CHALMERS
UNIVERSITY OF TECHNOLOGY

Excessive Ethanol Oxidation Versus Efficient Chain Elongation Processes

Downloaded from: https://research.chalmers.se, 2024-10-12 08:07 UTC

Citation for the original published paper (version of record):
Quintela, C., Peshkepia, E., Grimalt-Alemany, A. et al (2024). Excessive Ethanol Oxidation Versus Efficient Chain Elongation Processes. Waste and Biomass Valorization, 15(4): 2545-2558.
http://dx.doi.org/10.1007/s12649-023-02323-0

N.B. When citing this work, cite the original published paper.

research.chalmers.se offers the possibility of retrieving research publications produced at Chalmers University of Technology. It covers all kind of research output: articles, dissertations, conference papers, reports etc. since 2004. research.chalmers.se is administrated and maintained by Chalmers Library

(article starts on next page)

Waste and Biomass Valorization
https://doi.org/10.1007/s12649-023-02323-0

ORIGINAL PAPER

Excessive Ethanol Oxidation Versus Efficient Chain Elongation Processes

Cesar Quintela[1] · Evi Peshkepia[1] · Antonio Grimalt-Alemany[1] · Yvonne Nygård[2] · Lisbeth Olsson[2] · Ioannis V. Skiadas[1] · Hariklia N. Gavala[1]

Received: 6 June 2023 / Accepted: 19 October 2023
© The Author(s) 2023

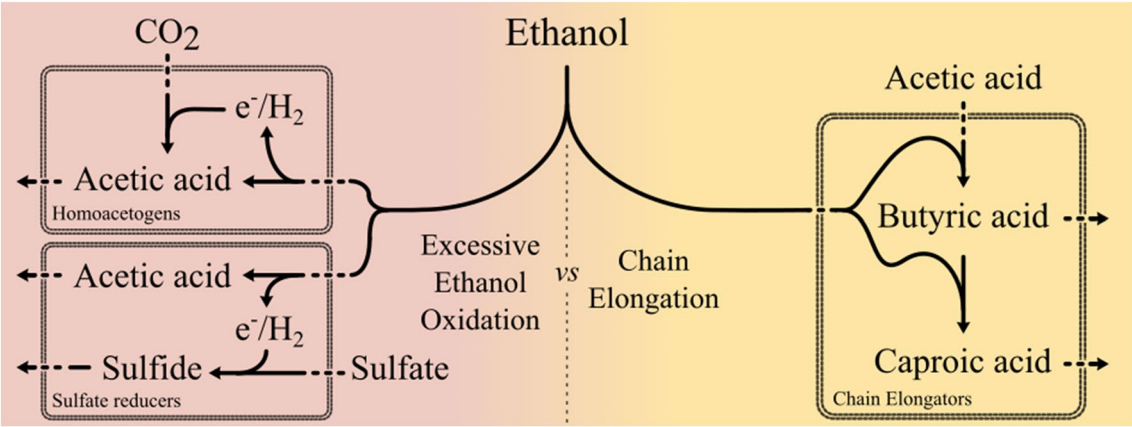
Abstract

Purpose Chain elongation is a metabolic feature that consists of the elongation of short-chain fatty acids to longer and more valuable acids when ethanol is available. To lower the operational costs, the process can also be performed using mixed microbial cultures. However, certain microorganisms in the mixed cultures can use the ethanol provided in competing reactions, which is usually termed excessive ethanol oxidation (EEO). Although minimizing ethanol use is essential, there is a lack of studies analyzing the extent, causes, and solutions to excessive ethanol oxidation processes.

Methods To address this knowledge gap, ethanol, and acetic acid mixtures, at a molar ratio of 5 to 2, were fermented, and the following were analyzed: the fermentation profile at different (1) pH and (2) headspace gas compositions, (3) a 16S analysis of the headspace gas composition fermentations, and (4) a thermodynamic analysis of the reactions involved.

Results and Conclusions: All fermentations, except the ones at the lowest pH (5.3), exhibited a significant EEO activity that reduced the yield of chain-elongated products. It was demonstrated that neither the inhibition of methanogenic activity nor the increased H_2 partial pressure is an efficient method to inhibit EEO. It was also shown that CO_2 can act as an electron acceptor for EEO, promoting the growth of acetogenic bacteria. In the absence of CO_2, sulfate was used as an electron acceptor by sulfate-reducing bacteria to facilitate EEO. Methods such as low pH operation with in-line extraction, and the use of alternative sulfur salts, are proposed to increase the ethanol use efficiency in chain elongation processes.

Graphical Abstract



Keywords Chain elongation · Ethanol oxidation · H_2 partial pressure · CO_2 reduction · Sulfate reduction · Mixed culture fermentation

Extended author information available on the last page of the article

Published online: 06 December 2023

Statement of Novelty

This study investigated the conditions that favor excessive ethanol oxidation versus efficient chain elongation reactions, the factors that affect these phenomena, and the strategies that can be applied to prevent it. To the best of our knowledge, there is a lack of studies looking at the competing reactions that may lower the efficiency of the chain elongated products. In this work, the analysis of the fermentation profiles at different operating conditions were supplemented with bacterial community analysis as well as thermodynamic analysis of the reactions involved. Consequently, it could be shown how neither the inhibition of the methanogenic activity, nor the supplementation with extra H_2 partial pressure, prevents excessive ethanol oxidation, as it is generally stated in recent literature in the field.

Introduction

The impact the current fossil-fuel-based industries and economies have on the climate [1] demands a shift towards renewable and circular alternatives. The chemical industry is one of the activities most distant from carbon–neutral goals, with most products still coming from petrol refining processes [2]. The few bioprocesses implemented, such as ethanol production from corn or sugarcane, require high amounts of fertilizers and compete directly or indirectly with food and feed supply [3, 4]. To promote a sustainable chemical industry, further efforts should focus on the utilization of waste streams and other renewable feedstocks which would increase the circularity of our economies [5]. In this context, chain elongation could prove to be a useful process to incorporate waste streams into the production of green platform chemicals. Chain elongation allows bacteria to elongate fatty acids, using the reducing power from an electron donor such as ethanol or lactic acid. Short-chain fatty acids, such as acetic and butyric acid, are common products of waste fermentation processes such as food waste fermentation and syngas fermentation. On the other hand, middle-chain fatty acids such as caproic and caprylic acid tend to hold higher market prices. Chain elongation seems therefore to be a promising technology to increase the revenue of resource recovery processes and to enlarge the product portfolio [6]. Chain elongation has also an important role in the fermentation of C1 compounds, i.e., CO_2, CO, and/or syngas to medium chain fatty acids [7–10].

The elongation of carboxylic acids using ethanol as an electron donor takes place intracellularly through the reverse β-oxidation pathway. Through this pathway, ethanol is first oxidized to Acetyl-CoA, with concomitant reduction of NAD^+ to NADH (reaction 1). A rather small fraction of Acetyl-CoA is transformed to acetic acid accompanied by ATP generation through substrate-level phosphorylation. NADH must be oxidized again to maintain the intracellular redox balance. To accomplish this, cells introduce a major fraction of acetyl-CoA into a reverse β-oxidation cycle, which elongates the present carboxylic acids by two carbons while NADH transfers electrons to ferredoxin and finally to protons, generating H_2 as shown in reaction 2. The reduced ferredoxin can also shuttle electrons back to NAD^+ in an exergonic reaction catalyzed by the ferredoxin:NAD oxidoreductase enzyme complex, Rnf. The energy generated by this redox reaction is used to pump protons out of the cell. This results in a proton motive force that is harnessed by the ATP synthase to produce extra ATP [11]. Depending on the environmental conditions, chain-elongating bacteria can potentially perform more ethanol oxidation, generating more ATP through substrate-level phosphorylation and then using more ferredoxin for H_2 production and NAD^+ regeneration; or introduce more ethanol into the reverse β-oxidation cycle, and then use the ferredoxin in the Rnf complex to generate ATP through the proton motive force. This results in a somewhat flexible ethanol-to-acetate consumption ratio (reaction 2) [12]. Nevertheless, a 6 to 4 ethanol-to-acetate molar ratio is usually given as the standard reverse β-oxidation stoichiometry (reaction 3) [6, 13]. This would mean that for every molecule of ethanol oxidized (reaction 1), five molecules of ethanol would enter the elongation cycle (reaction 2, a = 5).

