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

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Engineering in Biological and Environmental Engineering

by

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May 2013

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ABSTRACT

Fermentation of synthesis gas (syngas, which is a gas mixture including CO, H_2, and CO_2) from a renewable biomass source is gaining momentum. However, energy-intensive distillation of ethanol at dilute concentrations of 2-4% (w/w), which are anticipated for syngas fermentation with carboxydotrophic bacteria, is one impediment towards widespread adaptation. *n*-Caproic acid is a carboxylic acid with a chain length of six carbon atoms, and can be extracted more easily compared to ethanol. This because of its hydrophilic nature and its charge as *n*-caproate beyond a p*K*a of 4.88 while ethanol is completely miscible due to its short 2-carbon chain and hydrogen-bonding interactions. *n*-Caproic acid can be produced from ethanol in an anaerobic open culture (reactor microbiome) by chain elongation *via* the reversed β oxidation pathway. Here, we show a proof-of-concept to utilize diluted ethanol and acetic acid in real syngas fermentation effluent as the sole substrate for chain elongation into the product *n*-caproic acid. This concept, therefore, integrates the syngas platform and the carboxylate platform within a biorefinery. We observed with a bioreactor study that lowering the pH to slightly acidic conditions was necessary to shift the metabolic flux from production of methane to production of *n*-caproic acid. The highest concentration of *n*-caproic acid of ~ 1 g L^{-1} was produced at pH 5.44.

ACKNOWLEDGMENTS

I sincerely thank Dr. Largus Angenent, who is an Associate Professor in the Department of Biological and Environmental Engineering for guiding me through every phase of the project and providing inputs that have been invaluable for the successful completion of the project.

I would also like to extend my gratitude to Dr. Hanno Richter, who is a Research Associate in the Angenent Lab for advising and providing constructive criticism during the project.

I would also like to thank all members of the Angenent lab for providing support during the course of the project.

HIGHLIGHTS

- *n*-caproic acid was produced from a novel source (syngas fermentation effluent).
- The carboxylate platform was integrated with the syngas platform.
- Detrimental effect of methanogenesis on *n*-caproic acid concentration was established.

KEYWORDS: *N*-CAPROIC ACID, CHAIN ELONGATION, SYNGAS, CARBOXYLATE

1. INTRODUCTION

Biobased 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, 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 distillation as a method for product recovery. An anaerobic fermentation process with open cultures (reactor microbiomes) and fed with dilute, undistilled ethanol can catalyze a chain elongation reaction *via* a well-understood reversed β oxidation pathway, leading to the production *n*-caproic acid (Kenealy, Cao et al. 1995, Agler, Wrenn et al. 2011). *n*-Caproic acid is a 6 carbon chain carboxylic acid that is more hydrophobic in comparison to ethanol and easier to extract. A recent study has shown that *n*-caproic acid was separated by in-line liquid-liquid extraction from a recycle line in a bioreactor (Agler, Spirito et al. 2012), providing a potential method to avoid or reduce the use of ethanol distillation. Production of *n*-caproic acid has several other benefits compared to ethanol. First, *n*-caproic acid is more energy dense than ethanol (Steinbusch, Hamelers et al. 2011). Second, *n*-caproic acid is a more valuable commodity that is used: 1. in animal feed rations; 2. as an antimicrobial (Skřivanová and Marounek 2007); and 3. as a feedstock for esterification into products with artificial flavoring (Minich 1960).

An anaerobic fermentor, which is optimized for chain elongation, has a different reactor microbiome compared to an anaerobic digester. For efficient chain elongation, the acetoclastic methanogens must be mostly inhibited to prevent the intermediate acetate of being converted into methane. It is important to maintain high enough hydrogen partial pressures to prevent short- and medium-chain carboxylic acid to be oxidized, 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 inhibition can be achieved by the addition of an antimicrobial such as 2-Bromoethanesulfonate (Zinder, Anguish et al. 1984). 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 (VanKessel and Russell 1996).

