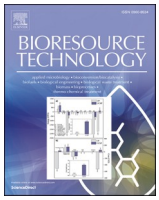
Bioresource Technology 394 (2024) 130192

Contents lists available at ScienceDirect

Bioresource Technology

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



Carbon dioxide as key player in chain elongation and growth of *Clostridium kluyveri*: Insights from batch and bioreactor studies

Carla Fernández-Blanco, María C. Veiga, Christian Kennes *

Chemical Engineering Laboratory, Faculty of Sciences and Interdisciplinary Centre of Chemistry and Biology, Centro Interdisciplinar de Química e Bioloxía (CICA), BIOENGIN Group, University of A Coruña, E-15008-A Coruña, Spain

HIGHLIGHTS

- *C. kluyveri* managed to grow in a medium with different levels of available CO_2.
- 11.0 g/L C_6 was reached from acetate and ethanol in a bioreactor with CO_2 supply.
- There is a direct correlation between CO_2 concentration and *C. kluyveri*'s growth.
- CO_2 uptake by *C. kluyveri* at bioreactor level was demonstrated.

ARTICLE INFO

Keywords:
Clostridium kluyveri
Carbon dioxide
Ethanol-based chain elongation
n-Butyrate
n-Caproate

ABSTRACT

Chain elongation technology allows medium-chain fatty acids (MCFAs) production as an alternative to fossil resources. *Clostridium kluyveri* generates *n*-caproate primarily from ethanol and acetate, presumably requiring CO_2 for growth. Here, the impact of CO_2 on *C. kluyveri* was explored. Bottle studies revealed the bacterium's adaptability to low CO_2 levels, even in conditions with minimal dissolved $NaHCO_3$ (0.0003 M) and unfavorable pH (below 6) under 1 bar CO_2. Bioreactor investigations demonstrated a direct correlation between CO_2 availability and bacterial growth. The highest *n*-caproate production (11.0 g/L) with 90.1 % selectivity was achieved in a bioreactor with continuous CO_2 supply at 3 mL/min. Additional bottle experiments pressurized with 1 bar CO_2 and varying ethanol:acetate ratios (1:1, 2:1, 4:1) also confirmed CO_2 consumption by *C. kluyveri*. However, increasing the ethanol:acetate ratio did not enhance *n*-caproate selectivity, likely due to overly acidic pH conditions. These findings provide insights into chain-elongators responses under diverse conditions.

1. Introduction

Chain elongation technology has emerged in recent years as a more sustainable way to produce MCFAs such as *n*-caproate and *n*-caprylate (Angenent et al., 2016), which, among other applications, can be used for aviation fuels, lubricants, detergents, or antimicrobial agents (Ahn et al., 2023; Harvey and Meylemans, 2014). It is a bioprocess catalyzed by an anaerobic bacterium, commonly *Clostridium kluyveri*, where an electron donor (e.g., ethanol) and an electron acceptor (e.g., acetate) elongate the hydrocarbon chain by 2 carbon units per cycle (Spirito et al., 2014). In this way, it is possible to increase the added value of the product and its hydrophobicity, thus facilitating its separation from the culture broth compared to the substrates (Grootscholten et al., 2013). At present, most of the studies reported in this field are based on mixed cultures (Sakarika et al., 2023; Tang et al., 2023; Wang et al., 2023; Yu et al., 2023) and hardly any assessed the behavior of pure chain elongating microorganisms. However, there are a few studies available in the literature that evaluate the growth and MCFAs production performance in *C. kluyveri* in serum bottles and at larger bioreactor scale. These studies tested the strain's ability to utilize different electron acceptors, such as acetate or *n*-butyrate (San-Valero et al., 2019), the effect of pH and yeast extract (San-Valero et al., 2020), different ethanol:acetate molar ratios (Yin et al., 2017), its behavior in co-culture with acetogenic (Diender et al., 2016, 2019; Fernández-Blanco et al., 2022; Richter et al., 2016) and propionigenic (Parera Olm and Sousa, 2023) strains, and even its use in continuous fermentations with in-line product extraction (Gildemyn et al., 2017).

In the middle of the past century, it was revealed that CO_2 uptake by

* Corresponding author.
E-mail address: Kennes@udc.es (C. Kennes).

https://doi.org/10.1016/j.biortech.2023.130192
Received 31 October 2023; Received in revised form 7 December 2023; Accepted 8 December 2023
Available online 9 December 2023

C. kluyveri is related to amino acids synthesis (Andrew and Morris, 1965; Tomlinson, 1954a,b; Tomlinson and Barker, 1954). In turn, this consumption is directly correlated with growth of the strain (Tomlinson and Barker, 1954). In a medium with yeast extract, it was observed that bacterial growth increased proportionally with carbonate concentration up to approximately 0.003 M. Also, in a synthetic medium, it was determined through ^{14}C labeling that approximately 25 % of the cellular carbon originated from CO_2, while 11 % was derived from the carboxylic carbon found in acetate. Likewise, during the growth phase, a minor fraction of CO_2 is assimilated into the acetate's methyl group, and less than 1 % of the acetate's carboxylic carbon is released as CO_2. These findings were corroborated by another publication from the same year (Tomlinson, 1954a), which demonstrated that by utilizing labeled $^{14}CO_2$ as a substrate, the carboxylic carbon atoms of the five synthesized amino acids (i.e., alanine, serine, glycine, aspartic acid, and threonine), were primarily, if not exclusively, sourced from CO_2. Fourteen years after the previous papers were published, it was concluded that C_1 units in *C. kluyveri* could be formed through CO_2 reduction and to a lesser extent, serine cleavage (Jungermann et al., 1968). CO_2 was also noted to be integrated into the S-methyl group of methionine, as well as the positions 2 and 8 of purines and potential pathways for carbon incorporation into reduced one-carbon units were discussed by Decker et al. (1967).

The renewed recent biotechnological interest in producing MCFAs in a more sustainable way, combined with the above findings, make studies on the effect of CO_2 on the growth of *C. kluyveri* interesting at bioreactor scale. A decade ago, Zhang et al. (2013) demonstrated for the first time the *in-situ* formation of MCFAs from H_2 and CO_2 in a hollow-fiber membrane biofilm reactor with a mixed culture. In their work, the authors suggest that *C. kluyveri* is a microorganism capable of utilizing acetate, H_2 and CO_2 to yield MCFAs. Specifically, the highest concentrations of *n*-caproate and *n*-caprylate achieved in such a system were 0.98 g/L and 0.42 g/L, respectively. In a much more recent study, researchers explored the influence of CO_2 on the ethanol-based chain elongation (Huo et al., 2023). They suggested that CO_2 plays a role as an initiator of chain elongation. Specifically, they observed that low CO_2 doses (i.e., 0.5 $L_{CO2}/(L \cdot d)$) promoted the production of *n*-caproate and the relative abundance of *C. kluyveri* on the microbiome, while higher CO_2 doses (i.e., 2.0 $L_{CO2}/(L \cdot d)$) not only led to increased excessive ethanol oxidation (EEO), as stated before by Roghair et al. (2018a), but also reduced *n*-caproate yield from 28.1 % to 41.8 %, depending on the electron acceptors used. Furthermore, isotopic studies using $^{13}CO_2$ unequivocally reveal the assimilation of carbon from CO_2 into microbial biomass and extracellular products (Huo et al., 2023). On the other hand, in the framework of microbial electrosynthesis (MES), CO_2 has been used together with different electron donors such as ethanol (Li et al., 2022) and CO (Chu et al., 2020) to produce MCFAs over the last few years.