$$C_2H_6O + H_2O + 2NAD^+ \rightarrow C_2H_4O_2 + 2NADH \quad (1)$$

$$(a+1)C_2H_6O + (a-1)C_2H_4O_2 + 2\,NADH \rightarrow aC_4H_8O_2 + (a-1)H_2O + 2H_2 + 2NAD^+ \quad (2)$$

$$(a=5)6C_2H_6O + 4C_2H_4O_2 \rightarrow 5C_4H_8O_2 + 4H_2O + 2H_2 \quad (3)$$

However, in mixed culture fermentations, some microbial groups may perform ethanol oxidation without coupling it to chain elongation [14–17]. This often leads to ethanol over acetic acid consumed ratios being significantly higher than 6 to 4. Grootscholten [18] defined this as "excessive ethanol oxidation" (EEO). Ethanol utilization for either EEO or chain elongation is especially relevant for fermentations based on undefined mixed microbial cultures. For the latter, a highly biodiverse inoculum is subject to conditions, that, over time, result in a microbial community performing the biological conversion. As this process does not require sterile conditions, it allows for low operational costs. However, in cases where competing

reactions co-exist, suppressing unwanted microbial groups can sometimes be challenging. Ethanol oxidation with the production of H_2 is thermodynamically sensitive to H_2 partial pressures. Several studies based on mixed methanogenic cultures mention that by inhibiting hydrogenotrophic methanogens, alcohols, and acids oxidation accompanied H_2 production becomes thermodynamically unfeasible[19, 20]. However, methanogens are not the only group that consume H_2 and facilitate oxidation processes in chain-elongating mixed cultures. Sulfate reducing bacteria (SRB) [21] and acetogenic bacteria [22] can both consume H_2 generated from oxidation processes when working in syntrophic relation with other bacteria. This leads to EEO occurring in chain elongating processes even when inhibiting methanogenesis [23]. In some studies, researchers have applied H_2 partial pressures above 10^{-2} atm to inhibit any H_2-mediated oxidation, including the syntrophic ethanol oxidations with SRB and acetogenic bacteria [18]. However, bacteria performing the oxidation and reduction reactions intracellularly [24], and microbes involved in direct intraspecies electron transfer (DIET) mediated syntrophic ethanol oxidation [25], would not produce H_2, and would therefore not be inhibited by high H_2 partial pressures.

The aim of this paper is to assess the impact EEO has in chain elongation processes, investigate the factors responsible, and furthermore, propose strategies to improve the efficiency of ethanol utilization in this pathway. This is fundamental for economically feasible chain elongation processes. In this study, two batch experimental series were performed to determine the effect of firstly pH and secondly headspace gas composition on the degree of ethanol oxidation and chain elongation in mixed cultures fermenting acetic acid and ethanol. Ethanol consumption efficiency towards chain elongation and carbon yield were analyzed, while a 16S rRNA analysis of the microbial communities was applied to support the macroscopic observations. Finally, thermodynamic analyses were performed to find the feasibility boundaries of the reactions involved under relevant environmental conditions. EEO was found to be a challenge when designing efficient chain elongation processes using mixed cultures, as it was only found to be suppressed by low pH (5.3), which may not be optimal for chain elongation processes [26]. Both acetogenic and sulfate-reducing activities were found to enable EEO in the absence of methanogens and under relatively high H_2 partial pressures. Our results challenge the norm, i.e., that methanogenic activity inhibition and/or H_2 supplementation are measures enough to prevent EEO, as there are other routes, often overlooked, that make EEO possible even at high H_2 partial pressures and in the absence of methanogens. Strategies to optimize the efficiency of ethanol use in chain elongation processes are also discussed.

Materials and Methods

Medium and Inoculum

A modified basal anaerobic (BA) medium was used in all experiments, to supply the culture with the necessary nutrients for microbial growth. The medium was prepared as described by Grimalt–Alemany [27], using the following stock solutions: macronutrients (NH_4Cl, 100 g L^{-1}; NaCl, g L^{-1}; $MgCl_2{\cdot}6H_2O$, 10 g L^{-1}; $CaCl_2{\cdot}2H_2O$, 5 g L^{-1}), dipotassium hydrogen phosphate solution ($K_2HPO_4{\cdot}3H_2O$, 200 g L^{-1}), sodium sulfate solution (Na_2SO_4, 100 g L^{-1}), sodium sulfide solution (Na_2S, 24.975 g L^{-1}), vitamin solution (biotin, 10 mg L^{-1}; folic acid, 10 mg L^{-1}; pyridoxine HCl, 50 mg L^{-1}; riboflavin HCl, 25 mg L^{-1}; thiamine HCl, 25 mg L^{-1}; cyanocobalamin, 0.5 mg L^{-1}; nicotinic acid, 25 mg L^{-1}; p-aminobenzoic acid, 25 mg L^{-1}; lipoic acid, 25 mg L^{-1}; d-pantothenic acid hemicalcium salt, 25 mg L^{-1}), and modified ATCC 1754 trace metal (micronutrients) solution (nitrilotriacetic acid, 2000 mg L^{-1}; $MnSO_4{\cdot}H_2O$, 1119 mg L^{-1}; $Fe(SO_4)_2(NH_4)_2{\cdot}6H_2O$, 800 mg L^{-1}; $CoCl_2{\cdot}6H_2O$, 200 mg L^{-1}; $ZnSO_4{\cdot}7H_20$, 200 mg L^{-1}; $CuCl_2{\cdot}2H_2O$, 20 mg L^{-1}; $NiCl_2{\cdot}6H_20$, 20 mg L^{-1}; $Na_2MoO_4{\cdot}2H_2O$, 20 mg L^{-1}; $Na_2SeO_3{\cdot}5H_2O$, 27 mg L^{-1}; $Na_2WO_4{\cdot}2H_2O$, 25 mg L^{-1}; H_3BO_3, 10 mg L^{-1}; $AlCl_3$, 10 mg L^{-1}). The stock solutions were added to deionized water in the following amounts: macronutrients, 20 ml L^{-1}; dipotassium hydrogen phosphate solution, 5 ml L^{-1}; sodium sulfate solution, 10 ml L^{-1}; sodium sulfide solution, 0.2 ml L^{-1}; vitamin solution, 10 ml L^{-1}; and trace metal solution, 10 ml L^{-1}. Yeast extract was also supplemented to the media to a final concentration of 0.5 g L^{-1}. In difference with Grimalt–Alemany [27], the sodium sulfide concentration was reduced four times, to limit precipitation and darkening of the media and the reactor and ease the OD measurements.

The source of inoculum for the batch experiments was a continuously stirred tank reactor (CSTR) with 4 L active volume and 1 L headspace, fed with 25 ml min^{-1} syngas (45% H_2, 25% CO_2, 20% CO, 10% N_2). The reactor was operated at a temperature of 37 °C, pH of 6, and a hydraulic retention time of 3 days, and it was originally inoculated with anaerobic sludge from the Lyngby–Taarbæk wastewater treatment plant (Denmark). Prior to inoculation, the sludge was subjected to a heat pretreatment to remove methanogenic archaea, by setting a temperature of 95 °C for 15 min, while continuously flushing with N_2 to keep anaerobic conditions. The CSTR was producing a mixture of acetic, butyric, and caproic acid, and therefore containing both acetogenic and chain elongating communities.

Experimental Methodology

All batch experiments were performed in triplicates, in 550 ml serum vials with 150 ml active volume and sealed with rubber stoppers. After adding the medium and flushing it with N_2, a 10% v/v inoculum was added to the vials. The headspace gas composition was set by injecting additional N_2, H_2, or CO_2, and the pH was adjusted using 5 M KOH and 1 M HCl solutions.

The first series of experiments was set up to investigate the effect of pH on the chain elongation and ethanol oxidation process. The inoculum was first adapted to the experimental conditions by running a series of vials at different pH values (5.3, 6, 6.8, and 7.5), with N_2 (1.5 atm) as headspace, and a mixture of ethanol and acetic acid at a 1:1 ratio as substrate. The experimental vials were set up at the same pH conditions using 10% inoculum from the adapted vials, N_2 as headspace, and an ethanol-to-acetate ratio of 5:2, to match the reverse β-oxidation reaction stoichiometry [12] and allow for some caproic acid production.

To study the effect of the gas composition on the ethanol oxidation phenomenon, a second series of batch experiments was set up using 10% inoculum directly from the CSTR, and an ethanol-to-acetate ratio of 5:3. A pH of 6.8 was chosen, as it was the one exhibiting the highest degree of ethanol oxidation. Three headspace gas compositions were tested: N_2: CO_2 (1:0.5 atm), N_2:H_2 (1.4:0.1 atm), and N_2 (1.3 atm) as a control (same conditions as the pH 6.8 vials of the pH-effect experiment). The rationale for the choice of the gas used was 1) to test the influence of CO_2 partial pressure, which could affect the oxidation of ethanol or volatile fatty acids, by scavenging the H_2 emitted in these processes [22, 24] and b) to test the influence of H_2 addition in the headspace, which, according to literature, should inhibit ethanol oxidation [18, 28]. As the conditions of the gas composition experiments were tailored around ethanol oxidation activity, possible associations between the ethanol oxidation activity and the microbial community were investigated as well. To obtain representative DNA samples of the experiment, the microbial cultures from the gas composition effect experiment were re-activated. New vials were prepared with the same conditions and inoculated with 10% v/v inoculum from the old vials. 10 ml liquid samples were then collected from each of the new vials for 16S rRNA analysis. The samples were centrifuged and the pellet frozen until the DNA extraction was performed.

