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Short Communication

Upgrading dilute ethanol from syngas fermentation to *n*-caproate with reactor microbiomes



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HIGHLIGHTS

- *n*-Caproic acid was produced from actual syngas fermentation effluent.
- The carboxylate platform was integrated with the syngas platform.
- Detrimental effect of methanogenesis on *n*-caproic acid concentration established.

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ABSTRACT

Fermentation of syngas from renewable biomass, which is part of the syngas platform, is gaining momentum. Here, the objective was to evaluate a proof-of-concept bioprocessing system with diluted ethanol and acetic acid in actual syngas fermentation effluent as the substrate for chain elongation into the product *n*-caproic acid, which can be separated with less energy input than ethanol. Chain elongation is performed with open cultures of microbial populations (reactor microbiomes) as part of the carboxylate platform. The highest concentration of *n*-caproic acid of $\sim 1 \text{ g L}^{-1}$ was produced at a pH of 5.44 and a production rate of $1.7 \text{ g L}^{-1} \text{ day}^{-1}$. A higher *n*-butyrate production rate of $20 \text{ g L}^{-1} \text{ day}^{-1}$ indicated that product toxicity was limiting the chain elongation step from *n*-butyric acid to *n*-caproic acid. This result shows that the syngas and carboxylate platforms can be integrated within a biorefinery, but that product separation is necessary.

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1. Introduction

Bio-based fuels research and development has flourished during the last decade with the goal to develop renewable and sustainable alternatives to fossil fuels. Although solar and wind energy have proven to be a sustainable alternative to fossil fuels, there is still a need for sustainable liquid fuels. Worldwide, ethanol has remained the premier biofuel. However, ethanol distillation is an energy-intensive process that considerably impacts the ratio of energy returned vs. energy invested (Schrier, 2012). It is, therefore, desirable to circumvent ethanol distillation as a method for product recovery (Agler et al., 2012).

An anaerobic fermentation process with open cultures (reactor microbiomes) and fed with dilute, undistilled ethanol can catalyze a chain-elongating reaction via a well-understood reversed β -oxidation pathway, leading to the production of *n*-caproic acid (Agler et al., 2011; Kenealy et al., 1995). This

process is part of the carboxylate platform and includes bacteria in the reactor microbiome, such as *Clostridium kluyveri*, which are known to elongate carboxylic acid carbon chains (Agler et al., 2012). *n*-Caproic acid is a 6 carbon chain carboxylic acid that is more hydrophobic in comparison to ethanol, and therefore easier to extract (Steinbusch et al., 2011). A recent study has demonstrated *n*-caproic acid separation by in-line, liquid–liquid extraction (i.e., pertraction) from recycled liquor from a bioreactor (Agler et al., 2012), providing a potential method to avoid or reduce the use of ethanol distillation. Production of *n*-caproic acid has at least two other benefits compared to ethanol. First, *n*-caproic acid is more energy dense than ethanol (Steinbusch et al., 2011) and can be converted to liquid biofuels (Agler et al., 2011). Second, *n*-caproic acid is a more valuable commodity that is already used: (i) in animal feed rations; (ii) as an antimicrobial (Skřivanová and Marounek, 2007); and (iii) as a feedstock for esterification into food products (Minich, 1960).

An anaerobic fermentor, which is optimized for chain elongation, has a different reactor microbiome compared to an anaerobic digester (both in the carboxylate platform). For efficient chain

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elongation, the acetoclastic methanogens must be mostly inhibited to prevent the intermediate acetic acid from being converted into methane. In addition, it is important to maintain high enough hydrogen partial pressures to prevent oxidation of short- and medium-chain carboxylic acid, and thus hydrogenotrophic methanogens must be curtailed, possibly by limiting the availability of carbon dioxide. In conventional anaerobic digestion, the pH is maintained near neutral to increase methane production. In chain-elongating fermentors, methanogenesis can be suppressed by the addition of an inhibitor such as 2-bromoethanesulfonate (Zinder et al., 1984; Steinbusch et al., 2011). However, this is an expensive chemical and would not be economically feasible to be used for large-scale production of *n*-caproic acid. As an alternative, methanogens can be inhibited by maintaining the pH at 5.5 (Agler et al., 2012; VanKessel and Russell, 1996); or by completely excluding the presence of methanogens through the use of an inoculum devoid of methanogens and the use of synthetic substrates (Grootscholten et al., 2013a).

