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6. AUTHORS
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14. ABSTRACT
We envision producing medium-chain carboxylic acids from organic feedstocks with open cultures of microbial consortia (reactor microbiomes) via a bioprocess that is called chain elongation. Our vision is based on the carboxylate platform and combines two fermentation steps into one bioprocess: 1. producing short-chain carboxylic acids from variable feedstocks; 2. chain elongating these acids into hydrophobic, extractable medium-chain carboxylic acids (n-caproic acid and n-caprylic acid). We proposed three different objectives: 1. To understand

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Report Title

Final Report: Increasing the Conversion Efficiency of Biomass Into n-caproate With Reactor Microbiomes

ABSTRACT

We envision producing medium-chain carboxylic acids from organic feedstocks with open cultures of microbial consortia (reactor microbiomes) via a bioprocess that is called chain elongation. Our vision is based on the carboxylate platform and combines two fermentation steps into one bioprocess: 1. producing short-chain carboxylic acids from variable feedstocks; 2. chain elongating these acids into hydrophobic, extractable medium-chain carboxylic acids (n-caproic acid and n-caprylic acid). We proposed three different objectives: 1. To understand how hydrogen partial pressures can influence the production efficiencies of n-caproic acid at mesophilic temperatures; 2. To investigate whether changes in the hydrogen partial pressures alter the microbiome composition; and 3. To ascertain if reactor microbiomes that operate at thermophilic temperatures can produce n-caproic acid at sufficient rates. We found that hydrogen gas did influence the production rates of medium-chain carboxylic acids, and therefore also likely the microbiome (analysis still in progress), but that a higher temperature would not be advisable. At a lower temperature of 30 degrees C we found that lactic acid was converted efficiently to n-caproic acid in a continuously fed bioreactor that was operated at mildly acid conditions and with product extraction. This is opening the way to chain elongation with lactic acid.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
12/13/2016	11	Leo A. Kucek, Mytien Nguyen, Largus T. Angenent. Conversion of l-lactate into n-caproate by a continuously fed reactor microbiome, Water Research, ( ): 163. doi:
12/13/2016	12	Leo A. Kucek, Catherine M. Spirito, Largus T. Angenent. High n-caprylate productivities and specificities from dilute ethanol and acetate: chain elongation with microbiomes to upgrade products from syngas fermentation, Energy Environ. Sci., ( ): 3482. doi:
12/13/2016	13	Leo A. Kucek, Jiajie Xu, Mytien Nguyen, Largus T. Angenent. Waste Conversion into n-Caprylate and n-Caproate: Resource Recovery from Wine Lees Using Anaerobic Reactor Microbiomes and In-line Extraction, Frontiers in Microbiology, ( ): . doi:
12/13/2016	14	Hanno Richter, Bastian Molitor, Martijn Diender, Diana Z. Sousa, Largus T. Angenent. A Narrow pH Range Supports Butanol, Hexanol, and Octanol Production from Syngas in a Continuous Co-culture of Clostridium ljungdahlii and Clostridium kluyveri with In-Line Product Extraction, Frontiers in Microbiology, ( ): . doi:

TOTAL: **4**

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

1. Angenent L. T. Resource recovery from food waste: from storage to biorefineries, Food Waste-to-Low Carbon Energy Conference, April 27-28th, 2016, New Brunswick, NJ, United States.
2. Angenent L. T. Production of medium-chain carboxylic acid (MCCA) oil by reactor microbiomes, Nova Nordisk Foundation, Cell Factories & Biosustainability - Technologies for Cell Factory Construction Conference, May 17-21, 2015, Copenhagen, Denmark.
3. Angenent L. T. Using reactor microbiomes to produce chemicals from biomass: medium-chain carboxylic acid oil (keynote presentation), Environmental Technology for Impact 2015 (ETEI2015) conference, April 29-30, 2015, Wageningen, The Netherlands.
4. Angenent L. T. Chain elongation with reactor microbiomes, 114th General Meeting, May 17-20, 2014, Boston, Massachusetts.
5. Spirito C. M.*, Daley S. E., Werner J. J. and Angenent L.T. (2015). Time course analysis of the effects of antibiotic disturbances on anaerobic reactor microbiomes. 14th World Congress on Anaerobic Digestion, November 15-18, 2015, Viña del Mar, Chile.
6. Xu J.*, Guzman J. J., Spirito C. M., Andersen S., Rabaey K. and Angenent L. T. (2015). Oil production from complex substrates with microbiomes at ambient temperatures and pressures: electrochemically induced phase separation in the carboxylate platform. 14th World Congress on Anaerobic Digestion, November 15-18, 2015, Viña del Mar, Chile.
7. Xu J., Guzman J. J. L., Spirito C. M., Andersen S., Rabaey K. and Angenent L. T.* (2015). Oil production from complex substrates with microbiomes at ambient temperatures and pressures: electrochemically induced phase separation in the carboxylate platform. The 5th ISMET meeting, October 1-4, 2015, Arizona State University, Tempe, AZ.
8. Kucek L. A.* and Angenent L. T. (2015). The carboxylate platform: conversion of carbon-rich wastes into liquid fuels and chemicals. The Water Environment Federation Technical Exhibition and Conference (WEFTEC), September 26-30, 2015, Chicago, Illinois, USA.
9. Kucek L. A.* and Angenent L. T. (2014). The carboxylate platform: conversion of carbon-rich wastes into liquid fuels and chemicals. National Advanced Biofuels Conference & Expo, October 13, 2014, Minneapolis, MN.
10. Xu J.*, Guzman J. J., Andersen S., Rabaey K. and Angenent L. T. (2014). Continuous production of an oily stream of medium-chain carboxylic acids with a microbial electrochemical technology. North American regional meeting of the International Society for Microbial Electrochemistry and Technology (NA-ISMET meeting), May 13-15 2014, Penn State University, University Park, PA.