Calculations and Thermodynamic Analysis

The efficiency of ethanol consumption towards chain elongation was calculated considering the amount of butyric and caproic acids produced as well as the stoichiometric ethanol needed. This way the degree of excessive ethanol oxidation under different pH conditions was assessed. According to Angenent and colleagues [12], intracellular bioenergetics suggest a required ethanol-to-acetate ratio of around 1.5–1.7 for butyric acid production, depending on their titers. Assuming a similar pathway for acetic and butyric acid elongation [6], the ratio of ethanol-to-acetate required for caproic acid formation would be of around 3.3–3.9. We then calculated the ethanol needed for the butyric and caproic acid produced and evaluated the efficiency of ethanol consumption towards chain elongation according to Eq. (4).

$$\%\,efficiency = \frac{Amount\ of\ ethanol\ needed\ for\ chain\ elongation}{Total\ amount\ of\ ethanol\ consumed} \cdot 100 = \frac{n_{but} \cdot v_{but} + n_{cap} \cdot v_{cap}}{n_{eth,con}} \cdot 100 \tag{4}$$

where n_{but} and n_{cap} are the amounts of butyric and caproic acid produced in mmol, respectively, v_{but} and v_{cap} are the stoichiometric needs in ethanol for butyric and caproic acid production, in mmol ethanol per mmol acid, respectively, and $n_{eth,con}$ is the amount of ethanol consumed, in mmol. The values used for v_{but} and v_{cap} depend on the chain elongation stoichiometry used. Taking into account the same ethanol requirements for acetic and butyric acid elongation, a 6:4 ethanol consumed to acid consumed stoichiometry led to v_{but} and v_{cap} being 1.20 and 2.31, respectively; while for a 5:3 ethanol-to-acid ratio the values of v_{but} and v_{cap} are 1.25 and 2.39.

The thermodynamic analyses were performed as in Grimalt–Alemany [29]. Gibbs free energy exchanges ($\Delta_r G^0$) of the reactions studied were calculated using the Gibbs free energy of formation (G_f^0) of the species involved, corrected for temperature and ionic strength, according to the Debye–Hückel Eq. (5). They were then corrected for the concentration of substrates and products taking part in the reaction (reaction 6).

$$G_f = G_f^0 - RT\alpha \frac{z_i^2 \sqrt{I}}{1 + B\sqrt{I}} \tag{5}$$

$$\Delta_R G = \Delta_R G^0 + RTln \frac{[C]^c [D]^d}{[A]^a [B]^b} \tag{6}$$

where R is the ideal gas constant (8.31 J), T is the temperature in Kelvin, α is a constant calculated as a value of the temperature, z_i is the charge of compound i, I is the ionic strength of the solution, B is an empirical constant with a value of 1.6 $L^{1/2}$ $mol^{-1/2}$ within a range of ionic strength of 0.05–0.25 M [30]. In Eq. (6), [C] and [D], and [A] and B], are the concentrations of products and substrates,

respectively, raised to the power of their respective stoichiometric coefficients. In the cases where acids were involved in the reactions, acid–base equilibriums were introduced within the correction for substrates and products, so that the effect of the pH is considered in the calculations. The detailed methodology and steps for the thermodynamic calculations are included in the Supplementary material.

Analytical Methods

Headspace composition was determined in a gas chromatograph (8610C, SRI Instruments, Germany) equipped with a thermal conductivity detector, and two packed columns, a 6′ × 1/8″ Molsieve 13 × column and a 6′ × 1/8″ silica gel column, connected in series through a rotating valve. The columns were kept at 65 °C for 3 min, followed by a 10 °C min^{-1} ramp till 95 °C, and a 24 °C min^{-1} till 140 °C. 50 µl gas samples were collected and injected with a gas-tight syringe (model 1750SL, Hamilton) [29]. Volatile fatty acids (VFAs) and alcohols were determined through a High-Performance Liquid Chromatograph (Shimadzu, USA) equipped with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, Denmark) maintained at 60 °C. 12 mM H_2SO_4 was used as eluent at a flow rate of 0.6 ml min^{-1}. The biomass concentration was monitored by measuring the optical density at a wavelength of 600 nm (OD_{600}) using a spectrophotometer (DR3900, Hach Lange). The pH of the broth was measured by taking 3-ml samples and using a PHM210 pH meter (Hach, USA).

DNA Isolation, Amplicon Sequencing and Microbial Community Analysis

The DNA was isolated from the samples using a DNeasy Powersoil Kit (Qiagen, Denmark) according to the manufacturer's recommendations. DNA samples were submitted to Macrogen Inc. (Korea) for 16S amplicon library preparation and sequencing using Illumina Miseq (300 bp paired-end sequencing). The libraries were constructed according to the 16S Metagenomic Sequencing Library Preparation Protocol (Part #15,044,223, Rev. B) using Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2. Regions V4–V5 of 16S rRNA gene were amplified with primers 515F (5′-GTG YCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGYCA ATTYMTTTRAGTTT-3′) [31]. Raw reads were primer-trimmed with cutadapt, discarding all untrimmed reads [32]. Low-quality tails were trimmed by a fixed length of 20 bases in forward reads and 60 bases in reverse reads. Trimmed reads were merged, quality filtered and denoised using DADA2 within the Qiime2 pipeline [33]. Taxonomic assignment to ASVs was performed using classify-sklearn algorithm and a taxonomic classifier based on MiDAS 4.81 database [33, 34]. Downstream analyses were performed using the Phyloseq (version 1.28.0), ggpubr (version 0.4.0), and R packages (version 3.6.0). The raw sequences obtained in this study are available in the NCBI SRA database with BioProject accession number PRJNA1013019.

Results and Discussion

Excessive Ethanol Oxidation Versus Chain Elongation at Different pH Conditions

To assess the influence pH has on the chain elongation reaction, four different pH were tested, and ethanol and acetic acid were added at a molar ratio of 5–2, with only N_2 in the headspace. It is important to note that, to ensure that methanogenic activity was effectively suppressed, samples were taken occasionally during the experiment, and methane was never detected. Figure 1 shows the concentration of the main extracellular metabolites during the experiments. In the first days of fermentation, a significant amount of ethanol was converted to acetic acid before the butyric acid production started, which was more evident at the highest pH conditions. A 6 ± 4%, 13 ± 2%, 36% ± 3%, and 31% ± 1% of the electron equivalents present in ethanol were diverted to the competing EEO pathway before the chain elongation starts at pH 5.3, 6, 6.8, and 7.5, respectively (Table S1). The pH remained rather stable in all experimental vials, within 0.5 pH units of the initial value.

The efficiency of ethanol consumption towards chain elongation was evaluated for the time interval where chain elongation took place (Fig. 1, Table 1). The lowest pH tested, 5.3, showed the highest ethanol consumption efficiencies towards chain elongation. This implies a suppression of the EEO reactions, which may be due to an inherent metabolic response of the cells towards less acidic products that is very commonly observed at acidic pH values [35, 36]. In turn, the suppression of EEO increased the ethanol availability for chain elongation, leading to higher caproic to butyric acid ratios [23, 37]. In overall, chain elongation generates less acid molecules per molecule ethanol (Eq. 3) compared to ethanol oxidation to acetic acid. Chain elongation reactors are usually not operated at low pH, as it increases the concentration of undissociated, longer fatty acids, which can be inhibitory for microbes at relatively low concentrations. This inhibition was not seen in the present study, as the low substrate concentrations used resulted in concentration of caproic acid below inhibitory levels. Nonetheless, it may become a severe obstacle in a process resulting in high concentrations of organic acids. In that case, operating reactors at low pH, with in-situ extraction of carboxylic acids [38, 39], might be a way