Besides, the presence of CO_2 limits the partial pressure of H_2 (pH_2). An excessively high pH_2 could lead to the reduction of acids to their respective alcohols, making it advisable to maintain an appropriate H_2:CO_2 ratio. Weimer and Kohn (2016) established this optimal ratio at 1 bar of H_2 and 0.3 bar of CO_2 to favor the production of longer chain fatty acids. It is also crucial to take into account that when dissolving CO_2 in the fermentation medium, this leads to acidification. This should be approached with caution, especially when dealing with a pH-sensitive strain like *C. kluyveri*, which thrives at a pH close to neutral for optimal growth (Barker and Taha, 1941). Therefore, the aim of this work was to study the effect of $NaHCO_3/CO_2$ on the chain elongation process with pure cultures of *C. kluyveri* in batch studies at bioreactor scale. A first study in bottles with different concentrations of available CO_2 was set up to test the effectiveness on the activity of *C. kluyveri*, followed by three studies in bioreactors, two of them with continuous CO_2 feeding and another one with an initial dissolved $NaHCO_3$ concentration of 0.03 M. Also, an additional assay was performed in bottles pressurized with 1 bar CO_2 evaluating the chain elongation performance with different ethanol:acetate ratios.

2. Material and methods

2.1. Microorganism and culture media

C. kluyveri DSM555 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The composition of the growth medium, per liter distilled water, was as follows: (a) carbon substrates, 13.6 g Na-acetate·$3H_2O$, and 21 mL ethanol (96 %); (b) growth and metabolic rate promoters, 0.31 g K_2HPO_4, 0.23 g KH_2PO_4, 0.25 g NH_4Cl, 0.20 g $MgSO_4 \cdot 7H_2O$, 2.5 g $NaHCO_3$, and 1 g yeast extract. Also, 1 mL SL-10 trace metal solution, 1 mL selenite-tungstate solution, 0.5 mL Na-resazurin (0.1 % w/v), 0.25 g L-cysteine-HCl·$3H_2O$, 0.25 g $Na_2S \cdot 9H_2O$, and 1 mL of the seven vitamins solution were incorporated in the medium.

The makeup of the SL-10 trace metal solution included the following components (per liter distilled water): 10 mL HCl (25 %), 1.5 g $FeCl_2 \cdot 4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2 \cdot 2H_2O$, 2 mg $CuCl_2 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, and 36 mg $Na_2MoO_4 \cdot 2H_2O$. The selenite-tungstate solution contained the following compounds (per liter deionized water): 0.5 g NaOH, 3 mg $Na_2SeO_3 \cdot H_2O$, and 4 mg $Na_2WO_4 \cdot 2H_2O$. The seven vitamins solution consisted of (per liter deionized water): 100 mg vitamin B_{12}, 80 mg *p*-aminobenzoic acid, 20 mg D-(+)-biotin, 200 mg nicotinic acid, 100 mg calcium pantothenate, 300 mg pyroxidine hydrochloride, and 200 mg thiamine-HCl·$2H_2O$.

After completely dissolving all components in 40 mL distilled water, the serum bottles were purged with pure N_2 for six minutes (i.e., three minutes in the liquid phase and three minutes in the headspace), while simultaneously adjusting the pH to 6.8 using either 2 M HCl or 2 M NaOH. Following this, 0.83 mL ethanol (96 %) was added just before sealing the bottles with butyl rubber septa and aluminum crimps. Finally, the bottles were autoclaved at 121 °C for 20 min and incubated at 37 °C under constant shaking conditions at 100 rpm.

2.2. Bottle studies

Sterilized bottles containing the aforementioned culture medium for *C. kluyveri* were pressurized, if applicable, with 1 bar CO_2 using a manometer (i.e., pressure relative to atmospheric pressure). Then, all bottles were inoculated (10 % v/v) with a seed culture of *C. kluyveri* in exponential growth phase and incubated at 37 °C and 100 rpm. Every day, a 2 mL liquid sample was taken for metabolites analysis, optical density measurements at 600 nm (OD_{600nm}) and pH measurement, together with another 1 mL gaseous sample for CO_2 and H_2 measurement. All samples were taken through two 3-way valves (1 for liquids and 1 for gases) stuck through a needle into the septum of the bottles. All bottle studies were performed in triplicate unless otherwise specified. For the experiment aiming to study the effect of different $NaHCO_3/CO_2$ concentrations, the ethanol:acetate molar ratio was always 3.5:1. However, for the study on the effect of the ethanol:acetate molar ratio, other, 1:1, 2:1 and 4:1 ratios were analyzed, keeping the acetate concentration fixed.

2.3. Bioreactor studies

The three bioreactors utilized in the experiments (i.e., bioreactors 1, 2, and 3) were 2 L Eppendorf BIOFLO 120 automated bioreactors with a working volume of 1.2 L, maintained at a constant temperature of 37 °C (Eppendorf AG, Hamburg, Germany). In all bioreactors, the medium for *C. kluyveri* described above, excluding L-cysteine-HCl, $Na_2S \cdot 9H_2O$, ethanol, and vitamins, was introduced into the vessel and then subjected to autoclaving 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 1.5 h while it was being stirred at 250 rpm. Subsequently, the gas supply was stopped, and for bioreactors 2 and 3, a continuous supply of pure CO_2 at a rate of 3 mL/min, monitored using a

mass gas flow meter (Aalborg GFC 17, Müllheim, Germany), was initiated. The flow units displayed by the GFC 17 were in mL/min. L-cysteine and $Na_2S{\cdot}9H_2O$ were then anaerobically and aseptically added, and for bioreactor 3, 4.8 mL of a 15 % (w/v) titanium citrate solution, another reducing agent, was also introduced. The pH was then adjusted to 6.8 through the addition of HCl and/or NaOH using peristaltic pumps, and ethanol was also added. Finally, vitamins were supplied, and the bioreactor was inoculated with a seed culture of *C. kluyveri* (10 % v/v) in the early exponential growth phase. The pH value was continuously monitored on-line and adjusted when it deviated from the set point by ± 0.02 unit. In all experiments, the gas flow rate at the reactor outlet was measured using a MilliGasCounter (Ritter, Germany).

On a daily basis, a minimum of 4 mL liquid samples were taken from the bioreactors using a sterile syringe for OD_{600nm} measurements and metabolites analysis. Similarly, duplicates of 1 mL gas samples were also withdrawn from the bioreactor outlet to determine the concentration of H_2 and CO_2.

2.4. Analytical methods

Bacterial growth was assessed by measuring the OD_{600nm} in unfiltered samples and using a cuvette with a path length of 10 mm. This measurement was carried out using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain).

The concentrations of all metabolites were determined through High Performance Liquid Chromatography (HPLC) using an HP1100 system from Agilent Co., Palo Alto, USA. The HPLC system was equipped with an Agilent Hi-Plex Column (300 × 7.7 mm) and featured both a diode array detector (DAD) and a refractive index detector (RID) maintained at 50 °C. A 0.005 M H_2SO_4 solution served as the mobile phase, with a flow rate of 0.80 mL/min, and the column was kept at a constant temperature of 45 °C. Prior to analysis, all liquid samples were filtered through 0.22 μm PTFE syringe filters.

H_2 and CO_2 concentrations were determined by gas chromatography (GC) (Agilent Technologies, Madrid, Spain). The GC for H_2 measurements was equipped with a thermal conductivity detector (TCD) and a 15-meter HP-PLOT Molecular Sieve 5 A column (0.53 mm ID, 50 μm film thickness). The GC equipment used for CO_2 determinations was an HP 5890 gas chromatograph (Agilent Technologies, Madrid, Spain), featuring a Porapak Q 80/100 (inox) column (2 m × 1/8′) connected to a thermal conductivity detector (TCD). Injection, oven, and detection temperatures were maintained at 90, 25, and 100 °C, respectively. In both cases, the carrier gas utilized was helium.