Number of Presentations: 10.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
12/13/2016	2.00	Catherine M. Spirito, Hanno Richter, Korneel Rabaey, Alfons J. M. Stams and Largus T. Angenent. Chain elongation with anaerobic reactor microbiomes to recover resources from waste, Current Opinion in Biotechnology (10 2013)
12/13/2016	1.00	Dyvia Vasudevan, Hanno Richter, Largus T. Angenent. Upgrading dilute ethanol from syngas fermentation to n-caproate with reactor microbiomes, Bioresource Technology (09 2013)
12/13/2016	5.00	Uwe Schroeder, Falk Harnisch, Largus T Angenent. Microbial Electrochemistry and Technology: terminology and classification, Energy & Environmental Science (10 2014)
12/13/2016	10.00	Largus T. Angenent et al.. Chain elongation with reactor microbiomes: open-culture biotechnology to produce biochemicals, Environmental Science & Technology (09 2015)

TOTAL: **4**

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

US Provisional Patent Application 7298-01-US "Production of primarily caprylic acid (C8) in chain elongation bioreactors". Filing date: May 26, 2016.

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Catherine Spirito	0.25	
Phandanouvong Lozano	0.05	
FTE Equivalent:	**0.30**	
Total Number:	**2**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Hanno Richter	0.02
FTE Equivalent:	**0.02**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Largus T. Angenent	0.03	
FTE Equivalent:	**0.03**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Chase Brett	0.25	Environmental Engineering
FTE Equivalent:	**0.25**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	1.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:	1.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME	
Leo Kucek	
Total Number:	**1**

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment

Technology Transfer

Final report: Proposal Number: 62324-EV, Agreement Number: W911NF-12-1-0555

For the period beginning August 15, 2012 and ending August 14, 2016

Largus T. Angenent, PhD – Cornell University

la249@cornell.edu

Statement of the problem studied:

We envision producing medium-chain carboxylic acids from organic feedstocks with open cultures of microbial consortia (reactor microbiomes) *via* a bioprocess that is called chain elongation. Our vision is based on the carboxylate platform and combines two fermentation steps into one bioprocess: 1. producing short-chain carboxylic acids from variable feedstocks; 2. chain elongating these acids into hydrophobic, extractable medium-chain carboxylic acids (*n*-caproic acid (C6) and *n*-caprylic acid (C8)). We proposed three different objectives: 1. *To understand how hydrogen partial pressures can influence the production efficiencies of* n-*caproic acid at mesophilic temperatures*; 2. *To investigate whether changes in the hydrogen partial pressures alter the microbiome composition*; and 3. *To ascertain if reactor microbiomes that operate at thermophilic temperatures can produce* n-*caproic acid at sufficient rates.*

Summary of the most important results:

Our approach was organized similarly to the three approaches (Approach 1-3), and below we will outline the results from these three approaches during the reporting period.

Approach 1:

We designed three stainless steel reactors (5 L) with stainless steel tubing to prevent hydrogen leakage (**Figure 1**). These reactors were used to investigate the effect of hydrogen partial pressures on *n*-caproic production and will be used for approach 1 and 2. The setup of the reactors is based on the current *n*-caproic acid production reactor operating in the Angenent lab, which is made of glass, with the exceptions that these reactors (and the associated parts and components of the entire system: pumps, extraction system, etc.) have been designed and selected to prevent the loss of hydrogen gas from the system. All connections and piping in contact with the headspace gas are made out of stainless steel. Many modifications have been made to the reactors to make the system work properly. The system is the most elaborate system that we have ever built in our laboratory, and at every step, the circumvention of hydrogen gas leakage and diffusion out of the system has been taken in consideration.