Several reactor configurations have been used to study chain elongation with reactor microbiomes, including a batch reactor (Kenealy et al., 1995), an upflow fixed bed reactor (Ding et al., 2010), an anaerobic sequencing batch reactor (Agler et al., 2012), and the anaerobic filter (AF) reactor (Grootscholten et al., 2013a). The highest production rates, thus far, have been achieved with continuously-fed AFs. Grootscholten et al. (2013a) achieved an *n*-caproic acid production rate of $16 \text{ g L}^{-1} \text{ day}^{-1}$ with a product selectivity of 85%, and in a later study even an *n*-caproic acid production rate of $\sim 55 \text{ g L}^{-1} \text{ day}^{-1}$ with a product selectivity of $\sim 80\%$ (Grootscholten et al., 2013b). It is important to realize that the substrate for these two studies consisted of a synthetic substrate that is a mixture of procured ethanol and acetic acid with added growth factors and nutrients.

On the other hand, the study by Agler et al. (2012) fed a $\sim 1/6$ diluted stream of real, complex substrate (fermentation beer with 15% [w/w] ethanol) to a 5-L anaerobic fermentor. This substrate was withdrawn from the beer well at a corn kernel-to-ethanol plant and contained leftover corn kernel solids and yeast cells (Agler et al., 2012). Besides base (NaOH), no other growth factors or nutrients were added to the influent. In the quest to find other actual substrates, dilute ethanol and acetic acid obtained in effluent from a synthesis gas fermentation setup was identified as a potential substrate for the chain-elongating process.

Synthesis gas, which is referred to as syngas and comprises of a mixture of carbon monoxide, hydrogen, and carbon dioxide, can be produced sustainably with the syngas platform through a thermochemical step with biomass feedstock such as wood waste or willow (Munasinghe and Khanal, 2010). The produced syngas is converted to ethanol and acetic acid using carboxydutrophic bacteria, including *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Acetobacterium woodii*, *Clostridium carboxidivorans*, or *Peptostreptococcus productus*. Syngas fermentation setups with a pure culture of one of these carboxydutrophic bacteria can achieve ethanol concentrations of 2–4% (w/w) (Richter et al., 2013). The two-stage, 5-L syngas fermentation system that was operated in the Angenent Lab has achieved ethanol concentrations of up to 3% and a volumetric production rate of $7.3 \text{ g L}^{-1} \text{ day}^{-1}$ ($\sim 0.3 \text{ g L}^{-1} \text{ h}^{-1}$) (Richter et al., 2013). It is anticipated that effluent from syngas fermentation systems could consist of many of the growth factors and nutrients for chain elongation, even though further studies are necessary to ascertain this.

Here, a proof-of-concept study was evaluated to answer the question whether real, filtered syngas fermentation effluent, including dilute ethanol and acetic acid, can be used as a substrate to a continuously fed, chain-elongating bioreactor to circumvent ethanol distillation. In this project, it has been shown that the inte-

gration of the syngas platform with the carboxylate platform may be technically feasible after system improvements are made, and a discussion of these improvements is provided.

2. Methods

2.1. Inoculum and substrate for the bioreactor

The inoculum for the chain-elongating bioreactor was obtained from the effluent of a 5-L chain-elongating fermentor, which was fed corn beer for ~ 2 years and included a liquid–liquid extraction system (Agler et al., 2012). The substrate for the chain-elongating bioreactor was the effluent from a two-stage, 5-L syngas fermentor with a pure culture of *C. ljungdahlii* strain ERI-2 (Richter et al., 2013). The synthetic syngas to the syngas fermentor consisted of a procured gas mixture of 65% carbon monoxide, 5% carbon-dioxide, and 30% hydrogen (Airgas East, Ithaca, NY). The substrate that was fed to the chain-elongation bioreactor during days 0–23 of the operating period (Batch I) included ethanol and acetic acid concentrations of 11.4 and 2.3 g L^{-1} , respectively. The substrate that was fed during Days 24–29 (Batch II) consisted of considerably lower ethanol and acetic acid concentrations that were artificially maintained at 1.8 and 1.2 g L^{-1} , respectively, with procured chemicals, because of an unanticipated crash in the clostridia production rates during that period in the syngas fermentation system. Both Batch I and II substrate solutions were supplemented with 10 mL L^{-1} trace metal solution (Rajagopalan et al., 2002), 5 mL L^{-1} $2\times$ vitamin solution (Rajagopalan et al., 2002), 1 mL L^{-1} yeast extract, and 2.9 g L^{-1} of potassium bicarbonate (it was not investigated whether these growth factors and nutrients were necessary). Bicarbonate was added because *C. kluyveri* (one of the microbial species that performs reversed β -oxidation pathway) requires carbon dioxide for its metabolism (Kenealy et al., 1995). 5 mM resazurin was added as an oxygen indicator.