Various substrates, such as ethanol, acetate, succinate (Kenealy, Cao et al. 1995), glucose (Ding, Tan et al. 2010), and corn beer (Agler, Spirito et al. 2012) have been used to study biological *n*-caproic acid production. Here, we investigated whether we can utilize dilute ethanol and acetate obtained in effluent from a 6-L synthesis gas fermentation setup as a substrate for the chain elongation process. Synthesis gas, which is referred to as syngas is a mixture of carbon monoxide, hydrogen, and carbon dioxide), is converted to ethanol and acetate using carboxydotrophic bacteria, including *Clostridium lj ungdahlii*, *Clostridium autoethanogenum*, *Acetobacterium woodii*, *Clostridium carboxidivoran*

s, and *Peptostreptococcus productus* (Munasinghe and Khanal 2010). Syngas fermentation setups with a pure culture of one of these carboxydotrophic bacteria can achieve ethanol concentrations of 2-4% (w/w), and the highest ethanol concentration that was achieved was 4.8% with a pressurized system (James L. Gaddy 2007
)The two-phase, 6-L syngas fermentation system that we have operated has achieved ethanol concentrations of up to 3% at a volumetric production rate of 0.3 g L^{-1} day^{-1} (Richter 2012). We anticipate that effluent from syngas fermenters would consist of many of the growth factors and nutrients for chain elongation, even though further studies are necessary to ascertain this. Syngas for sustainable ethanol production can be produced with the syngas platform through a thermochemical step from biomass feedstock such as wood waste or willow.
Several reactor configurations have been used to study chain elongation with reactor microbiomes, including the anaerobic sequencing batch reactor (ASBR) (Agler, Spirito et al. 2012), batch reactor (Kenealy, Cao et al. 1995), fed-batch reactor (Steinbusch, Hamelers et al. 2011), and the anaerobic filter (AF) reactor (Grootscholten, Steinbusch et al. 2012). The fed-batch reactor achieved a *n*-caproic acid production rate of 8.17 g L^{-1} day^{-1} with a product specificity of 62% for *n*-caproic acid (Steinbusch, Hamelers et al. 2011), whereas the AF achieved a *n*-caproic acid production rate of 16.6 g L^{-1} day^{-1} with a product specificity of 85% (Grootscholten, Steinbusch et al. 2012), which represents a nearly two-fold increase in the *n*-caproic acid production rate. The substrate for these latter two studies consisted of a synthetic substrate that is a mixture of procured ethanol and acetic acid with added growth factors and nutrients. We fed a ~1/6 diluted stream of real, complex substrate (fermentation beer with 15% (w/w) ethanol) to a 5-L ASBR system, which was withdrawn from the beer well at a corn kernel-to-ethanol plant (Agler, Spirito et al. 2012). This influent stream contained left over corn kernel solids and yeast cells and besides base (NaOH), no other growth factors or nutrients were added. Here, we evaluated as a proof-of-concept study whether real, filtered syngas fermentation effluent including dilute ethanol and acetic acid can be used as a substrate to a chain elongation fermentor to circumvent ethanol distillation. We show that the integration of the syngas platform with the carboxylate platform may be technically feasible after system improvements are made, and we discuss what these improvements may consist of.

2. MATERIALS AND METHODS

2.1 INOCULUM

The inoculum used was obtained from the effluent of a 5-L chain-elongating fermentor that has been fed corn beer, that has been operated in our lab for ~ 2 years, and that includes product separation with liquid-liquid extraction (Agler, Spirito et al. 2012).

2.2 MEDIUM

Effluent from a two-phase, 6-L syngas fermenting system with a pure culture of *Clostridium ljungdahlii* strain ERI-2 was used as liquid feed medium for the chain elongation bioreactor. The synthetic syngas to the syngas fermentor consisted of a gas mixture of 65% carbon monoxide, 5% carbon-dioxide, and 30% hydrogen (Airgas East, Ithaca, NY) (Richter 2012). The effluent that was fed as the first batch during Days 0 – 24 of the operating period contained ethanol and acetic acid concentrations of 11.4 g L^{-1} and 2.3 g L^{-1}, respectively. These concentrations were artificially maintained in the second batch at 1.8 g L^{-1} and 1.2 g L^{-1} with procured chemicals, respectively. The concentrations in our syngas fermentation system, which was operated upstream from our chain elongation fermentation system, had considerably decreased due to unstable conditions. The syngas fermentation medium was supplemented with 10 mL L^{-1} trace metal solution (Rajagopalan, P. Datar et al. 2002), 5 mL L^{-1} 2X vitamin solution (Rajagopalan, P. Datar et al. 2002), 1 mL L^{-1} yeast extract, and 2.9 g L^{-1} of potassium bicarbonate (we did not investigate whether these growth factors and nutrients are necessary). Bicarbonate was added because *Clostridium kluyveri* requires carbon

dioxide for its metabolism. 5mM Resazurin was added as an oxygen indicator.