2.5. Calculations

n-Caproate selectivity over *n*-butyrate was calculated according to Eq. (1).

$$n\mathrm{C}_6\ \text{selectivity}(\%) = \frac{[n\mathrm{C}_6]_{\text{end}}\left(\frac{\text{mol}}{\text{L}}\right)}{[n\mathrm{C}_6]_{\text{end}}\left(\frac{\text{mol}}{\text{L}}\right) + [n\mathrm{C}_4]_{\text{end}}\left(\frac{\text{mol}}{\text{L}}\right)} \times 100 \quad (1)$$

where $[n\mathrm{C}_6]_{\text{end}}$ corresponds to the final concentration of *n*-caproate (in mol/L) in the fermentation broth.

To calculate the growth rate (μ) in h^{-1}, a minimum of three OD_{600nm} data points from the exponential growth phase were taken and plotted on an exponential time scale, as shown in Eq. (2).

$$y = Ae^{\mu x} \quad (2)$$

where y refers to the OD_{600nm} values, A is a constant, μ is the growth rate (in h^{-1}) and x is the time (in h).

3. Results and discussion

3.1. Growth of C. kluyveri in bottles with different available CO_2 concentrations

In the bottle assay conducted in this work, *C. kluyveri* was grown on three media with different concentrations of available CO_2. The first medium contained an initial dissolved $NaHCO_3$ concentration of 0.03 M and was not supplemented with CO_2. Under these conditions, the strain managed to grow after a lag phase of almost 150 h, as shown in Fig. 1A, while the CO_2 concentration in the headspace consequent to the $NaHCO_3/CO_2$ equilibrium remained slightly below 1000 g/m^3 throughout the experiment (Fig. 2A). The final concentrations of *n*-butyrate and *n*-caproate after 257.75 h, were 2.75 ± 0.05 g/L and 6.10 ± 0.25 g/L, respectively, while the consumption of acetate and ethanol corresponded to 45.5 ± 1.0 % and 66.7 ± 0.5 %, respectively.

For the other two media, two transfers were made from a first inoculum of *C. kluyveri* grown in a complete medium (containing 0.03 M $NaHCO_3$) to a medium without dissolved $NaHCO_3$, in order to reduce the $NaHCO_3$ concentration by 10 % with each transfer, thus leaving the initial $NaHCO_3$ concentration at merely trace levels of approximately 0.0003 M (~25 ppm) before starting the experiment. When this medium was pressurized with 1 bar CO_2, inevitably the initial, approximately neutral, pH decreased to a value of 5.85 ± 0.03, which is, theoretically, well below the optimum pH of the strain. In fact, previous research has shown that a pH of 5.5 was detrimental to the growth of this microorganism (Gildemyn et al., 2017). Despite this, the strain succeeded in growing with almost no lag phase, although its activity did not last long, probably inhibited by the final pH reached of 5.53 ± 0.01 (Fig. 1B). The final concentrations of *n*-butyrate and *n*-caproate were in this case, 3.64 ± 0.07 g/L and 3.16 ± 0.06 g/L, respectively, whereas the acetate and ethanol consumptions were only of 55.0 ± 0.9 % and 31.1 ± 0.3 %, respectively. In this instance, the concentration of *n*-caproate was not as high as in the complete medium probably due to several factors. Firstly, the aforementioned problem of acidification of the medium, which cannot be solved since it is not possible to control the pH inside the bottles. Secondly, it is likely that the CO_2 concentration in the headspace of the bottle (i.e., close to 5000 rpm, Fig. 2B) was so high that it caused some kind of inhibition on the microorganism. This production profile of MCFAs, where the concentration of *n*-butyrate is equal to or higher than the concentration of *n*-caproate, is also typical of chain elongation processes where there is no adequate ethanol:acetate ratio (Spirito et al., 2018).

In the medium with very low dissolved $NaHCO_3$ and without CO_2 pressurization, *C. kluyveri* also managed to grow with almost no lag phase. In this case, the initial pH was 6.46 ± 0.01 (Fig. 1C), closer to the pH for optimal growth. At the end of the experiment, *n*-butyrate and *n*-caproate concentrations were 3.39 ± 0.06 g/L and 4.34 ± 0.46 g/L, respectively, with acetate and ethanol consumptions of 58.0 ± 0.3 % and 39.5 ± 1.5 %, respectively. Although the estimated initial soluble $NaHCO_3$ concentration was very low (i.e., 0.0003 M), CO_2 concentrations in the headspace equal to or less than 250 g/m^3 could be found (Fig. 2C), which could contribute positively to bacterial growth. Also, the differences in terms of lag phase between the experiments with the complete media and the media with low $NaHCO_3$ concentration were speculated to be due to the fact that the *C. kluyveri* inoculum used with the complete media was a couple of days older and therefore less active.

Based on these results, it was demonstrated that *C. kluyveri* can thrive in media with varying CO_2 concentrations, ranging from low (~200 g/m^3) to high (~6000 g/m^3).

3.2. n-Caproate production in pressurized bottles at 1 bar CO_2 with varying ethanol:acetate molar ratios

After establishing that *C. kluyveri* can grow in the presence of 1 bar CO_2 in bottles, it was decided to initiate another study to assess the

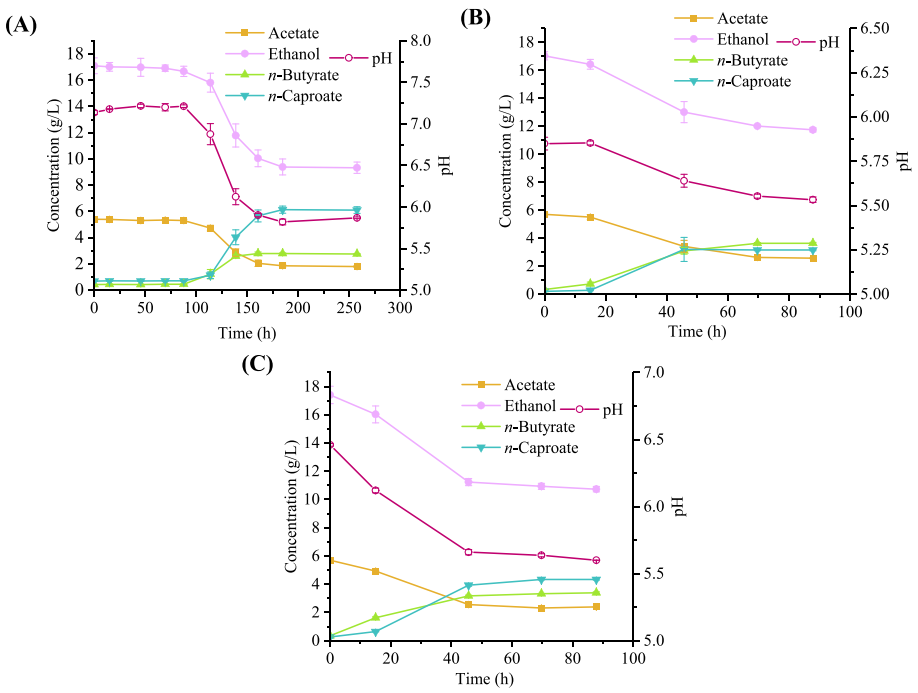


Fig. 1. Profile of MCFAs production by *C. kluyveri* from ethanol and acetate in: (A) a complete medium with $NaHCO_3$ 0.03 M; (B) a medium with very low $NaHCO_3$ (0.0003 M) and with 1 bar of CO_2; (C) a medium traces $NaHCO_3$ and without CO_2.