2.2. Operating conditions for the anaerobic filter

The AF, with a total volume of 700 mL, was made from plastic with a cone at the bottom, and contained porcelain berl saddles with an outside diameter of 8 mm (CG-1284, Chemglass Life Sciences, Vineland, NJ), resulting in an active volume of 425 mL. The bioreactor was operated in upflow mode and the feed substrate was pumped with a peristaltic pump (model 7528-10, Cole-Parmer, Vernon Hills IL) from a refrigerator (4°C) to the bottom of the AF at a flow rate of 0.5 mL min^{-1} , resulting in a hydraulic retention time (HRT) of 14 h (Fig. 1). The mixed liquor of the AF was continuously recirculated using a peristaltic pump (model 7553-10, Cole-Parmer) at a flow rate of 22.7 mL min^{-1} . The temperature was maintained at 30°C with a custom-built recirculating water heater and a hose wrapped around the bioreactor. The pH of the system was maintained at 5.5–5.7 for the first 6 days of the operating period, was increased to close to 6.5 between days 6 and 10, and was decreased again to below 5.5 after day 10. 0.5 M KOH or 0.5 M HCl was pumped for maintaining the pH at the set point by using a controller (alpha-pH 8000, Eutech Instruments, Singapore).

The offgas was passed through a bubbler and into a gas meter (MilliGascounter type MGC-1 PVDF, Ritter, Bochum, Germany) to determine the gas production rate (Fig. 1). At the start of the operating period, the bioreactor was completely filled with inoculum and the recirculation pump was operated for 24 h without the addition of substrate. After 24 h, the bioreactor was fed with substrate and sample volumes of 2 mL were collected daily during the operating period and centrifuged at $9.3g$ for 10 min. The supernatant was frozen at -20°C for further analysis.

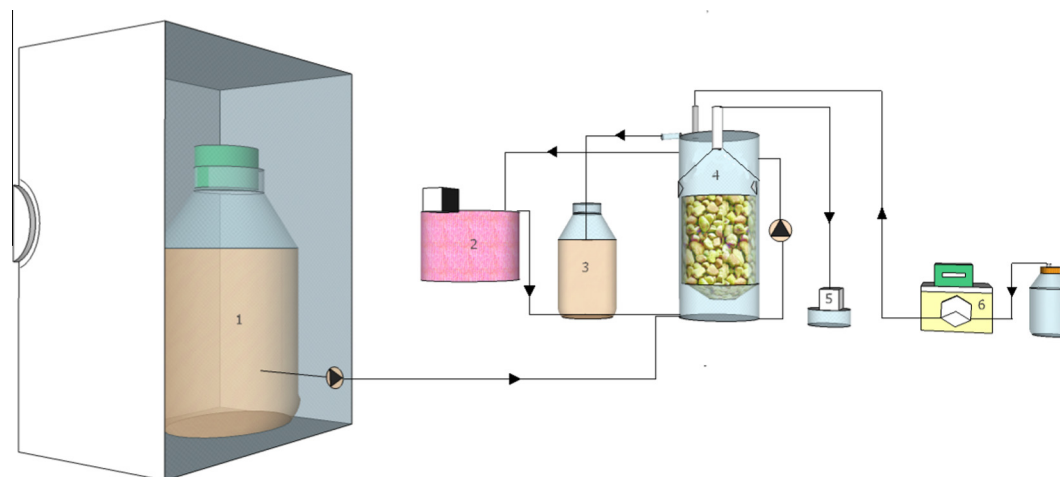


Fig. 1. Schematic of the bioreactor setup: (1) feed in refrigerator; (2) recirculating water bath with temperature controller; (3) effluent storage container; (4) anaerobic filter (AF) with internal recirculation; (5) gas flow meter; and (6) pH controller with KOH storage container.