2.3 OPERATION OF THE REACTOR

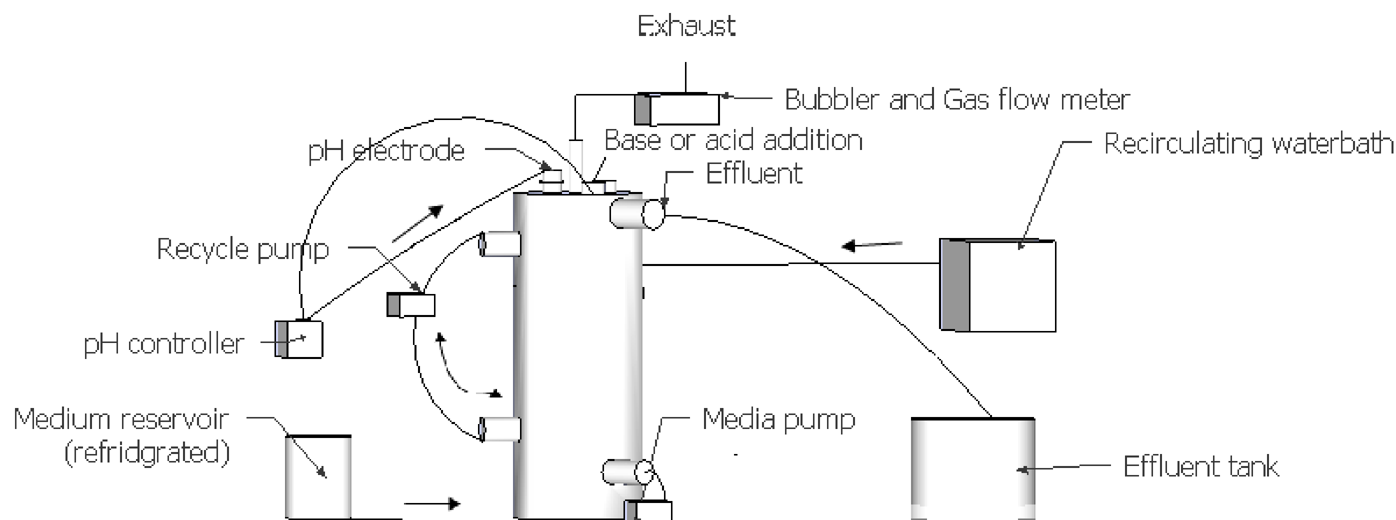

Figure 1: Schematic of the reactor setup.

The AF reactor had a total volume of 700 ml, was made of plastic with a cone at the bottom, and contained ceramic saddles resulting in a liquid volume of 425 ml. The reactor was operated in upflow mode and the feed medium was pumped with a peristaltic pump (7528-10, COLE-PARMER, Illinois) from a refrigerator (4°C) to the bottom of the reactor at a flow rate of 0.5 mL min^{-1}, resulting in a hydraulic retention time (HRT) of 14 h. The reactor was continuously recycled using a peristaltic pump (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 reactor. The pH of the system was maintained at 6.5 for the first 5 days of the operating period and it was decreased to 5.5 after that day. Next, the pH was further decreased to 5.2 after Day 24 of the operating period. 0.5 M KOH or 0.5 M HCl was pumped for maintaining the pH at the set point by using a controller (alpha-pH8000, EUTECH, Singapore).

At the start of the experiment, the reactor was completely filled with inoculum and recycled for 24 h without the addition of substrate. After 24 h, the reactor was fed with medium and sample volumes of 2 ml were collected daily during the operating period of 44 days and centrifuged at 10,000 rpm for 10 min. The supernatant was frozen at -20°C for further analysis.

2.4 ANALYTICAL TECHNIQUES

2.4.1 ANALYSIS OF CARBOXYLIC ACIDS AND ETHANOL

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), which was equipped with a 7673 autoinjector and flame ionization detector, was used for the quantification of carboxylic acids and alcohols. The flow rates of hydrogen, air, and helium were 35, 380, and 30 mL min^{-1}. For the carboxylic acids, the column was a capillary GC column (Nukol); 15 m x 0.53 mm i.d. Supelco, St. Louis, MO). The temperature program was 708°C for 2 min, a ramp of 12°C min^{-1} to 200°C where the temperature was held for 2 min. Injection port and detector were set at 200°C and 275°C, respectively. For the quantification of alcohols, a custom-made packed bed glass column was used, 1.8 m x 2 mm i.d.