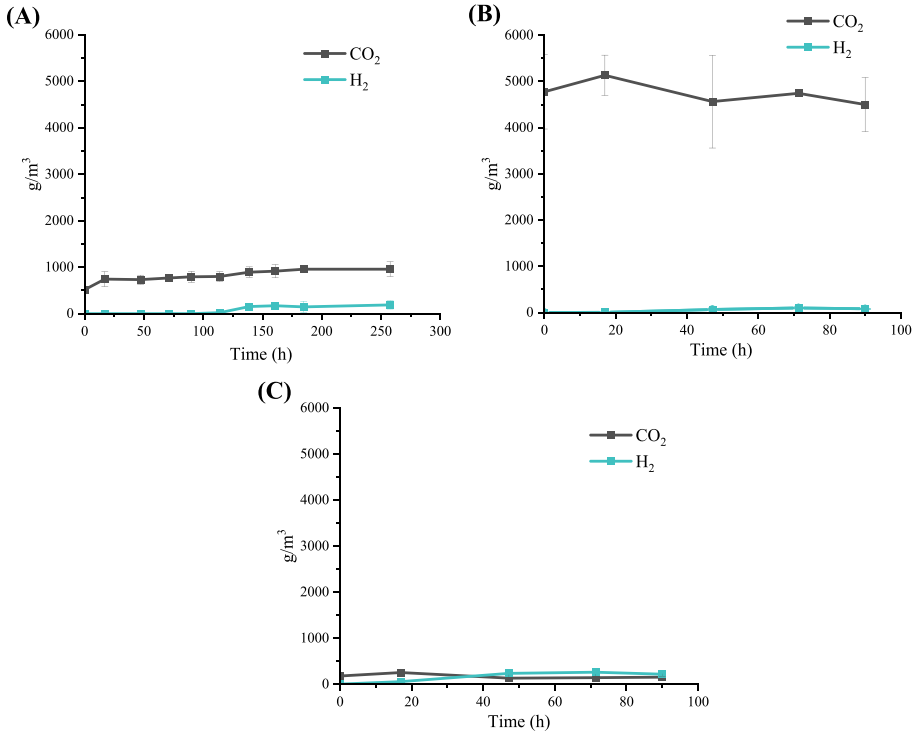


Fig. 2. Concentration of CO_2 and H_2 in the headspace of the bottles using: (A) a complete medium with $NaHCO_3$ 0.03 M; (B) a medium with very low $NaHCO_3$ (0.0003 M) and with 1 bar of CO_2; (C) a medium without $NaHCO_3$ and without CO_2.

impact of the ethanol:acetate molar ratio on the strain's behavior. For this, the acetate concentration was kept constant while the ethanol concentration was variable. Fig. 3 shows the MCFAs production profile, as well as the pH changes experienced throughout fermentation and the gas composition in the headspace of the bottles as a function of time.

In the three conditions studied, the initial pH dropped to about 5.9 as a consequence of CO_2 pressurization, so the starting pH was not optimal for the strain, even quite close to the pH 5.5 limit established by Gildemyn et al. (2017). When the ethanol:acetate molar ratio used was 1:1 (Fig. 3A), a lag phase of 65 h was observed and the final achieved concentrations of *n*-butyrate and *n*-caproate after 308 h were 4.39 ± 0.10 g/L and 3.41 ± 0.16 g/L, respectively (*n*-caproate selectivity of

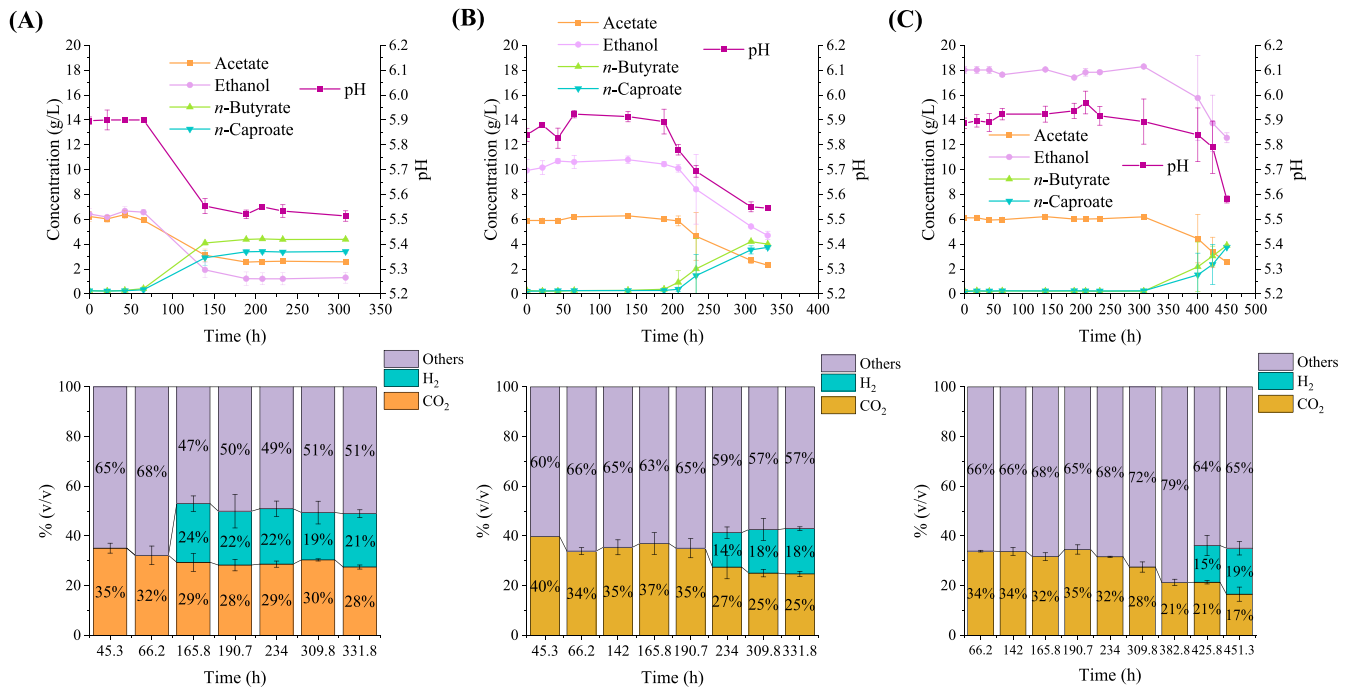


Fig. 3. MCFAs production profile by *C. kluyveri* from ethanol and acetate in bottles pressurized with 1 bar CO_2 (up) and percentage gas composition of the headspace over time using: (A) an ethanol:acetate molar ratio of 1:1; (B) an ethanol:acetate molar ratio of 2:1; and (C) an ethanol:acetate molar ratio of 4:1. Note: "others" in the legend mainly refers to N_2.

37.1 %, molar basis). At an ethanol:acetate molar ratio of 2:1, the lag phase was extended until 208 h and the final concentration of *n*-butyrate and *n*-caproate were, respectively, 4.00 ± 0.05 g/L and 3.74 ± 0.13 g/L after 331 h (*n*-caproate selectivity of 41.5 %, molar basis) (Fig. 3B). Finally, by increasing the ratio up to 4:1, the lag phase was the longest at 331 h, with final concentrations of *n*-butyrate and *n*-caproate after 450.7 h from the start of the experiment of 3.90 ± 0.03 g/L and 3.76 ± 0.01 g/L, respectively (*n*-caproate selectivity of 42.2 %, molar basis) (Fig. 3C). At first sight, it would be expected that as the ethanol:acetate ratio increases, higher concentrations of *n*-caproate and lower concentrations of *n*-butyrate would be obtained (Spirito et al., 2018); however, what is observed here is a final *n*-caproate concentration that is practically the same in all three cases. It should be noted that the initial pH is a conditioning factor in the performance of *C. kluyveri*, so the fermentations may have been inhibited prematurely by the low pH, since, as can be seen in Fig. 3A,B,C, the final pH was always slightly higher than 5.5. On the other hand, the fact that the lag phases were longer as the ethanol concentration increases is consistent, since it exerts greater toxicity on the microorganism, as previously stated for other species from the genus *Clostridium* (Fernández-Naveira et al., 2016).