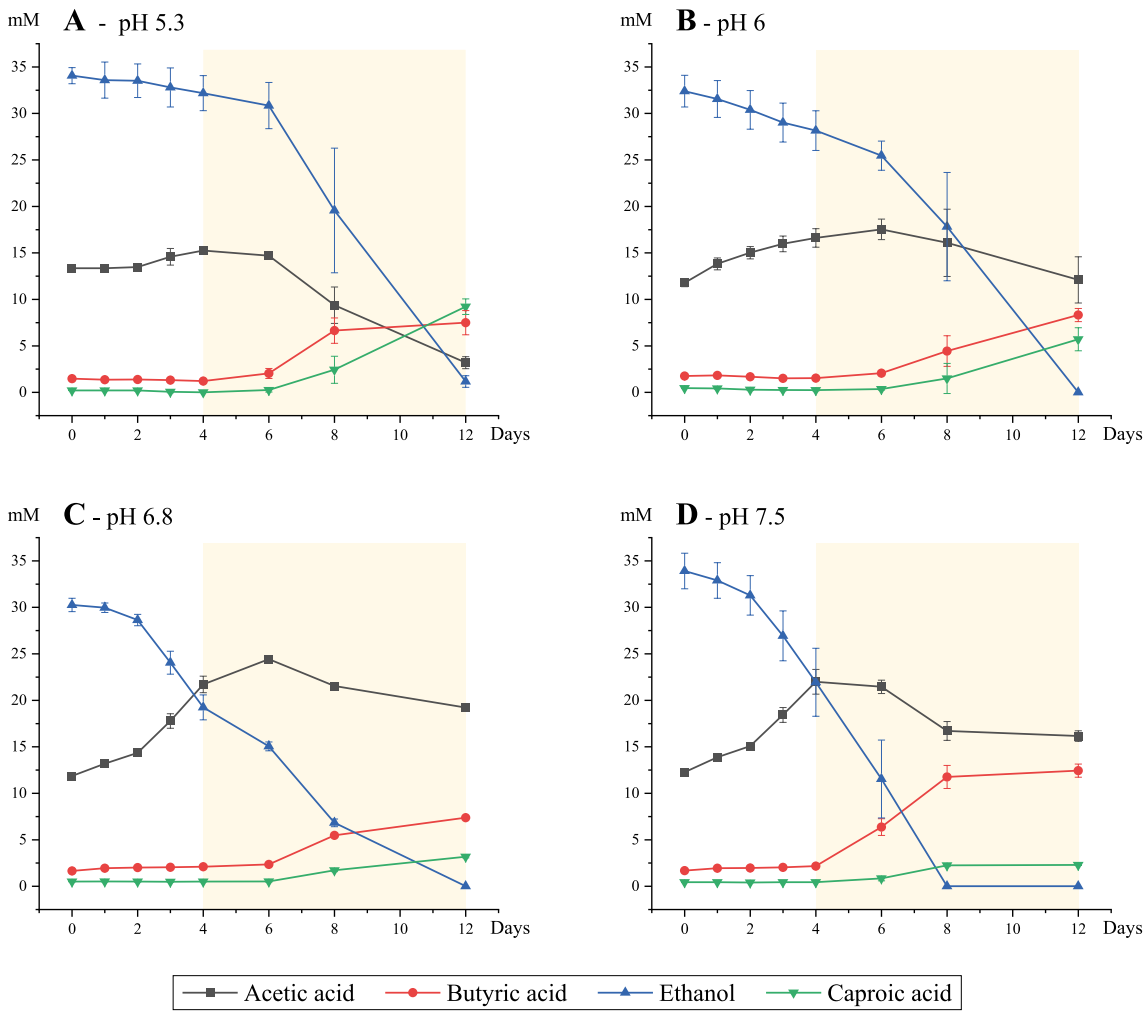


Fig. 1 Concentration of the main extracellular metabolites along the batch experiments at pH 5.3 (**A**), pH 6 (**B**), pH 6.8 (**C**), and pH 7.5 (**D**). The yellow-colored area indicates the part of the batch experiments when chain elongation took place. (Color figure online)

Table 1 Ratios of substrates and products calculated from the pH study batch experiment and resulting efficiencies of ethanol use in chain elongation. Ethanol consumption efficiencies were calculated using a molar stoichiometry of ethanol: acetic acid of 6:4[1] and 5:3[2], according to the formula described in "Calculations and Thermodynamic Analysis" section.

pH Condition	5.3	6	6.8	7.5
Ethanol consumed/acetic acid consumed	2.57	6.25	7.70	3.76
Caproic acid produced/butyric acid produced	1.47	0.81	0.51	0.18
Ethanol consumption efficiency (%)	93[1]–96[2]	74[1]–76[2]	65[1]–67[2]	76[1]–79[2]

to benefit from a reduced EEO and efficient chain elongation yield while avoiding the product inhibition from the protonated acids. At pH 5.3, the ethanol consumption towards elongated acids was matching the stoichiometrically predicted (1.2–1.25 and 2.3–2.4 mmol ethanol consumed per mmol butyric and caproic acid produced, respectively). On the other hand, at higher pH values, the efficiencies of ethanol use towards chain elongation were considerably lower than the theoretical values. Table 1 shows that at a pH range of 6–7.5, when considering only the chain elongation phase, 20–35% of the electron equivalents of the ethanol consumed are not coupled to butyric or caproic acid production. This underlines the importance of investigating the conditions that favor ethanol oxidation in a chain elongation process.

The Effect of H_2 and CO_2 on the Degree of Excessive Ethanol Oxidation in the Chain Elongation Process

One of the most established methods to suppress EEO is the increase of H_2 partial pressure. As H_2 is one of the main products of EEO, increasing its partial pressure would make the free energy change of the reaction less negative until the reaction becomes thermodynamically unfeasible. Several studies report the inhibition of methanogens as a sufficient measure to prevent EEO [19, 20]. Hydrogenotrophic methanogens consume H_2 and CO_2 to produce methane, thus keeping H_2 partial pressures low and allowing for excessive ethanol oxidizers to thrive. This symbiotic relationship is also called syntrophic ethanol oxidation. The suppression of methanogens via the heat-pretreatment of the inoculum, performed in this study, should have been an efficient method to prevent excessive ethanol oxidation from happening. However, other metabolic reactions could also play a role in keeping H_2 partial pressures low, such as homoacetogenesis, i.e., the generation of acetic acid from CO_2 and H_2 [22, 40]. Although CO_2 was not added to the experiment, yeast extract was added, as it is needed for an efficient chain elongation process [41], representing around 13% of the total organic carbon in the substrates. Fermentation of the organic compounds present in the yeast extract inevitably generates CO_2 that could act as an electron acceptor for homoacetogens.

To test the effect of additional H_2 and CO_2 in the excessive ethanol oxidation reaction, a second batch experiment was designed to test different headspace gas partial pressures, at a pH of 6.8. As it can be seen in Fig. 2, EEO was present in all three conditions. The vials with solely N_2 in the headspace (Fig. 2A, control) replicated successfully the first 6 days of the pH 6.8 condition in the pH-effect experiment (Fig. 1C). In the N_2: CO_2 vials (Fig. 2B), CO_2 addition seemed to accelerate ethanol uptake. Here, the ethanol was consumed in merely 6 days, in contrast to 12 days when only N_2 was present (Fig. 2A). Lastly, H_2 addition did not seem to be an efficient method to prevent the excessive ethanol oxidation pathway in this study (Fig. 2C), as a significant portion of the ethanol was oxidized to acetic acid within 6 days. Additionally, gas samples were also taken occasionally, and methane was not detected in the headspace.

The vials with N_2: CO_2 in the headspace were the only ones in which the carbon yield surpassed 100% (Fig. 3A). This was probably due to acetogenic metabolism and would indicate that CO_2 acted as an electron acceptor, facilitating the oxidation of ethanol to acetic acid. The addition of CO_2 and the subsequent production of acetic acid drastically lowered the pH of the fermentation, which reached values below 5 (Fig. 2B). This favored the chain elongation pathway over excessive ethanol oxidation, which can be seen in the higher ratio of C4–C6 acids produced per ethanol consumed (standardized to c-mol), and higher C6 acid produced per C4 acid produced (standardized to c-mol) (Fig. 3B). It should also be noted that the Chain Elongation phase of the N_2: CO_2 series accounted for 65–68% ethanol consumption efficiency. This means that a significant percentage of the ethanol oxidized was still not coupled to chain elongation, but probably to acetogenic metabolism.

To conclude, the fact that EEO was maintained at high H_2 partial pressures and in the absence of additional CO_2 (Fig. 2C), implied that another electron sink for EEO, able to shuttle electrons without H_2 evolution, was present in the fermentation broth. Apparently, sulfate ions added with the growth medium allowed sulfate reducing strains to thrive on ethanol as carbon and energy source[42, 43]. In the following sections, different routes, enabling excessive ethanol oxidation without H_2 production, are discussed from microbial community analysis and thermodynamics perspectives.

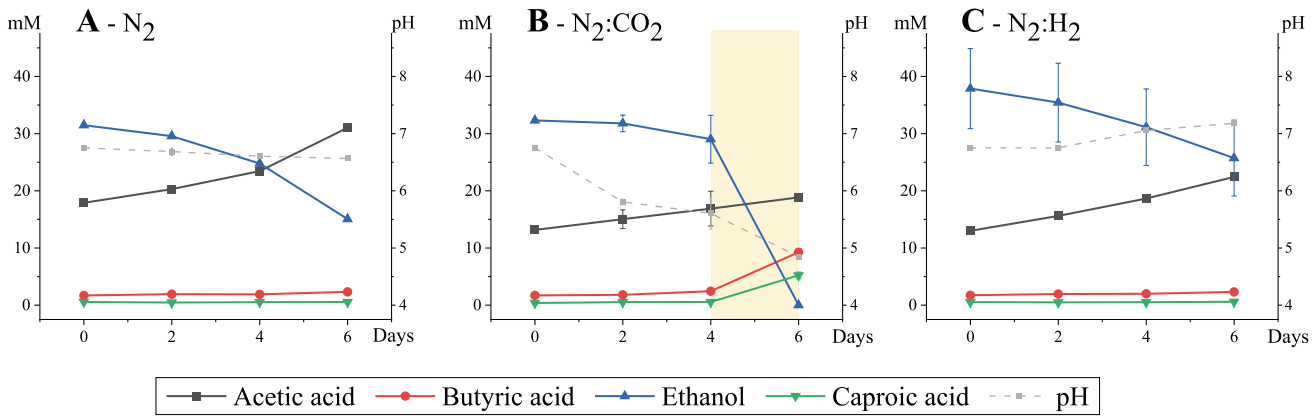


Fig. 2 Concentration in mM of the main extracellular metabolites along the batch experiments, with headspace gas compositions being N_2 (**A**), N_2: CO_2 (**B**), and N_2:H_2 (**C**). The pH value along the fermentation is shown as grey dashes. (Color figure online)

