10.1099/00221287-133-12-3557.

Lonkar, S., Fu, Z., Holtzapple, M., 2016. Optimum alcohol concentration for chain elongation in mixed-culture fermentation of cellulosic substrate. Biotechnol. Bioeng. 113, 2597–2604. https://doi.org/10.1002/bit.26024.

Mayer, A., Schädler, T., Trunz, S., Stelzer, T., Weuster-Botz, D., 2018. Carbon monoxide conversion with *Clostridium aceticum*. Biotechnol. Bioeng. 115, 2740–2750. https://doi.org/10.1002/bit.26808.

Perez, J.M., Richter, H., Loftus, S.E., Angenent, L.T., 2013. Biocatalytic reduction of short-chain carboxylic acids into their corresponding alcohols with syngas fermentation. Biotechnol. Bioeng. 110, 1066–1077. https://doi.org/10.1002/bit.24786.

Phillips, J.R., Atiyeh, H.K., Tanner, R.S., Torres, J.R., Saxena, J., Wilkins, M.R., Huhnke, R.L., 2015. Butanol and hexanol production in *Clostridium carboxidivorans* syngas fermentation: medium development and culture techniques. *B*ioresour. Technol. 190, 114–121. https://doi.org/10.1016/j.biortech.2015.04.043.

Reddy, M. Venkateswar, Kumar, G., Mohanakrishna, G., Shobana, S., Al-Raoush, R.I., 2020. Review on the production of medium and small chain fatty acids through waste valorization and CO_2 fixation. Bioresour. Technol. 309, 123400. https://doi.org/10.1016/j.biortech.2020.123400.

Reddy, Motakatla Venkateswar, ElMekawy, A., Pant, D., 2018a. Bioelectrochemical synthesis of caproate through chain elongation as a complementary technology to anaerobic digestion. Biofuels, Bioprod. Biorefining 12, 966–977. https://doi.org/10.1002/bbb.1924.

Reddy, Motakatla Venkateswar, Hayashi, S., Choi, D., Cho, H., Chang, Y.C., 2018b. Short chain and medium chain fatty acids production using food waste under non-augmented and bio-augmented conditions. J. Clean. Prod. 176, 645–653. https://doi.org/10.1016/j.jclepro.2017.12.166.

Richter, H., Molitor, B., Diender, M., Sousa, D.Z., Angenent, L.T., 2016. A narrow pH range supports butanol, hexanol, and octanol production from syngas in a continuous co-culture of *Clostridium ljungdahlii* and *Clostridium kluyveri* with in-line product extraction. Front. Microbiol. 7, 1773. https://doi.org/10.3389/fmicb.2016.01773.

San-Valero, P., Abubackar, H.N., Veiga, M.C., Kennes, C., 2020. Effect of pH, yeast extract and inorganic carbon on chain elongation for hexanoic acid production. Bioresour. Technol. 300, 122659. https://doi.org/10.1016/j.biortech.2019.122659.

Sarria, S., Kruyer, N.S., Peralta-Yahya, P., 2017. Microbial synthesis of medium-chain chemicals from renewables. Nat. Biotechnol. 35, 1158–1166. https://doi.org/10.1038/nbt.4022.

Schuchmann, K., Müller, V., 2014. Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. Nat. Rev. Microbiol. 12, 809–821. https://doi.org/10.1038/nrmicro3365.

Siles, J.Á., Martín, M.D.L.Á., Martín, A., Raposo, F., Borja, R., 2007. Anaerobic digestion of wastewater derived from the pressing of orange peel generated in orange juice production. J. Agric. Food Chem. 55, 1905–1914. https://doi.org/10.1021/jf0630623.

Song, Y., Hwang, S., Cho, B.-K., 2015. Draft genome sequence of *Clostridium aceticum* DSM 1496, a potential butanol producer through syngas fermentation. Genome Announc. 3, e00258. https://doi.org/10.1128/genomeA.00258-15.

Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J.M., Angenent, L.T., 2014. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Curr. Opin. Biotechnol. 27, 115–122. https://doi.org/10.1016/j.copbio.2014.01.003.

Steinbusch, K.J.J., Hamelers, H.V.M., Plugge, C.M., Buisman, C.J.N., 2011. Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass. Energy Environ. Sci. 4, 216–224. https://doi.org/10.1039/c0ee00282h.

Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41, 100–180.