(Supelco). The support matrix of this column was Chromosorb W/AW80 over 100 mesh; phases were preconditioned: phase A was 10% Carbowax-20M; phase B was 0.1% phosphoric acid. Glass Purecol inlet liners, 2 mm i.d. were installed (Supelco). The inlet and detector temperatures were 220°C and 240°C, respectively. The column temperature program was 100°C for 2 min, a temperature ramp of 40°C min^{-1} to 180°C where the temperature was kept for 5 min.

2.4.2. ANALYSIS OF HEADSPACE GAS

The gas was allowed to pass through a bubbler and into a Milligascounter type MGC-1 PVDF (Ritter, Bochum, Germany) for the measurement of the daily volumetric production. Gas samples were analyzed with two Gow Mac gas chromatographs, series 580 (Bethlehem, PA), which were equipped with thermal conductivity detectors. For carbon dioxide and methane quantification, the gas chromatograph was equipped with a 1.8-m Supelco 80/100 Hayesep Q column at 25°C and helium was the carrier gas.

3. RESULTS AND DISCUSSION

3.1. CONCENTRATIONS OF CARBOXYLIC ACIDS AND ETHANOL

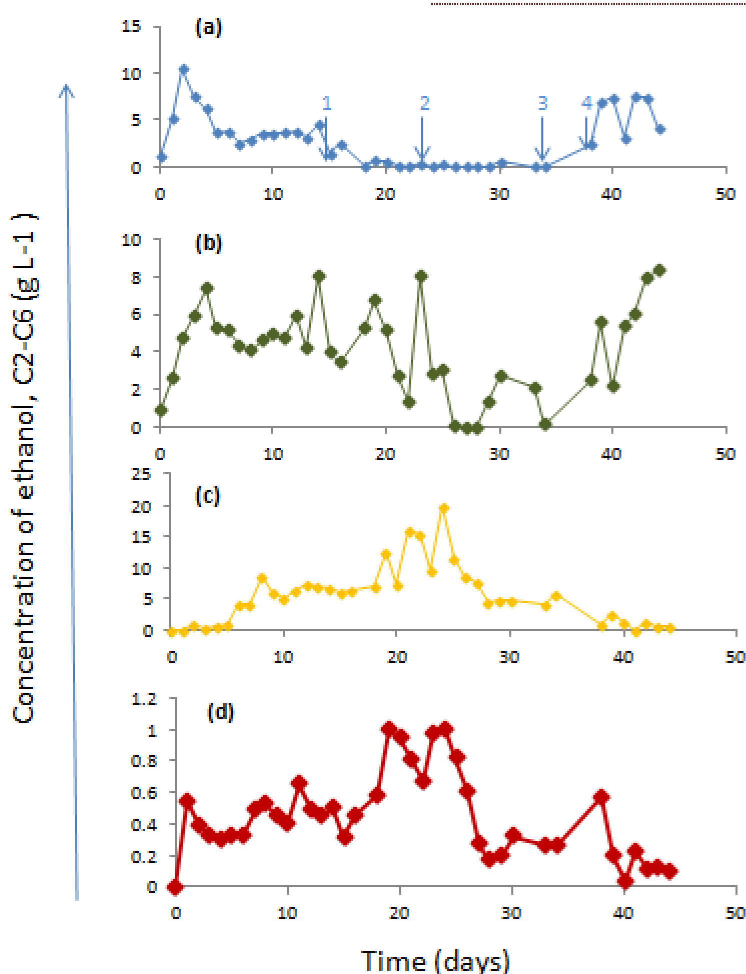

Figure 2. Composition of chain elongation reactor effluent over the operating period: a. Concentration of ethanol in g L^{-1}; b. Concentration of acetic acid in g L^{-1}; c. Concentration of *n*-butyric acid in g L^{-1}; and d. Concentration of *n*-caproate in g L^{-1}. Unplanned perturbation: 1. and 3. Air was introduced inside the reactor due to problem with the media pump; 2. Additional synthetic ethanol and acetate solutions were added to feed medium due to loss of ethanol production in syngas fermentation system (the feed ethanol concentration was 11.4 g L^{-1} until day 24 of the operating period and 1.8 g L^{-1} ethanol on day 25); and 4. Ethanol concentration increased due to increased production of ethanol from syngas fermentation setup.