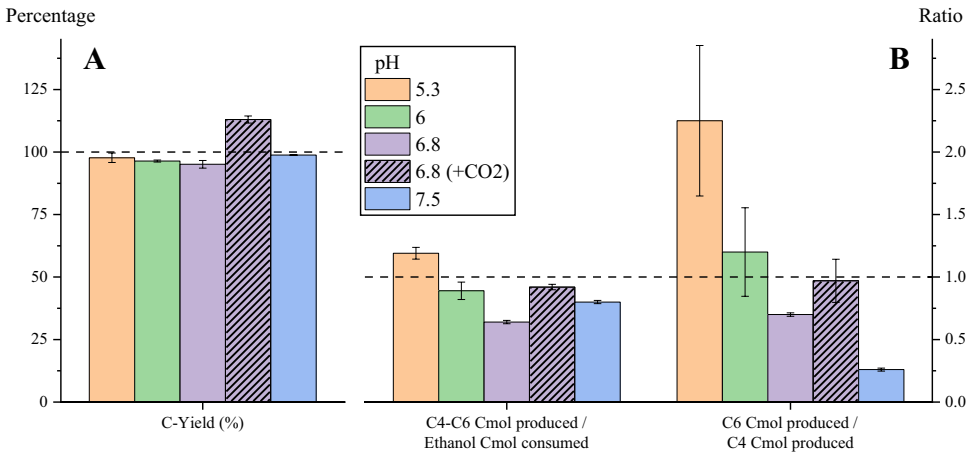


Fig. 3 Additional parameters describing the batch fermentations, for the four pH values tested and the supplementary CO_2 addition: % carbon yield excluding cell biomass and yeast extract (**A**); ratios of elongated acids produced over ethanol consumed, and of caproic acid produced over butyric acid produced (**B**)

The Effect of H_2 and CO_2 Addition on the Chain Elongating and Ethanol Oxidizing Bacterial Community

The presence of excessive ethanol oxidation even at relatively high H_2 partial pressures (0.1 atm) implies the existence of routes that enable ethanol oxidation without concomitant production of hydrogen. Such routes are usually not considered in studies addressing chain elongation reactions. The two main routes that will be discussed are acetogenesis and sulfate reduction, which offer alternative electron acceptors to facilitate ethanol oxidation without the terminal production of H_2. A thermodynamic analysis of these alternatives is shown in "Thermodynamic Analysis of Possible Coexisting Routes" section.

Some bacteria, such as *Acetobacterium woodii*, can couple ethanol degradation with acetogenesis, maintaining the intracellular redox balance without generating H_2 [24]. Through this route, gaseous carbon is fixed into liquid products through the reduction of CO_2 into acetic acid, following the stoichiometry proposed by Bertsch and colleagues [24] (reaction 9, Table 2). Other bacteria, such as *Desulfovibrio* spp., are capable of oxidizing ethanol in the presence of sulfate, by reducing it to hydrogen sulfide, thus avoiding H_2 generation [44, 45] (reaction 10, Table 2). A third option enabling excessive ethanol oxidation in the presence of relatively high H_2 partial pressures is the occurrence of direct interspecies electron transfer (DIET). DIET allows for redox syntrophic relations to happen, without the need for H_2 production as an intermediary. This means that microbes that can only perform the oxidation or reduction part of reactions (8) or (9) can still grow in syntrophic association with other microorganisms which can only perform the complementing reaction, by shuttling electrons through electrical contacts between bacteria [46]. Ethanol oxidation is one of the most studied DIET oxidation reactions [25, 47], and acetogenic bacteria have also shown potential to participate in DIET reactions [48].

To study whether the differences in the excessive ethanol oxidation profiles could be explained by changes in the microbial community, the 16S rDNA of the "gas composition effect" experiment vials was analyzed and presented in Fig. 4. Notably, the enrichment of the *Desulfovibrio* genus in the vials with N_2/H_2 (Fig. 4C) is especially relevant, as strains within this genus could drive ethanol oxidation without H_2 production by reducing sulfate. Sodium sulfate was present in the medium at a concentration of 1 g L^{-1} (7 mM) and, according to Eq. 10 (Table 2), it would allow for maximum 14 mM ethanol (out of the 30–35 added) to be oxidized into acetic acid. SRB can also oxidize acetate to CO_2 using

Table 2 Reaction stoichiometries used in the thermodynamic analysis and referenced throughout the article

Process	Reaction	Equ.
Chain Elongation	$6C_2H_6O + 4C_2H_4O_2^- \rightarrow 5C_4H_8O_2^- + H^+ + 4H_2O + 2H_2$	(7)
EEO with H_2 production	$C_2H_6O + H_2O \rightarrow C_2H_4O_2^- + H^+ + 2H_2$	(8)
EEO coupled with acetogenesis	$2\ C_2H_6O + 2\ CO_2 \rightarrow 3C_2H_3O_2^- + 3\ H^+$	(9)
EEO with sulfate reduction	$2C_2H_6O + SO_4^{2-} \rightarrow 2C_2H_3O_2^- + H_2S + 2H_2O$	(10)

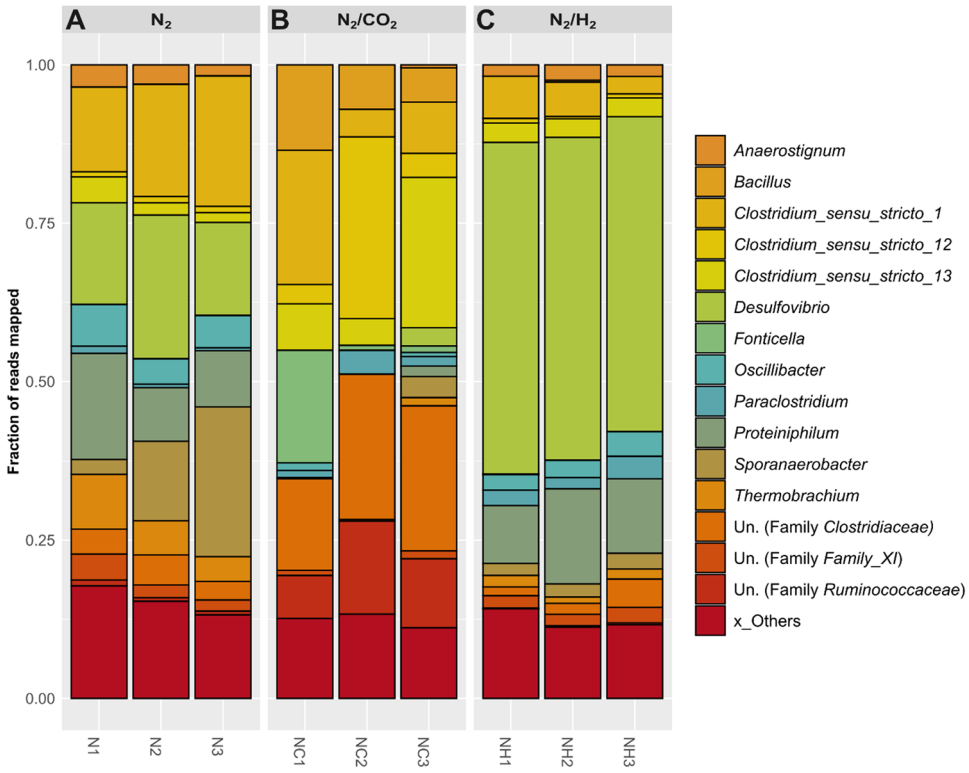


Fig. 4 Relative abundance of microbes identified to genus level for each vial of the gas composition effect experiment

sulfate as the electron acceptor, and this would reduce the amount of ethanol that SRB can oxidize. However, several studies have shown that mixed cultures of SRB growing on substrates such as ethanol or butyric acid produced mainly acetic acid (also seen in Fig. 2), as the oxidation of the latter may happen very slowly [49]. The fact that the relative abundance of *Desulfovibrio* was higher in the vials with H_2 partial pressures could be explained by the consumption of H_2 as an additional electron donor, but also by the inhibition of any H_2-based syntrophic growth i.e., syntrophic ethanol oxidation. This would give an advantage to some *Desulfovibrio* spp., which were able to use sulfate as an electron acceptor for ethanol oxidation, and thus avoid the production of H_2. In accordance with that, *Desulfovibrio* had the least relative abundance in the N_2/CO_2 condition, when the availability of electron acceptors was the highest (Fig. 4B). In this case, it may be that other microbes outcompeted *Desulfovibrio* spp. by performing acetogenic-coupled syntrophic ethanol oxidation.In fact, the N_2/CO_2 samples showed a consistent increase in unannotated genera from the *Clostridiaceae* and *Ruminococcaceae* families. Many species of the *Clostridiaceae* family, such as *Clostridium ljungdhalii* and *Clostridium kluyveri* are known as acetogens and reverse beta-oxidizers, respectively. *Ruminococcaceae* members are less studied for industrial purposes, but several species have been found involved in H_2 production [50], interspecies H_2-transfer [51], and butyrate production [52]. It is inferred that the presence of CO_2 might therefore have promoted acetogenic and chain elongating growth, which lowered the pH and prevented the growth of sulfate-reducing – ethanol oxidizing species. In the absence of CO_2, and especially in the presence of H_2, sulphate reducing bacteria (SRB) seem to be the main drivers of ethanol oxidation.