As for the gaseous composition of the headspace, its time-dependent evolution is also plotted in Fig. 3A,B,C. It can be noticed that towards the end of fermentation the percentage CO_2 in the headspace is always lower than at the beginning, and this is especially remarkable for the 2:1 and 4:1 ethanol:acetate molar ratios. The fact that the proportion of the majority gases (designated as other gases, and being mostly N_2) remains practically constant with these two molar ratios suggests that this decrease is due to the consumption of CO_2 by the bacteria or its elimination in the presence of H_2.

Nevertheless, bottle systems come with numerous limitations, primarily due to the inability to continuously adjust pH, which poses a significant challenge when attempting to draw clear conclusions about optimal conditions. For this reason, further experiments in automated bioreactors, with on-line pH control, were carried out and are detailed in the following sections.

3.3. Bioreactor studies with pure cultures of *C. kluyveri*

3.3.1. Preliminary trials with $NaHCO_3$-rich medium or with a feeding of 3 mL/min CO_2

After testing the CO_2 consumption by *C. kluyveri* in previous assays, it was desired to evaluate the behavior of the strain with a CO_2 feeding at bioreactor level. Therefore, a series of experiments were conducted. Initially, an attempt was made to start up a control reactor without CO_2 feeding and with a reduced concentration of $NaHCO_3$ (0.0003 M). However, these trials proved unsuccessful, as the strain failed to grow in the reactor. On the other hand, the strain did grow in a reactor with complete medium (0.03 M $NaHCO_3$), as shown in Fig. 4 (bioreactor 1). From 6.77 g/L acetate and 14.4 g/L ethanol, the final achieved concentration of *n*-butyrate and *n*-caproate, after 279.2 h, were 3.10 g/L and

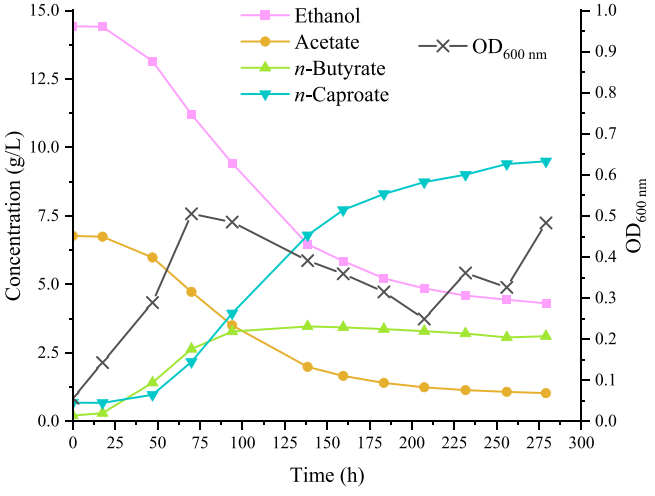


Fig. 4. Production profile of MCFAs by *C. kluyveri* from ethanol and acetate in bioreactor 1 without CO_2 feeding and with an initial dissolved $NaHCO_3$ concentration of 0.03 M.

9.50 g/L, respectively, with acetate and ethanol consumptions of 84.8 % and 70.4 %, respectively. The maximum OD_{600nm} reached was 0.51 at 70 h after starting the experiment, which is not particularly high. A batch bioreactor experiment set up by San-Valero et al. (2019) using similar initial ethanol, acetate, and $NaHCO_3$ concentrations, with a pure culture of *C. kluyveri*, led to a maximum OD_{600nm} of 0.77, which is slightly higher than the results reported here, but still comparable and of the same order of magnitude.

In a second bioreactor (bioreactor 2), *C. kluyveri* was grown in a medium with very low $NaHCO_3$ levels of about 0.0003 M, which was carried over from the inoculum, and a continuous CO_2 feeding rate of 3 mL/min (8.03 mmol CO_2/h). This relatively low CO_2 feeding rate was chosen because higher rates would over-acidify the medium, with consequent NaOH consumption, leading to cellular stress and a considerable rise in the reactor volume level. Under these conditions, a lag phase of 423.4 h was observed (Fig. 5), which is attributed to initial operational difficulties in maintaining anaerobic conditions within the reactor. Following the exponential growth stage, the concentrations of *n*-butyrate and *n*-caproate stabilized at 3.10 g/L and 9.16 g/L, respectively, with 76.5 % acetate and 100 % ethanol being consumed. At the beginning of the experiment, a rather low CO_2 outlet rate was observed (0.50 mmol CO_2/h, t = 21.95 h), as can be seen in Fig. 5, and which is expected since the CO_2 molecules, fed from the bottom of the reactor, need to occupy the whole space and the outlet is located on the reactor headplate. Following this initial period, the CO_2 outlet rate remained relatively constant, aside from minor fluctuations attributable to the accuracy of the measurements and analytical errors. However, in alignment with bacterial growth, a declining trend is noticeable between t = 304.75 h (1.93 mmol CO_2/h) and t = 474.08 h (0.80 mmol CO_2/h). This is also evidenced by the simultaneous production of H_2, reaching a maximum value of 2.92 mmol H_2/h at t = 423.58 h. After 476.08 h of experiment and having seen that the activity of the strain was declining (gradual drop in OD_{600nm} value from maximum 1.3 at t = 423.42 to 0.71 at t = 476.08 h), a partial replacement of the medium inside the reactor was decided, in order to check and circumvent possible limitation of some exhausted nutrients. For this purpose, 500 mL fermentation broth was aseptically removed, and 500 mL fresh medium was then added. Simultaneously, an abrupt rise in the CO_2 rate up to 3.02 mmol CO_2/h was observed at the reactor outlet, precisely in the period of inactivity of *C. kluyveri*. After the replacement of the medium, CO_2 at the outlet decreased drastically again. This was presumably because of medium removal and fresh medium addition, allowing CO_2 solubilization again in the aqueous phase. Thereafter, the CO_2 outlet rate remained constant, while new chain elongation activity was also observed, given the new H_2 production and the slight upward trend in the production of *n*-butyrate (up to 2.25 g/L) and *n*-caproate (7.09 g/L).