We observed that when ethanol concentration decreased (due to consumption) an increase in the carboxylic acid concentration occurred as anticipated in a functioning chain elongation fermentor (Figure 2). The highest carboxylic acid concentrations were observed on Day 23 of the operating period when ethanol concentrations were depleted and acetic acid, *n*-butyric acid, and *n*-caproic acid concentrations were 7.9, 19.4, and 1.0 g L^{-1}, respectively. This resulted in a product selectivity for *n*-caproic acid from ethanol and acetate of 3%. The ethanol in the feed medium was 11.4 g L^{-1} until Day 24 of the operating period. After this day, the ethanol production in the syngas fermentation system was hampered, and 1.8 g L^{-1} ethanol was added externally to the syngas effluent to maintain the chain elongation reactor in continuous operation. In a previous study by Grootscholten et al., the *n*-caproate concentration reached 11.1 g L^{-1}. However, the reactor had been operated at a neutral pH as opposed to our reactor operating conditions that included an acidic pH of 5.5. With a p*K*a of 4.88 for *n*-caproic acid, a much lower concentration of undissociated *n*-caproic acid existed of 11.02g L^{-1} at a pH of 7.0 (Grootscholten et al. 2012), which resulted in a lower inhibiting effect than a with a pH of 5.5 that is close to the p*K*a (0.8 gL^{-1}; Figure 4). However, there are disadvantages to running the reactor at a neutral pH, as described in the following section.

3.2. CORRELATION BETWEEN METHANE COMPOSITION AND CAPROATE CONCENTRATION AND PH

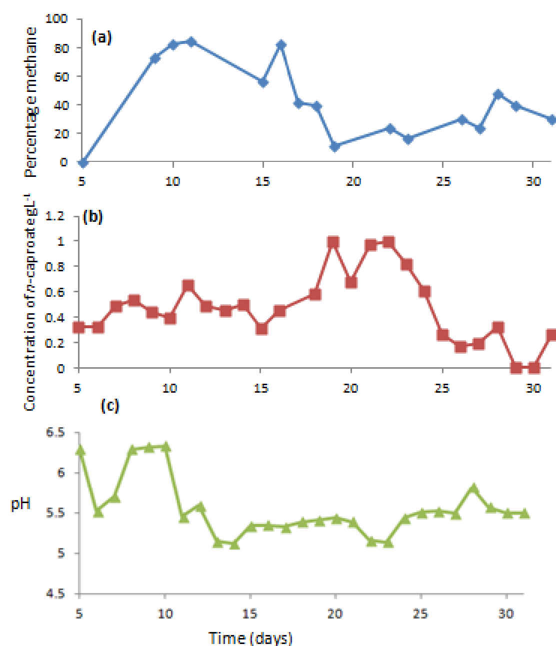

Figure 3. Operating and performance parameters during the operating period: a. poercentage of methane in the headspace gas; b. *n*-caproic acid concentration; and c. pH.

Prevention of acetoclastic methanogenesis has been the primary concern in the chain elongation processes to prevent acetic acid cleavage towards methane production. A simple, and yet efficient method of controlling acetoclastic methanogenesis is decreasing the pH to 5.5 (VanKessel and Russell 1996). Our reactor was inoculated with the effluent from a mesophilic (30°C) bioreactor to convert beer into *n*-caproic acid, which was maintained at a pH of 5.5 for close to a 2-year operating period. A similar concept of inoculating the AF reactor with the effluent of the bioreactor from an operating chain elongation system was performed by Grootscholten *et al.*, (2012). Their operating conditions of 7.0 while preventing methanogenesis was possible due to utilizing an inoculum that lacked methanogens, because the methanogen inhibitor 2-Bromoethanesulfonate (2-BES) was introduced during the entire operating period of the *n*-caproic fermentor from which the inoculum came from. Our inoculum did include methanogens (methane was produced mainly through hydrogenotrophic methanogenesis) and operating the AF reactor at a pH of 6.5 resulted in the production of considerable amounts of methane. We observed a relationship between pH and methane concentration (Figure 3 a,c). – a pH closer

to neutral conditions (6.5) resulted in a 80% methane content of the headspace gas for nearly 5 days of the operating period. Subsequently, the pH was decreased to a pH value of 5.5 and this led to a decrease in the methane concentration, confirming that methanogens were inhibited by the lower pH. We also observed that the concentration of *n*-caproic acid was inversely correlated to the methane composition (Figure 3 b, c).