Thermodynamic Analysis of Possible Coexisting Routes

To find the feasibility boundaries of these pathways, a thermodynamic analysis of possible EEO routes was performed. Four reactions were analyzed: chain elongation of ethanol and acetic acid (Eq. 3); and ethanol oxidation coupled with H_2 production (Eq. 8), acetogenesis via CO_2 reduction (Eq. 9), and sulfate reduction (Eq. 10). It is important to note that thermodynamics do not affect the rate of a reaction, but only determine whether the reaction could potentially occur. For biological reactions to happen, a thermodynamic boundary lower than zero must be surpassed to allow the cells to grow or maintain cell functions [50, 53]. Thermodynamic boundaries at − 20 and − 10 kJ are thus shown in Fig. 5 and 6. Figure 5 shows the profiles of the free energy change of the 4 reactions

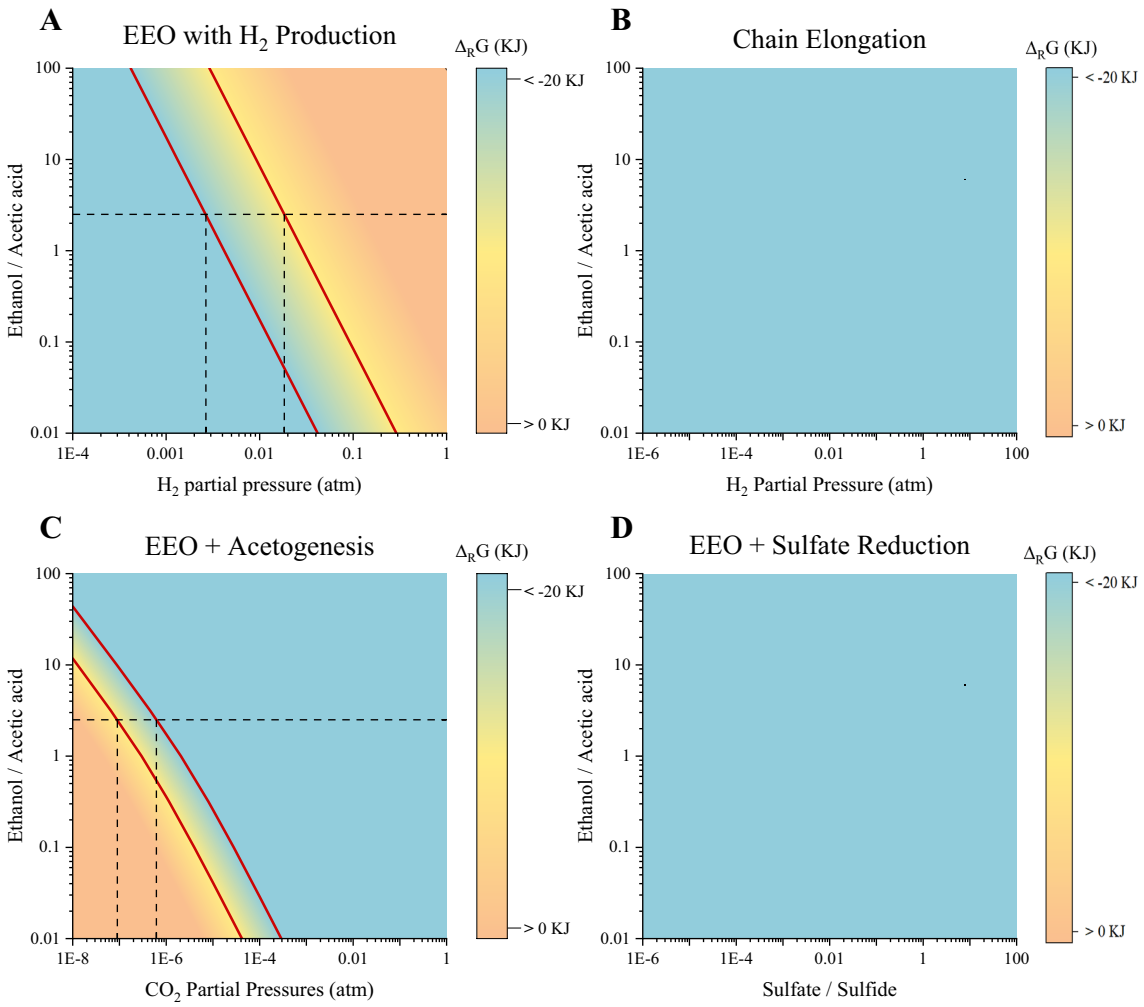


Fig. 5 $\Delta_r G$ of the reactions listed in Table 2 across different conditions. For these calculations, the concentration of ethanol plus acetic acid was kept constant at 0.1 M, the pH value was considered equal to 6.8, and the temperature equal to 310 K. Additionally, the butyric acid concentration used for the chain elongation reaction was 1 M, and the sulfate plus sulfide for the sulfate reduction reaction was kept constant at 7.8 mM (equal to the concentration in the batch experiments) The red lines correspond to the thermodynamic boundaries of − 20 and − 10 kJ

(Table 2) under different conditions. The chain elongation reaction (Fig. 5B) is feasible within the overall studied range of substrate ratios and H_2 partial pressures. On the other hand, ethanol oxidation with H_2 production (Fig. 5A) shows a clear thermodynamic feasibility boundary that falls within the range of conditions studied. At an ethanol-to-acetate ratio of 5–2, which is the initial ratio for all experiments, a H_2 partial pressure above 10^{-2} atm would make this reaction unfeasible. Therefore, to oxidize ethanol at H_2 partial pressures above 10^{-2} atm, bacteria must find a route to regenerate NAD^+ without producing H2.

Coupling ethanol oxidation with homoacetogenesis that consumes the excess electrons is one way to allow EEO to proceed. Figure 5C shows that carbon dioxide partial pressures as low as 10^{-6} already allow this combined pathway to happen, at a molar ethanol-to-acetate ratio of 5–2, and 0.1 M concentration of ethanol plus acetic acid. Another possible route is ethanol oxidation with sulfate as an external electron acceptor, which is exergonic over a wide range of conditions (Fig. 5D), even at low ethanol-to-acetate ratios and low sulfate concentrations. Therefore, both sulfate and carbon dioxide can potentially be used as electron sinks to facilitate EEO in the presence of high H_2 partial pressures. This applies when the coupling of the EEO and the reduction reactions (acetogenesis and sulfate reduction) happens intracellularly, or when the coupling with acetogenesis happens via DIET. For EEO and acetogenesis to be coupled through intraspecies H_2 transfer, both reduction and oxidation reactions must be thermodynamically feasible at the conditions studied.

Figure 6 shows the thermodynamic boundaries of the combined ethanol oxidation and acetogenic reactions. H_2

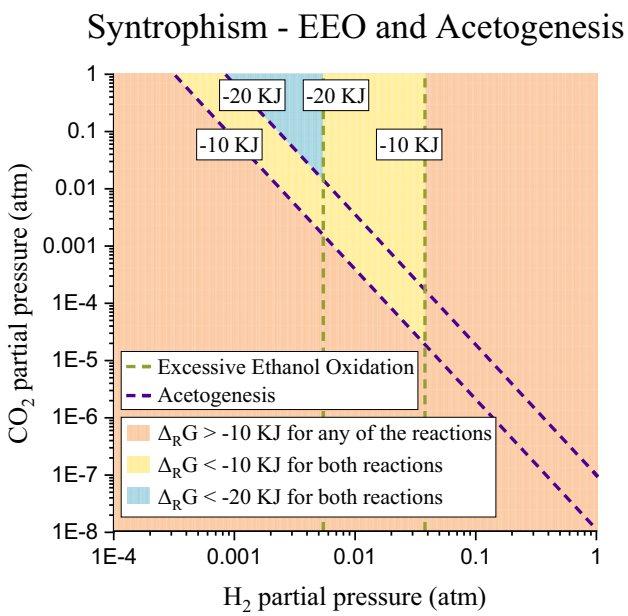


Fig. 6 Thermodynamic boundaries of the reactions considered for syntrophic ethanol oxidation (green and purple dashed lines) across different conditions. Colored areas show conditions enabling both reactions for a − 20 kJ boundary (blue) and a − 10 kJ (yellow). (Color figure online)

partial pressures above $4{\cdot}10^{-3}$–$4{\cdot}10^{-2}$ atm (depending on the feasibility boundary) would make any H_2-mediated syntrophism thermodynamically unfeasible (Fig. 6) for an ethanol-to-acetate ratio of 5:2 and a 0.1 M concentration of ethanol plus acetic acid. In addition, CO_2 partial pressures must also be above 0.02–$2{\cdot}10^{-6}$ atm (depending on the feasibility boundary), and a low H_2 partial pressure boundary, dependent on the CO_2 concentration, must also be surpassed. Therefore, CO_2 partial pressures surpassing 0.02 atm would allow acetogenic microbes to consume H_2 and keep its partial pressure low, facilitating the EEO with H_2 production. Chain elongating processes in the presence of CO_2, should therefore be operated at high ethanol-to-acetate ratios in the influent stream, to compensate the anticipated EEO [54].