2.3. Analytical techniques

For the analysis of short-chain and medium-chain carboxylic acids, the supernatant was diluted 2 times or 10 times with 2% formic acid. The sample was diluted 5 times in deionized water for the measurement of ethanol. A gas chromatography system (HP 5890, Hewlett Packard, Palo Alto, CA) was used, as detailed in the study by Perez et al. (2013) to measure carboxylic acids or ethanol. Gas samples were analyzed with two gas chromatographs (series 580, Gow Mac, Bethlehem, PA) (Perez et al., 2013).

3. Results and discussion

3.1. Concentrations of carboxylic acids and ethanol

When the ethanol concentration decreased in the continuously-fed AF (due to consumption) an increase in the carboxylic acid concentration was observed as anticipated in a functioning chain-elongating bioreactor (Fig. 2a–d). The highest carboxylic acid concentrations were observed on day 23–24 of the operating period when the ethanol concentration was depleted from 11.4 g L^{-1} in Batch I to below the detection limit and the acetic acid, *n*-butyric acid, and *n*-caproic acid concentrations were 7.9, 19.4, and 1.0 g L^{-1} , respectively (Fig. 2a–d). Chain elongation had increased over the initial operating period of 24 days by mainly producing *n*-butyric acid and some *n*-caproic acid (Fig. 2c and d). This resulted in *n*-butyric acid and *n*-caproic acid production rates of 20 and $1.7 \text{ g L}^{-1} \text{ day}^{-1}$ (36 and $3.8 \text{ g COD L}^{-1} \text{ day}^{-1}$, respectively, with a volumetric loading rate for ethanol and acetate of $45 \text{ g COD L}^{-1} \text{ day}^{-1}$). The system achieved an *n*-butyric acid selectivity of 88% and an *n*-caproic acid selectivity of 9.6% (based on COD). Chain elongation was diminished when ethanol and acetate concentrations dropped in Batch II as anticipated (Fig. 2). In a recent study, a much lower *n*-caproic acid production rate of $2.5 \times 10^{-2} \text{ g L}^{-1} \text{ day}^{-1}$ was reported by feeding H_2 and CO_2 directly into a chain-elongating biofilm system (Zhang et al., 2013). The far superior production rates here may indicate that ethanol and acetate production in a separate syngas fermentation system followed by a chain elongation bioprocess is advantageous, possibly because ethanol production through acetate reduction in a reactor microbiome has been characterized as a slow biological process (Agler et al., 2011). Syngas fermentation systems, on the other hand, have been optimized to specifically accelerate acetate reduction into ethanol (Richter et al., 2013).

3.2. Correlation between methane composition and *n*-caproic acid concentration and pH

Avoidance of acetoclastic methanogenesis is one of the primary concerns in chain-elongation with reactor microbiomes to prevent acetic acid cleavage towards methane production rather than medium-chain carboxylic acid production. The bioreactor that was operated in this study had been inoculated with the effluent from a mesophilic (30°C) chain-elongating fermentor, which was maintained at a pH of 5.5 during a 2 year operating period, and which had been characterized to produce methane by hydrogenotrophic methanogenesis rather than acetoclastic methanogenesis. A similar concept of inoculating an AF reactor with the effluent of the bioreactor from an operating chain-elongating system had been successful (Grootscholten et al., 2013a). For the latter study, a pH 7.0 in the bioreactor was possible without the emergence of acetoclastic methanogenesis because the inoculum completely lacked methanogens after a long-term operation with the methanogen inhibitor 2-bromoethanesulfonate (2-BES).