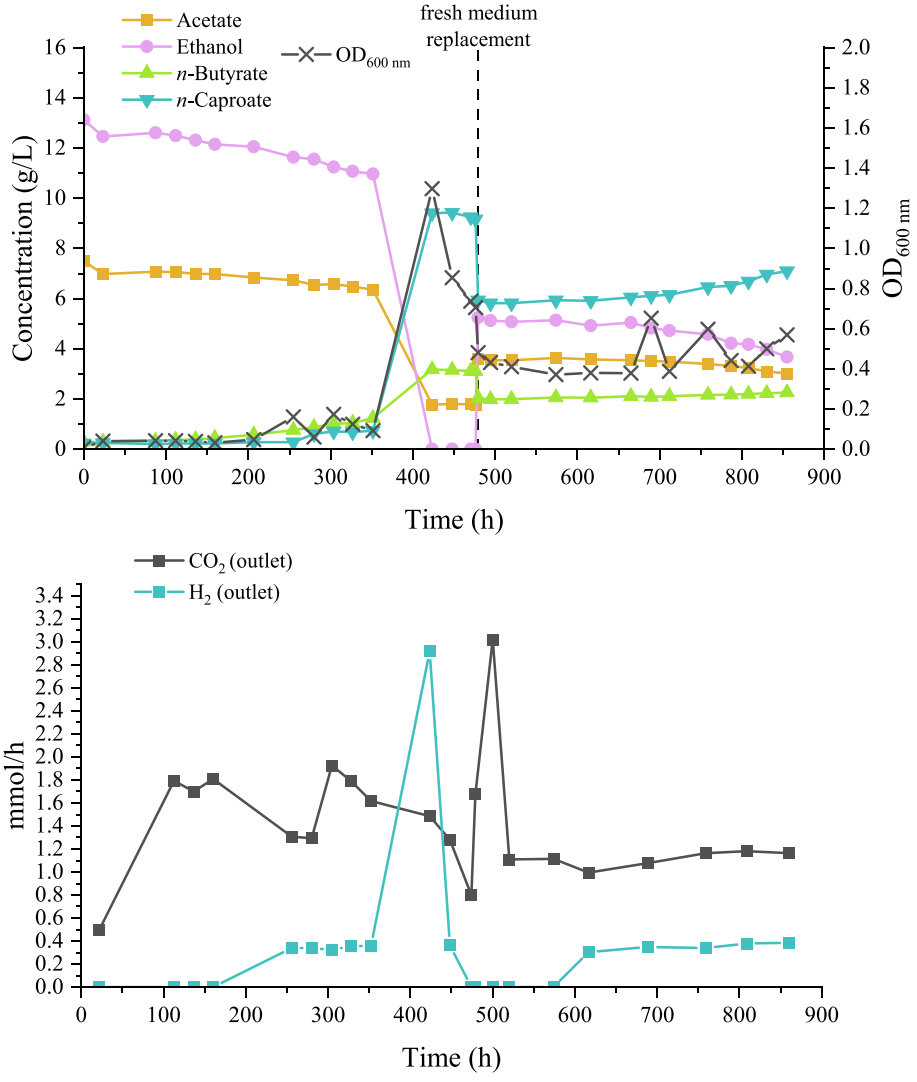


Fig. 5. Production profile of MCFAs by *C. kluyveri* from ethanol and acetate in bioreactor 2 with continuous CO_2 feeding of 3 mL/min and no initial dissolved $NaHCO_3$ (top); CO_2 and H_2 millimolar rates at the outlet of the bioreactor (bottom).

3.3.2. Optimized bioreactor with 3 mL/min CO_2 feeding

To assess the reproducibility of the prior experiment and considering the rapid exponential growth phase of the strain, there is a possibility that significant aspects of *C. kluyveri*'s activity profile were overlooked. Consequently, a new bioreactor, designated bioreactor 3, was started up. Also, a 15 % (w/v) titanium citrate solution was introduced into the reactoŕs medium in order to avoid potential issues related to oxygen infiltration, seen in bioreactor 2, which might have prolonged the lag phase and restricted somehow the bacterial performance. In this new experiment, the CO_2 feeding rate was also 3 mL/min (8.03 mmol CO_2/h), while the dissolved $NaHCO_3$ concentration was 0.0003 M. The production profile of MCFAs by *C. kluyveri* can be seen in Fig. 6, along with the CO_2 and H_2 rates at the bioreactor outlet.

In this scenario, there was hardly any lag phase following the inoculation of *C. kluyveri*, which is in stark contrast to the situation in bioreactor 2. Within less than a day of initiating the system, ethanol and acetate consumption, as well as *n*-butyrate and *n*-caproate production were observed. The OD_{600nm} value reached its peak at 1.89 after t = 43.3 h, and the fermentation was concluded after 88.75 h, resulting in final *n*-butyrate and *n*-caproate concentrations of 0.94 g/L and 11.0 g/L, respectively (with acetate and ethanol consumptions of 92.7 % and 87.6 %, respectively). With respect to H_2 production and CO_2 consumption, it can be seen in Fig. 6 that at the beginning of fermentation, the CO_2 outlet rate increased to a maximum of 2.1 mmol CO_2/h (t = 43.3 h). This is the same pattern as observed in bioreactor 2, since at such a low feeding flow rate, several hours are necessary for the reactor to get filled with CO_2 (solubilization in the liquid phase and occupation of the headspace). At this same point, the maximum H_2 production rate of 1.74 mmol H_2/h also occurred. From this point on, the outlet rate of CO_2 started to decrease, indicating that part of this gas was being used by *C. kluyveri*. A new addition of substrate (i.e., ethanol and acetate) at t = 92.25 h was done in an attempt to reactivate the strain, which did not happen though. This leads to suggest that it would also have been necessary to renew part of the medium, and thus the nutrients and vitamins, for *C. kluyveri* to recover its activity, as occurred in bioreactor 2. It is precisely after the addition of ethanol and acetate that a slight increase in the CO_2 rate is observed at the reactor outlet, as a sign that bacterial activity had ceased. The production of carbon chains longer than *n*-caproate (i.e., *n*-caprylate) is possible in any of the three bioreactors, as this strain showed minor *n*-caprylate production in other studies; however, this metabolite was not determined here.

Analyzing the data as a whole from the three bioreactors, it can be

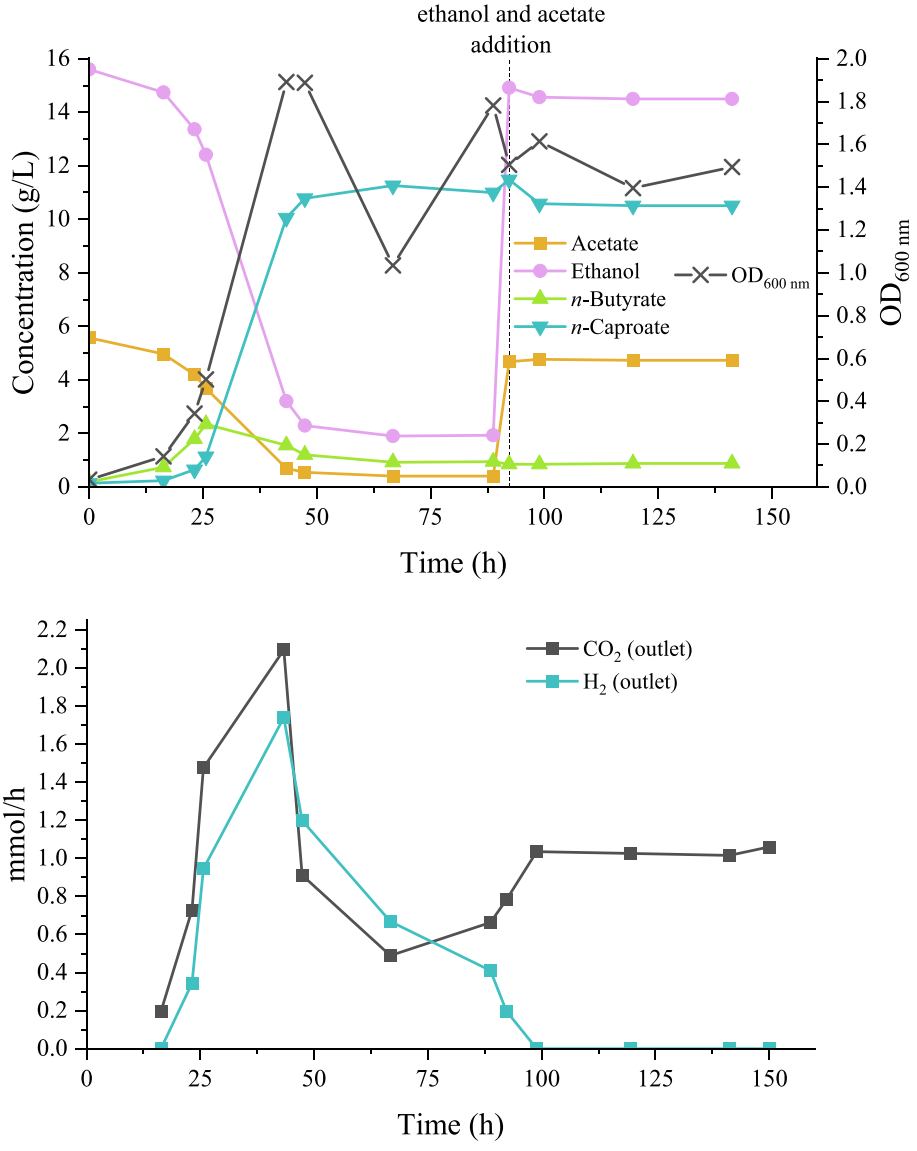


Fig. 6. Production profile of MCFAs by *C. kluyveri* from ethanol and acetate in bioreactor 3 with continuous CO_2 feeding of 3 mL/min and no initial dissolved $NaHCO_3$ (top). CO_2 and H_2 millimolar rates at the outlet of the bioreactor (bottom).