Regarding the use of sulfate as an electron acceptor, as no thermodynamic restrictions can be imposed to inhibit the growth of SRB on ethanol, there is a need to find alternative methods to optimize the use of ethanol in chain elongation processes. These could be either a) the inhibition of SRB following other non-thermodynamic-based strategies, b) the use of alternative sulfur sources, or c) the limitation of the sulfate added to the necessary amount supporting growth. Several studies have examined the inhibition of SRB, but the methods are usually not selective and end up affecting other microbial groups as well [55, 56]. Moreover, because of the complexity of mixed cultures, inhibiting SRB might have an entirely different effect on the overall performance depending on the process studied. More research examining the effect of SRB-inhibiting strategies in chain elongation processes is needed; to the best of our knowledge, such studies are lacking in international literature. One way to suppress SRB could be by substituting sulfur sources with other compounds containing sulfur that cannot be used as terminal electron acceptors, such as cysteine or bisulfide [57, 58]. Another strategy could be limiting the amount of sulfur added. According to the stoichiometry of reaction 10 (Table 2), each gram of sulfate would allow for 0.64 g of ethanol to be oxidized. To optimize a chain elongation process, special attention should therefore be paid to supply only the sulfate necessary for growth, as any additional sulfate could be used to oxidize ethanol into acetic acid, reducing the yield of chain elongated products. Nonetheless, because of the high thermodynamic drive of the sulfate reduction reaction, the affinity of SRB to sulfate ions must also be considered, as SRB bacteria could still oxidize the trace sulfate ions added preventing thus other microbial groups to use it. Moreover, as shown by the pH-effect batch experiment, a pH as low as 5.3 (Fig. 1A, Table 1) greatly reduced the extent of excessive ethanol oxidation and resulted in a very efficient chain elongation process. pH reduction may therefore be an efficient way to inhibit SRB.

Conclusions

In this work, excessive ethanol oxidation, EEO, was proven to be a challenge for efficient chain elongation processes using mixed microbial cultures. Contrary to what is most times stated in recent literature, this challenge remained even after the suppression of methanogenic activity, and the supplementation with extra H_2 partial pressure. Through EEO, a significant portion of the ethanol substrate was oxidized to acetic acid over a wide pH range (6–7.5) instead of being used in chain elongation. Only the fermentations at pH 5.3 exhibited an efficient chain elongation process, most probably due to a metabolic response to low pH which inhibited the excessive oxidation of ethanol to acetic acid and favored elongation to caproic acid. The toxic effect of the undissociated chain elongated products, usually seen at low pH, was not observed in this study, because of the low substrate concentration used, which led to product titers below the toxicity threshold.

EEO was shown to take place in experiments with N_2, N_2:CO_2, and N_2:H_2 in the headspace. The fermentation profile (together with the 16S analysis of the cultures throughout the experiment) implies that EEO could have been driven by SRB in the absence of CO_2, while acetogenic bacteria outcompeted SRB as ethanol scavengers in the presence of CO_2. The addition of CO_2 also triggered a pH drop which positively affected the chain elongation process, though a

significant portion of the ethanol was still diverged into acetogenesis. A thermodynamic analysis of the main reactions confirmed that ethanol oxidation with the production of H_2 is thermodynamically unfeasible at H_2 partial pressures above 10^{-2} atm, as it is usually discussed in chain elongation papers referring to EEO. However, using both CO_2 and sulfate as electron acceptors would make EEO feasible even at high H_2 partial pressures. Besides, H_2-mediated syntrophic growth of ethanol oxidizers and homoacetogens was also shown to be feasible. Although thermodynamic restrictions cannot be applied to prevent SRB-mediated EEO, other methods such as low pH operation (with in-line extraction), the use of alternative sulfur salts, and the limitation of sulfur addition are proposed.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12649-023-02323-0.

Author Contributions All authors contributed to the study conception, design and methodology. Material preparation, data collection and analysis were performed by Cesar Quintela Garcia and Evi Peshkepia. The first draft of the manuscript was written by Cesar Quintela Garcia and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open access funding provided by Technical University of Denmark. This study was supported by the DTU PhD alliance scheme and DTU Chemical Engineering.

Data Availability Data availability can be accessed through the external portal.

Declarations

Conflict of interest The authors have no relevant financial or non-financial competing interests to disclose.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Pörtner, H.-O., et al.: Climate change 2022: impacts, adaptation and vulnerability. IPCC Geneva, Switzerland (2022)
2. Skea, J., Shukla, P., Kilkiş, Ş: Climate change 2022: mitigation of climate change. Cambridge University Press, Cambridge (2022)
3. Muscat, A., De Olde, E.M., de Boer, I.J.M., Ripoll-Bosch, R.: The battle for biomass: a systematic review of food-feed-fuel competition. Glob. Food Sec. **25**, 100330 (2020)
4. Mohanty, S.K., Swain, M.R.: 'Bioethanol production from corn and wheat: food, fuel, and future', in bioethanol production from food crops, pp. 45–59. Elsevier, Amsterdam (2019)
5. Kümmerer, K., Clark, J.H., Zuin, V.G.: Rethinking chemistry for a circular economy. Science **367**(6476), 369–370 (2020). https://doi.org/10.1126/science.aba4979
6. Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J.M., Angenent, L.T.: Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Curr. Opin. Biotechnol. **27**, 115–122 (2014). https://doi.org/10.1016/j.copbio.2014.01.003
7. Diender, M., Stams, A.J.M., Sousa, D.Z.: 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 (2016)
8. Fernández-Blanco, C., Veiga, M.C., Kennes, C.: Efficient production of n-caproate from syngas by a co-culture of Clostridium aceticum and Clostridium kluyveri. J. Environ. Manage. **302**, 113992 (2022). https://doi.org/10.1016/J.JENVMAN.2021.113992
9. Baleeiro, F.C.F., Kleinsteuber, S., Neumann, A., Sträuber, H.: Syngas-aided anaerobic fermentation for medium-chain carboxylate and alcohol production: the case for microbial communities. Appl. Microbiol. Biotechnol. **103**(21–22), 8689–8709 (2019)
10. Batlle-Vilanova, P., et al.: Microbial electrosynthesis of butyrate from carbon dioxide: production and extraction. Bioelectrochemistry **117**, 57–64 (2017). https://doi.org/10.1016/J.BIOELECHEM.2017.06.004
11. H. Seedorf et al., 'The genome of Clostridium kluyveri, a strict anaerobe with unique metabolic features' (2007). www.pnas.org/cgi/content/full/
12. Angenent, L.T., et al.: Chain elongation with reactor microbiomes: open-culture biotechnology to produce biochemicals. Environ. Sci. Technol. **50**(6), 2796–2810 (2016). https://doi.org/10.1021/acs.est.5b04847
13. Wu, Q., et al.: Medium chain carboxylic acids production from waste biomass: current advances and perspectives. Biotechnol. Adv. **37**(5), 599–615 (2019)
14. Seitz, H.-J., Schink, B., Pfennig, N., Conrad, R.: Energetics of syntrophic ethanol oxidation in defined chemostat cocultures: 1. Energy requirement for H_2 production and H_2 oxidation. Arch. Microbiol. **155**, 82–88 (1990)
15. Seitz, H.-J., Schink, B., Pfennig, N., Conrad, R.: Energetics of syntrophic ethanol oxidation in defined chemostat cocultures: 2. Energy sharing in biomass production. Arch. Microbiol. **155**, 89–93 (1990)
16. Feng, D., et al.: How can ethanol enhance direct interspecies electron transfer in anaerobic digestion? Biotechnol. Adv. **52**, 107812 (2021)
17. Konstantinidi, S., Skiadas, I.V., Gavala, H.N.: Microbial enrichment techniques on syngas and CO_2 targeting production of higher acids and alcohols. Molecules **28**(6), 2562 (2023)
18. Grootscholten, T.I.M., Strik, D.P.B.T.B., Steinbusch, K.J.J., Buisman, C.J.N., Hamelers, H.V.M.: Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol. Appl. Energy **116**, 223–229 (2014). https://doi.org/10.1016/J.APENERGY.2013.11.061
19. S. Shrestha, S. Xue, and L. Raskin, 'Effect of methanogenic inhibitor on competitive reactions during ethanol chain elongation. *Advancing Chain Elongation Technology for Medium Chain Carboxylic Acids Production from Waste Streams*, p. 44, (2021)
20. Zinder, S.H., Anguish, T., Cardwell, S.: Selective inhibition by 2-bromoethanesulfonate of methanogenesis from acetate in a thermophilic anaerobic digestor. Appl. Environ. Microbiol. **47**(6), 1343–1345 (1984)
21. Lovley, D.R., Dwyer, D.F., Klug, M.J.: Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl. Environ. Microbiol. **43**(6), 1373–1379 (1982)