However, in the study here, a pH level of close to 6.5 resulted in a 80% methane content of the headspace gas after only 10 days of the operating period even though hydrogen fluxes were anticipated to be low (only ethanol and acetate were fed) (Fig. 3a and b). A simple and yet efficient method of controlling acetoclastic methanogenesis is by decreasing the pH to 5.5 (VanKessel and Russell, 1996), and therefore the pH was decreased to this pH value on day 10 of the operating period (Fig. 3b). The anticipated inhibition of acetoclastic methanogenesis is not just because of a lower pH value but also due to inhibition by increased concentrations of undissociated carboxylic acids (the slightly acidic pH of 5.5 is close to the pK_a values of 4.84 and 4.88 for *n*-butyric acid and *n*-caproic acid, respectively). For example, the pH decrease resulted in an increase in the undissociated *n*-caproic acid concentration to 0.17 g L^{-1} (Fig. 2e), while the total concentration of *n*-caproic acid remained $\sim 0.4 \text{ g L}^{-1}$ until day 15 of the operating period (Fig. 2d). This, and the requirement to flush out headspace gas, explains the lag period of almost 9 days for the decrease in the methane percentage to occur due the drop in pH on day 10 of the operating period (Fig. 3a and b).

Alternatively to a pH decrease, the bioreactor could have been subjected to methanogen inhibitors, such as 2-BES (Zinder et al., 1984), which is an expensive chemical rendering a large-scale application economically unviable. Grootscholten et al. (2013a) showed that methanogens did not grow in their system over the operating period of 80 days even though the pH was maintained