established that there is a direct relationship between the concentration of available CO_2 and growth of *C. kluyveri* (Table 1), as suggested by the studies of Tomlinson and Barker (1954) several decades ago. The increase in the OD_{600nm} value is remarkable when using a continuous CO_2 feed of 3 mL/min compared to a culture medium with an initial dissolved $NaHCO_3$ concentration of 0.03 M (bioreactor 1). For example, in the experiments carried out by Gildemyn et al. (2017), with *C. kluyveri* grown on DSMZ52 medium (Na_2CO_3 0.0094 M), the OD_{600nm} in no case is higher than 0.5. Likewise, batch bioreactor studies carried out by San-Valero et al. (2019) with a pure culture of *C. kluyveri* showed that the maximum OD_{600nm} reached in a medium with 0.03 M $NaHCO_3$ was 0.77, using only ethanol and acetate as carbon sources. Similarly, the same authors, in another work, studied the effect of adding $NaHCO_3$ for pH control, instead of NaOH, observing higher rates of acetate, *n*-butyrate and ethanol consumption efficiencies (75 %, 69 %, and 70 %, respectively) with respect to the control (56 %, 52 %, and 56 %, respectively), which was attributed by the authors to the presence of a greater amount of inorganic carbon that can make up for lower deficiencies (San-Valero et al., 2020). The difference with respect to the study shown here is that in the work presented by San-Valero et al. (2020) bicarbonate was only added in dissolved form when the pH value deviated from the setpoint as a consequence of acid accumulation in the broth, whereas here the feeding of gas (i.e., CO_2) was done continuously, regardless of pH. This uninterrupted addition of CO_2 caused an acidification of the medium that needed to be counteracted by the addition of base (i.e., NaOH). Although initially, the solubility of CO_2 in the aqueous medium could pose a limitation, the use of a microporous diffuser promotes a more effective gas/liquid transfer rate. Additionally, the direct utilization of gaseous effluent is interesting as it replicates industrial gas emissions, demonstrating that its use offers potential improvements to the biotechnological chain elongation process. The growth rate of *C. kluyveri* is notably three times greater in bioreactor 3 when compared to bioreactor 1 (Table 1). In bioreactor 2, however, the growth rate was not computed due to the insufficient number of data points in the experimental phase, as illustrated in Fig. 5, which makes it unreliable to perform this type of calculation. Furthermore, the selectivity of *n*-caproate over *n*-butyrate was determined for all three bioreactor experiments, and as shown in Table 1, the *n*-caproate selectivity in bioreactor 3 is notably higher compared to the two preceding experiments. In principle, it could be postulated that a continuous supply of CO_2 might facilitate the generation of longer chain carboxylates over their shorter counterparts, considering the selectivity data obtained from bioreactor 1 and bioreactor 3. However, upon closer examination of the data from bioreactor 2, it becomes evident that the selectivity is quite comparable and even moderately lower than that observed in bioreactor 1. The explanation attributed to this fact is related to the ethanol:acetate ratio, and therefore, the amount ethanol (i.e., electron donor) available to perform chain elongation. Previous studies have shown that higher ratios lead to the formation of longer chains and thus favor the selectivity of *n*-caproate over *n*-butyrate (Spirito et al., 2018). It is noteworthy that the ethanol:acetate molar ratio in bioreactor 2 was slightly lower (i.e., 2.2:1) when contrasted with the ratios in bioreactor 1 (2.8:1) and bioreactor 3 (3.7:1). Actually, the highest selectivity of 90.1 % (molar basis) achieved in bioreactor 3 occurs at the highest ethanol:acetate molar ratio. This fact was also corroborated by Kucek et al. (2016), in a mixed culture system, where it was found that a higher ethanol:acetate molar ratio was necessary to achieve a high *n*-carpylate:*n*-caproate product ratio. Nevertheless, very recent research has also experimentally demonstrated the production of *n*-caproate solely from ethanol, without the addition of carboxylates as electron acceptors (Allaart et al., 2023). Additionally, until the growth of the strain in bioreactor 2 was achieved, several experimental problems were encountered, accentuated by the challenge of maintaining the reactor under fully anaerobic conditions, as evidenced by the somewhat pinkish color of the medium. This would explain the worse results than expected, since the growth conditions of *C. kluyveri* were far from optimal. In any case, these experimental data prove once again the importance of CO_2 for chain elongation and its direct relationship with the growth of the microorganism, evidenced in this work by the OD_{600nm} and the growth rate. The CO_2 supply employed here, at a rate of 3 mL/min (3.1 $L_{CO2}/(L \cdot d)$), is expected to promote the EEO. In a continuous ethanol-based fermentation with a microbiome, Roghair et al. (2018a) reported that a supply of 2.5 $L_{CO2}/(L \cdot d)$ stimulated chain elongation to as high as 29 % with a significant *n*-caproate productivity of 10.8 $g/(L \cdot d)$. However, the EEO cannot be quantitatively assessed due to the lack of data regarding *n*-caprylate production in all experiments, which is another product from chain elongation.

Overall, the results presented here corroborate the CO_2 requirements of *C. kluyveri*, either in gaseous form or in the form of carbonate or bicarbonate. As a future work, it is proposed to consider the data reported in this work to implement a bioreactor system operating in continuous mode with a pure culture of *C. kluyveri*, with CO_2 feeding, since all the studies available to date have only been carried out with mixed cultures (Roghair et al., 2018a; Roghair et al., 2018b). It would be expected to see some effect on EEO and, it would be interesting to know if there is any effect on chain elongation performance. However, one of the main limitations of this system is that the CO_2 feeding is constantly acidifying the medium, so that a continuous pH adjustment (e.g., addition of NaOH) would be necessary, which could to some extent be harmful to bacterial growth, whose optimum pH is 6.8. Therefore, the study of different CO_2 flow rates and their evaluation on the effect of the pH of the fermentation broth is also proposed.

Table 1
Summary of results in the three batch mode bioreactor studies with *C. kluyveri*.

	CO_2 availability	End *n*-C_4 (g/L)	End *n*-C_6 (g/L)	Max. $OD_{600\ nm}$ (A)	*n*-C_6 Selectivity (% molar basis)	μ (h^{-1})
Bioreactor 1	$NaHCO_3$ 0.03 M	3.10	9.56	0.51	70.1	0.0271 (R^2 = 0.995)
Bioreactor 2	$NaHCO_3$ 0.0003 M (i.e., traces) + CO_2 (3 mL/min)	3.10	9.16	1.30	69.1	–
Bioreactor 3	$NaHCO_3$ 0.0003 M (i.e., traces) + CO_2 (3 mL/min)	0.87	10.5	1.89	90.1	0.0811 (R^2 = 0.998)

4. Conclusions

The present study provides evidence that there is a direct relationship between available CO_2 and growth of *C. kluyveri*, denoted by higher values of OD_{600nm} and growth rates. In a first bottle study, it was proved that the strain is able to grow in a medium with different concentrations of available CO_2. At the bioreactor level, the growth of *C. kluyveri* was compared in a medium with $NaHCO_3$ 0.03 M and in a medium with negligible $NaHCO_3$ concentration of 0.0003 M with continuous feeding of 3 mL/min of CO_2, observing much higher optical densities and growth rates with the continuous CO_2 feeding.

CRediT authorship contribution statement

Carla Fernández-Blanco: Data curation, Investigation, Methodology, Writing – review & editing. **María C. Veiga:** Data curation, Resources, Supervision, Validation, Writing – review & editing. **Christian Kennes:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christian Kennes reports financial support was provided by Spain Ministry of Science and Innovation. Maria C. Veiga reports financial support was provided by Government of Galicia.