22. Zavarzina, D.G., et al.: Syntrophic growth of alkaliphilic anaerobes controlled by ferric and ferrous minerals transformation coupled to acetogenesis. ISME J. **14**(2), 425–436 (2019). https://doi.org/10.1038/s41396-019-0527-4
23. Bao, S., et al.: Effect of acid/ethanol ratio on medium chain carboxylate production with different VFAs as the electron acceptor: insight into carbon balance and microbial community. Energies (Basel) **12**(19), 3720 (2019)
24. Bertsch, J., Siemund, A.L., Kremp, F., Müller, V.: A novel route for ethanol oxidation in the acetogenic bacterium Acetobacterium woodii: the acetaldehyde/ethanol dehydrogenase pathway. Environ. Microbiol. **18**(9), 2913–2922 (2016). https://doi.org/10.1111/1462-2920.13082
25. Summers, Z.M., Fogarty, H.E., Leang, C., Franks, A.E., Malvankar, N.S., Lovley, D.R.: Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. Science **330**(6009), 1413–1415 (2010). https://doi.org/10.1126/SCIENCE.1196526/SUPPL_FILE/SUMMERS.SOM.PDF
26. Ganigué, R., Sánchez-Paredes, P., Bañeras, L., Colprim, J.: Low fermentation pH is a trigger to alcohol production, but a killer to chain elongation. Front. Microbiol. **7**, 702 (2016). https://doi.org/10.3389/FMICB.2016.00702/BIBTEX
27. Grimalt-Alemany, A., Etler, C., Asimakopoulos, K., Skiadas, I.V., Gavala, H.N.: ORP control for boosting ethanol productivity in gas fermentation systems and dynamics of redox cofactor NADH/NAD+ under oxidative stress. J. CO_2 Util. **50**, 101589 (2021). https://doi.org/10.1016/j.jcou.2021.101589
28. Steinbusch, K.J.J., Hamelers, H.V.M., Plugge, C.M., Buisman, C.J.N.: Biological formation of caproate and caprylate from acetate: fuel and chemical production from low grade biomass. Energy Environ. Sci. **4**(1), 216–224 (2011)
29. Grimalt-Alemany, A., Łężyk, M., Lange, L., Skiadas, I.V., Gavala, H.N.: Enrichment of syngas-converting mixed microbial consortia for ethanol production and thermodynamics-based design of enrichment strategies. Biotechnol. Biofuels **11**(1), 1–22 (2018). https://doi.org/10.1186/s13068-018-1189-6
30. Alberty, R.A.: Thermodynamics of biochemical reactions. Wiley, Hoboken (2005)
31. Walters, W., et al.: Improved bacterial 16S rRNA gene (V4 and V4–5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. mSystems **1**(1), e00009-15 (2016)
32. Martin, M.: Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. **17**(1), 10–12 (2011). https://doi.org/10.14806/EJ.17.1.200
33. Bolyen, E., et al.: Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. **37**(8), 852–857 (2019). https://doi.org/10.1038/s41587-019-0209-9
34. Dueholm, M.K.D., et al.: MiDAS 4: a global catalogue of full-length 16S rRNA gene sequences and taxonomy for studies of bacterial communities in wastewater treatment plants. Nat. Commun. **13**(1), 1–15 (2022). https://doi.org/10.1038/s41467-022-29438-7
35. Gottschalk, G.: Bacterial fermentations. In: Gottschalk, G. (ed.) Bacterial metabolism, pp. 208–282. Springer, New York (1986)
36. Beales, N.: Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. Compr. Rev. Food Sci. Food Saf. **3**(1), 1–20 (2004). https://doi.org/10.1111/j.1541-4337.2004.tb00057.x
37. Liu, Y., Lü, F., Shao, L., He, P.: Alcohol-to-acid ratio and substrate concentration affect product structure in chain elongation reactions initiated by unacclimatized inoculum. Bioresour. Technol. **218**, 1140–1150 (2016). https://doi.org/10.1016/j.biortech.2016.07.067
38. Baroi, G.N., Skiadas, I.V., Westermann, P., Gavala, H.N.: Continuous fermentation of wheat straw hydrolysate by Clostridium tyrobutyricum with in-situ acids removal. Waste Biomass Valoriz. **6**, 317–326 (2015)
39. Malanca, A.A., et al.: Variables and mechanisms affecting electro-membrane extraction of bio-succinic acid from fermentation broth. Membranes (Basel) **12**(5), 542 (2022)
40. Stams, A.J.M., Elferink, S.O., Westermann, P.: Metabolic interactions between methanogenic consortia and anaerobic respiring bacteria. Biomethanation **I**, 31–56 (2003)
41. San-Valero, P., Abubackar, H.N., Veiga, M.C., Kennes, C.: Effect of pH, yeast extract and inorganic carbon on chain elongation for hexanoic acid production. Bioresour. Technol. **300**, 122659 (2020). https://doi.org/10.1016/J.BIORTECH.2019.122659
42. Muyzer, G., Stams, A.J.M.: The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. **6**(6), 441–454 (2008). https://doi.org/10.1038/nrmicro1892
43. Zhao, C., Chen, N., Liu, T., Feng, C.: Effects of adding different carbon sources on the microbial behavior of sulfate-reducing bacteria in sulfate-containing wastewater. J. Clean. Prod. **392**, 136332 (2023). https://doi.org/10.1016/J.JCLEPRO.2023.136332
44. Postgate, J.R., Campbell, L.L.: Classification of Desulfovibrio species, the nonsporulating sulfate-reducing bacteria. Bacteriol. Rev. **30**(4), 732–738 (1966)
45. Kremer, D.R., Nienhuis-Kuiper, H.E., Hansen, T.A.: Ethanol dissimilation in Desulfovibrio. Arch. Microbiol. **150**, 552–557 (1988)
46. Lovley, D.R.: Syntrophy Goes electric: direct interspecies electron transfer. Annu. Rev. Microbiol. **71**, 643–664 (2017). https://doi.org/10.1146/annurev-micro-030117-020420
47. Zhao, Z., Zhang, Y., Yu, Q., Dang, Y., Li, Y., Quan, X.: Communities stimulated with ethanol to perform direct interspecies electron transfer for syntrophic metabolism of propionate and butyrate. Water Res. **102**, 475–484 (2016). https://doi.org/10.1016/J.WATRES.2016.07.005
48. Kato, S., Yumoto, I., Kamagata, Y.: Isolation of acetogenic bacteria that induce biocorrosion by utilizing metallic iron as the sole electron donor. Appl. Environ. Microbiol. **81**(1), 67–73 (2015). https://doi.org/10.1128/AEM.02767-14
49. Hansen, T.A.: Carbon metabolism of sulfate-reducing bacteria. In: Odom, J.M., Singleton, R. (eds.) The sulfate-reducing bacteria: contemporary perspectives, pp. 21–40. Springer, New York (1993)
50. Nishio, N., Nakashimada, Y.: hydrogen generation from food industry and biodiesel wastes. Food Ind. Wastes (2013). https://doi.org/10.1016/B978-0-12-391921-2.00009-3
51. Iannotti, E.L., Kafkewitz, D., Wolin, M.J., Bryant, M.P.: Glucose fermentation products of Ruminococcus albus grown in continuous culture with Vibrio succinogenes: changes caused by interspecies transfer of H_2. J. Bacteriol. **114**(3), 1231–1240 (1973)
52. Morrison, D.J., Preston, T.: Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut microbes **7**(3), 189–200 (2016). https://doi.org/10.1080/19490976.2015.1134082
53. Steinbusch, K.J.J., Hamelers, H.V.M., Buisman, C.J.N.: Alcohol production through volatile fatty acids reduction with hydrogen as electron donor by mixed cultures. Water Res. **42**(15), 4059–4066 (2008)
54. Roghair, M., et al.: Controlling ethanol use in chain elongation by CO_2 loading rate. Environ. Sci. Technol. (2018). https://doi.org/10.1021/acs.est.7b04904
55. Chidthaisong, A., Conrad, R.: Specificity of chloroform, 2-bromoethanesulfonate and fluoroacetate to inhibit methanogenesis and other anaerobic processes in anoxic rice field soil. Soil Biol. Biochem. **32**(7), 977–988 (2000). https://doi.org/10.1016/S0038-0717(00)00006-7

56. Zhang, Z., et al.: Competition and cooperation of sulfate reducing bacteria and five other bacteria during oil production. J. Pet. Sci. Eng. **203**, 108688 (2021). https://doi.org/10.1016/J.PETROL.2021.108688
57. Lonkar, S., Fu, Z., Holtzapple, M.: Optimum alcohol concentration for chain elongation in mixed-culture fermentation of cellulosic substrate. Biotechnol. Bioeng. **113**(12), 2597–2604 (2016)
58. Robles, A., Yellowman, T.L., Joshi, S., Mohana Rangan, S., Delgado, A.G.: Microbial chain elongation and subsequent fermentation of elongated carboxylates as H_2-producing processes for sustained reductive dechlorination of chlorinated ethenes. Environ. Sci. Technol. **55**(15), 10398–10410 (2021)

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Authors and Affiliations

Cesar Quintela[1] · Evi Peshkepia[1] · Antonio Grimalt-Alemany[1] · Yvonne Nygård[2] · Lisbeth Olsson[2] · Ioannis V. Skiadas[1] · Hariklia N. Gavala[1]

✉ Hariklia N. Gavala
hnga@kt.dtu.dk; hari_gavala@yahoo.com

[1] Department of Chemical and Biochemical Engineering, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

[2] Division of Industrial Biotechnology, Department of Life Sciences, Chalmers University of Technology, Gothenburg, Sweden