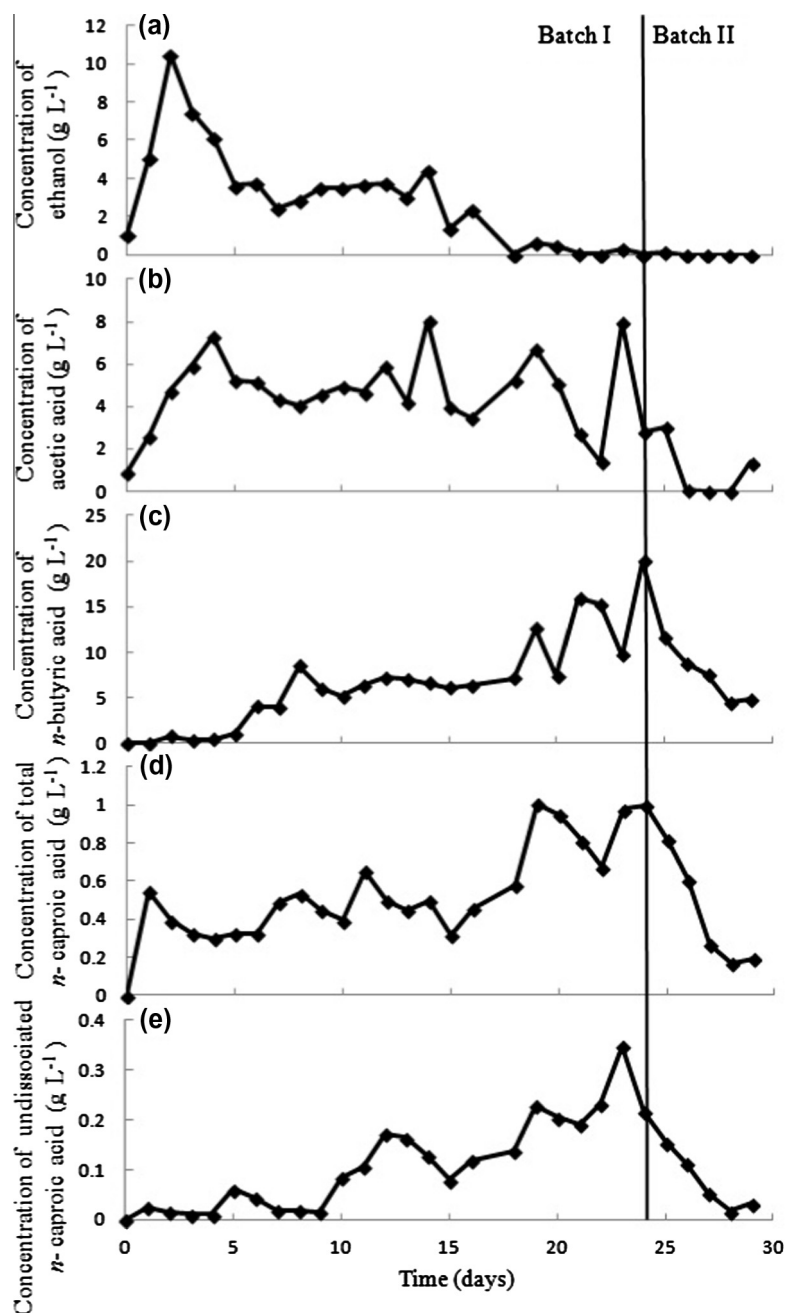


Fig. 2. Composition of the chain-elongating bioreactor effluent over the operating period: (a) concentration of ethanol; (b) concentration of acetic acid; (c) concentration of *n*-butyric acid; (d) concentration of total *n*-caproic acid; and (e) concentration of undissociated *n*-caproic acid. Batch I substrate contained an ethanol and acetic acid concentration of 11.4 and 2.31 g L⁻¹, respectively, while Batch II substrate contained considerably lower concentrations due to technical difficulties for the syngas fermentor.

at around 7.0 without 2-BES. However, this may not preclude methanogens to accumulate slowly during very long operating periods in an open-culture system with realistic influent streams (Luo et al., 2011). The lower pH value of 5.5 would preclude long-term establishment of acetoclastic methanogenesis (Agler et al., 2012), but operating a chain-elongation bioprocess at such a lower pH value results in lower product concentrations due to the toxicity of undissociated carboxylic acids.

3.3. Toxicity of *n*-caproic acid

The highest concentration of undissociated *n*-caproic acid was 0.35 g L⁻¹ at a pH level of 5.44 on day 24 of the operating period

(Fig. 2e). At this pH level, 22% of the total *n*-caproic acid was present in the undissociated form, while this would have been 0.8% at a pH of 7.0 (Fig. 2d and e). The concentration of undissociated *n*-caproic acid would have inhibited microbial activity and likely its own production (product inhibition) (Steinbusch et al., 2011), which is postulated here to be the reason why chain elongation resulted in mostly *n*-butyric acid production rather than to include a second elongation step towards *n*-caproic acid production. In agreement with this theory, higher production levels of *n*-caproic acid have only been observed for: (1) neutral pH values (Groot-scholten et al., 2013a); or (2) similar pH values of 5.5 but with a system that included the in-line extraction of the final product *n*-caproic acid (Agler et al., 2011). At a pH level of ~5.5, the *n*-caproic

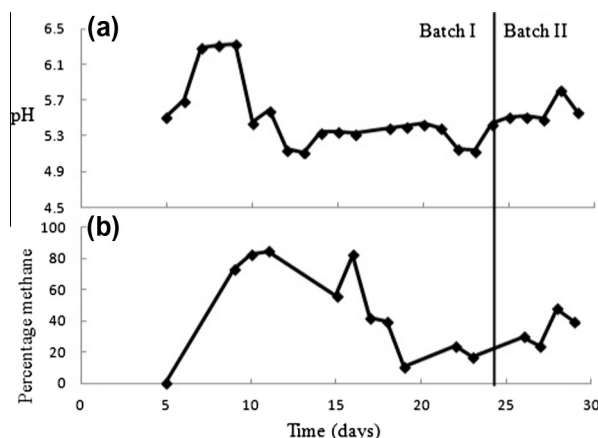


Fig. 3. Operating and performance parameters during the operating period: (a) pH level of the effluent; and (b) percentage of methane in the headspace gas. Batch I substrate contained an ethanol and acetic acid concentration of 11.4 and 2.31 g L⁻¹, respectively, while Batch II substrate contained considerably lower concentrations due to technical difficulties for the syngas fermentor.

acid concentration must be maintained below an inhibitory level by coupling the AF reactor with in-line extraction of *n*-caproic acid.

4. Conclusion

Here, the results of a proof-of-concept system show merit of coupling syngas fermentation with chain elongation from the carboxylate platform. Volumetric production rates for *n*-butyrate and *n*-caproic acid were 20 and 1.7 g L⁻¹ day⁻¹, respectively, by elongating acetic acid with ethanol. Decreasing the pH was a viable solution to inhibit acetoclastic methanogenesis and re-route production towards chain elongation. However, the relatively low product selectivity of 9.6% for *n*-caproic acid vs. 88% for *n*-butyric acid from ethanol and acetate indicates that in-line *n*-caproic acid extraction is necessary to circumvent product toxicity at a pH value of ~5.5.

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