Data availability

Data will be made available on request.

Acknowledgements

This study was supported by Spanish Ministry of Science and Innovation and European FEDER funds [TED2021-130055B-I00]. CFB thanks Xunta de Galicia for her doctoral contract (ED481A-2020/028). The authors, belonging to the BIOENGIN group, acknowledge the financial support from Xunta de Galicia to Competitive Reference Research Groups [ED431C 2021/55]. Funding for open access charge provided by Universidade da Coruña/CISUG.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.130192.

References

Ahn, J.H., Jung, K.H., Lim, E.S., Kim, S.M., Han, S.O., Um, Y., 2023. Recent advances in microbial production of medium chain fatty acid from renewable carbon resources: A comprehensive review. Bioresour. Technol. 381, 129147.

Allaart, M.T., Fox, B.B., Nettersheim, I.H.M.S., Pabst, M., Sousa, D.Z., Kleerebezem, R., 2023. Physiological and stoichiometric characterization of ethanol-based chain elongation in the absence of short-chain carboxylic acids. Sci. Rep. 13, 1–10.

Andrew, I.G., Morris, J.G., 1965. The biosynthesis of alanine by *Clostridium kluyveri*. Biochim. Biophys. Acta 97, 176–179.

Angenent, L.T., Richter, H., Buckel, W., Spirito, C.M., Steinbusch, K.J.J., Plugge, C.M., Strik, D.P.B.T.B., Grootscholten, T.I.M., Buisman, C.J.N., Hamelers, H.V.M., 2016. Chain elongation with reactor microbiomes: Open-culture biotechnology to produce biochemicals. Environ. Sci. Technol. 50, 2796–2810.

Barker, H.A., Taha, S.M., 1941. *Clostridium kluyverii*, an organism concerned in the formation of caproic acid from ethyl alcohol. J. Bacteriol. 43, 347–363.

Chu, N., Liang, Q., Zhang, W., Ge, Z., Hao, W., Jiang, Y., Zeng, R.J., 2020. Waste C_1 gases as alternatives to pure CO_2 improved the microbial electrosynthesis of C_4 and C_6 carboxylates. ACS Sustain. Chem. Eng. 8, 8773–8782.

Decker, K., Jungermann, K., Thauer, R.K., Hunt, S.V., 1967. Net CO_2 fixation into the S-methyl group of methionine and into the positions 2 and 8 of the purines in *Clostridium kluyveri*. Biochim. Biophys. Acta. 141, 202–204.

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.

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, 1–11.

Fernández-Blanco, C., Veiga, M.C., Kennes, C., 2022. Efficient production of *n*-caproate from syngas by a co-culture of *Clostridium aceticum* and *Clostridium kluyveri*. J. Environ. Manage. 302, 113992.

Fernández-Naveira, Á., Abubackar, H.N., Veiga, M.C., Kennes, C., 2016. Carbon monoxide bioconversion to butanol-ethanol by *Clostridium carboxidivorans*: kinetics and toxicity of alcohols. Appl. Microbiol. Biotechnol. 100, 4231–4240.

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.

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.

Harvey, B.G., Meylemans, H.A., 2014. 1-Hexene: A renewable C6 platform for full-performance jet and diesel fuels. Green Chem. 16, 770–776.

Huo, W., Ye, R., Shao, Y., Wang, H., Lu, W., 2023. Insight into the mechanism of CO_2 to initiate and regulate ethanol-driven chain elongation by microbial community and metabolic analysis. J. Environ. Chem. Eng. 11, 110537.

Jungermann, K., Thauer, R.K., Decker, K., 1968. The synthesis of one-carbon units from CO_2 in Clostridium kluyveri. Eur. J. Biochem. 3, 351–359.

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.

Li, Z., Cai, J., Gao, Y., Zhang, L., Liang, Q., Hao, W., Jiang, Y., Jianxiong Zeng, R., 2022. Efficient production of medium chain fatty acids in microbial electrosynthesis with simultaneous bio-utilization of carbon dioxide and ethanol. Bioresour. Technol. 352, 127101.

Parera Olm, I., Sousa, D.Z., 2023. Upgrading dilute ethanol to odd-chain carboxylic acids by a synthetic co-culture of *Anaerotignum neopropionicum* and *Clostridium kluyveri*. Biotechnol. Biofuels Bioprod. 16, 1–17.

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.

Roghair, M., Hoogstad, T., Strik, D.P.B.T.B., Plugge, C.M., Timmers, P.H.A., Weusthuis, R.A., Bruins, M.E., Buisman, C.J.N., 2018a. Controlling ethanol use in chain elongation by CO_2 loading rate. Environ. Sci. Technol. 52, 1496–1506.

Roghair, M., Liu, Y., Strik, D.P.B.T.B., Weusthuis, R.A., Bruins, M.E., Buisman, C.J.N., 2018b. Development of an effective chain elongation process from acidified food waste and ethanol into *n*-caproate. Front. Bioeng. Biotechnol. 6, 1–11.

Sakarika, M., Regueira, A., Rabaey, K., Ganigué, R., 2023. Thermophilic caproic acid production from grass juice by sugar-based chain elongation. Sci. Total Environ. 860, 160501.

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.

San-Valero, P., Fernández-Naveira, Veiga, M.C., Kennes, C., 2019. Influence of electron acceptors on hexanoic acid production by *Clostridium kluyveri*. J. Environ. Manage. 242, 515–521.

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.

Spirito, C.M., Marzilli, A.M., Angenent, L.T., 2018. Higher substrate ratios of ethanol to acetate steered chain elongation toward *n*-caprylate in a bioreactor with product extraction. Environ. Sci. Technol. 52, 13438–13447.

Tang, J., Yang, H., Pu, Y., Hu, Y., Huang, J., Jin, N., He, X., Wang, X.C., 2023. Caproic acid production from food waste using indigenous microbiota: Performance and mechanisms. Bioresour. Technol. 387, 129687.

Tomlinson, N., 1954a. Carbon dioxide and acetate utilization by *Clostridium kluyveri*. II. Synthesis of amino acids. J. Biol. Chem. 209, 597–603.

Tomlinson, N., 1954b. Carbon dioxide and acetate utilization by *Clostridium kluyveri*. III. A new path of glutamic acid synthesis. J. Biol. Chem. 209, 605–609.

Tomlinson, N., Barker, H.A., 1954. Carbon dioxide and acetate utilization by *Clostridium kluyveri*. I. Influence of nutritional conditions on utilization patterns. J. Biol. Chem. 209, 585–595.

Wang, Q., Yang, N., Cai, Y., Zhang, G., Wu, Y., Ma, W., Fu, C., Zhang, P., 2023. Advanced treatment and valorization of food waste through staged fermentation and chain elongation. Bioresour. Technol. 384, 129286.

Weimer, P.J., Kohn, R.A., 2016. Impacts of ruminal microorganisms on the production of fuels: how can we intercede from the outside? Appl. Microbiol. Biotechnol. 100, 3389–3398.

Yin, Y., Zhang, Y., Karakashev, D.B., Wang, J., Angelidaki, I., 2017. Biological caproate production by *Clostridium kluyveri* from ethanol and acetate as carbon sources. Bioresour. Technol. 241, 638–644.

Yu, P., Wu, M., Bao, W., Wang, H., 2023. Performance of a mixed inoculum of sludge and pit mud for short and medium-chain fatty acids production: Insight into key microbiome and functional potential in anaerobic fermentation inoculum. Chem. Eng. J. 466, 143142.

Zhang, F., Ding, J., Zhang, Y., Chen, M., Ding, Z.W., van Loosdrecht, M.C.M., Zeng, R.J., 2013. Fatty acids production from hydrogen and carbon dioxide by mixed culture in the membrane biofilm reactor. Water Res. 47, 6122–6129.