Alternatively, the reactor can be subjected to methanogen inhibitors such as 2-BES (Zinder, Anguish et al. 1984), which is an expensive chemical rendering a large-scale application economically unviable. Grootscholten *et al.* claimed that methanogens did not grow in their system over the operating period of 80 days, since the inoculum was the effluent of a reactor in which methanogens were not present. However, this does not preclude methanogens to accumulate slowly during very long operating periods in their open culture system (Luo, Karakashev et al. 2011), especially since they had already observed trace amounts of methane in their headspace gas.

3.3. TOXICITY OF CAPROIC ACID

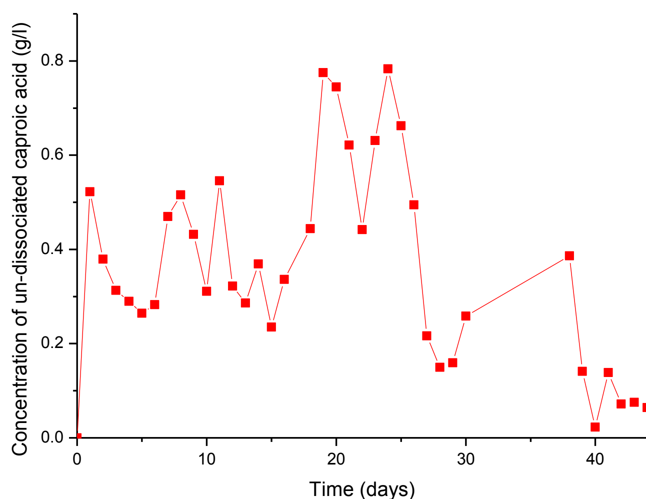

Figure 4: Concentration of undissociated *n*-caproic acid over the operating period.

The concentrations of undissociated *n*-caproic acid were calculated from pH and total n-caproic acid concentration data and plotted over the whole operation period (Figure 4). The highest level of undissociated *n*-caproic acid was 0.78 g L^{-1} at a pH level of 5.44 on Day 24 of the operating period (Figure 4). We believe that undissociated *n*-caproic acid concentrations at a pH of ~ 5.5 were inhibiting its own production at this condition. In agreement with this theory is that higher levels of *n*-caproic acid production have only been observed at: 1. neutral pH values (Grootscholten, Steinbusch et al. 2012); or 2. similar pH values but with a system that included the in-line extraction of the final product *n*-caproic acid (Agler et al., 2011). Therefore, the end-product inhibition from undissociated *n*-caproic acid determines possible final concentrations of total (dissociated and undissociated) *n*-caproic acid in the system. To keep *n*-caproic acid concentration below inhibitory levels, *n*-caproic acid must be removed by coupling our AF reactor with in-line extraction of *n*-caproic acid. The other operating conditions that should have been maintained at sufficiently high levels are the hydrogen partial pressure. Under a very low hydrogen partial pressure of 10^{-4}, which is common in anaerobic digesters, acetic acid and *n*-butyric acid, which are the intermediates for n-caproic production, would have been oxidized (Agler et al., 2011). It is, therefore, pertinent that hydrogen should be maintained in the headspace gas at sufficiently high concentrations (~0.5%) to prevent the loss of intermediate carboxylic acids. We cannot guarantee here that we maintained high enough hydrogen concentrations and further studies should always include measurement of hydrogen partial pressures.

4. OUTLOOK

Here, we have shown a proof-of-concept system that coupled syngas fermentation from the syngas platform to chain elongation from the carboxylate platform with the goal to circumvent the expensive distillation of dilute ethanol. More research is necessary to further study the ideal composition of the syngas fermentation effluent, including whether artificially added growth factors would be neccesary. Many of these artificial additions to our substrate solution to prevent nutrient limitations could have been avoided, and a medium optimization study is required. We concluded that

methanogenesis is a critical parameter in determining successful operation of the reactor. Decreasing the pH is the most economically viable solution to inhibit methanogenesis and re-route production towards *n*-caproic acid. However, we have shown a relatively low product selectivity of 3% and for increased production of *n*-caproic acid at an operating pH of 5.5, it is necessary to extract *n*-caproate from the system. Additional optimization studies are needed to: 1. find ideal ethanol/acetate ratio in the syngas fermentation effluent; 2. ascertain the stability of the performance and microbiome during prolonged operating periods; and 3. increase the selectivity and production rates of *n*-caproic acid.

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