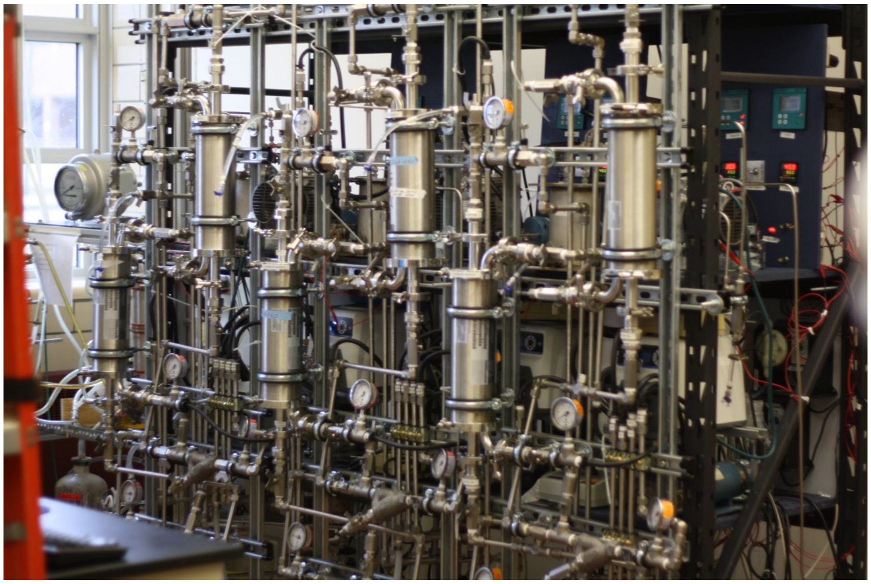


Figure 1: Stainless steel reactor systems designed to examine the effect of hydrogen partial pressures on medium-chain carboxylic acid production. The stainless-steel extraction modules are at the forefront (each bioreactor has two of these modules).

The graduate student Catherine Spirito operated these three, 5-L continuously stirred anaerobic bioreactors fed with soluble substrates for a period of nine months. The reactors were designed for medium chain carboxylic acid production. All conditions were kept the same in the reactors with the exception of the dissolved hydrogen concentrations, which was varied by sparging nitrogen or hydrogen gas. The organic loading rate was 7.8 g COD/L/day with a synthetic substrate consisting of ethanol and acetate (10:1 molar ratio). In R1, R2, and R3, the hydrogen composition in the headspace was 1.1±0.4, 15.1±7.1, and 4.2±1.3%, respectively. However, we did not find observe large differences in the production rates for *n*-caproic acid and *n*-caprylic acid - we observed a medium-chain production rate of 3.66, 3.01, and 3.09 g COD/L/day with C8/C6 ratios of 1.47, 1.27, and 2.21, respectively. The graduate student, who will graduate in May 2017, is currently performing an elaborate statistical analysis to find out whether the differences are statistically significant. The absence of very large differences in the production rates under different hydrogen partial pressures are in agreement with the predictions that we made in a recent review paper in *Environmental Science & Technology* [1] that followed after we had published an updated microbial pathway in a review paper in *Current Opinion in Biotechnology* [2]. From reviewing the literature and by performing an extensive stoichiometric and thermodynamic modeling approach for this review paper, we have found that two different microbial pathways can occur based on low and high concentrations of hydrogen gas within the type strain *Clostridium kluyveri* (**Figure 2A and 2B**). This is new information and explains discrepancies in the current literature. Now, we do not only theoretically understand why our chain elongation pathway (from short-chain carboxylic acids to medium-chain carboxylic acids) should work at different concentrations of hydrogen gas, but also which proteins we should monitor to proof that this shift in pathways can occur by changing the hydrogen partial pressure in the bioreactor system (for Approach 2). Information about which pathway is occurring is important for bioprocessing because at high hydrogen gas partial pressures the ATP production would be lower, resulting in a lower biomass growth and possibly a lower subsequent production rate of *n*- caproic acid and *n*-caprylic acid. The graduate student is, therefore, also including the biomass concentrations (a proxy for microbial growth) in the statistical analysis. The main thesis chapter and journal paper is in preparation.

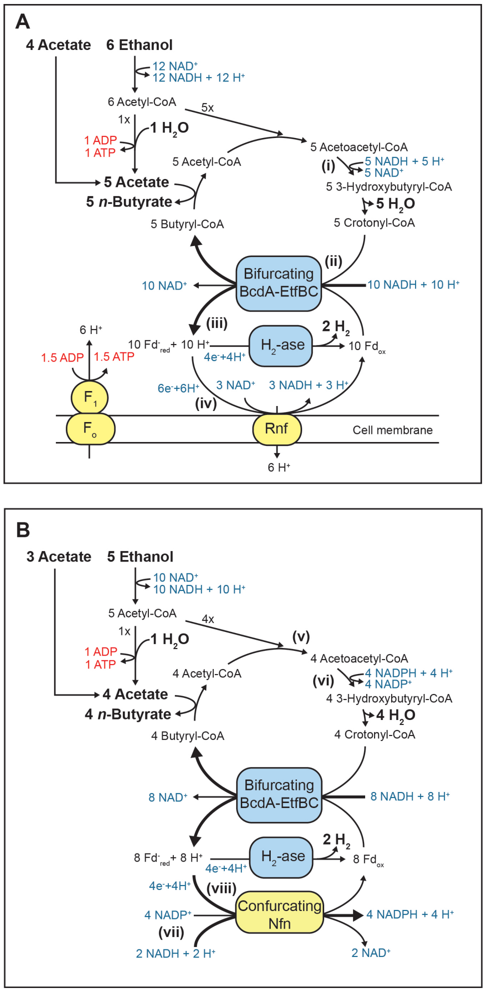
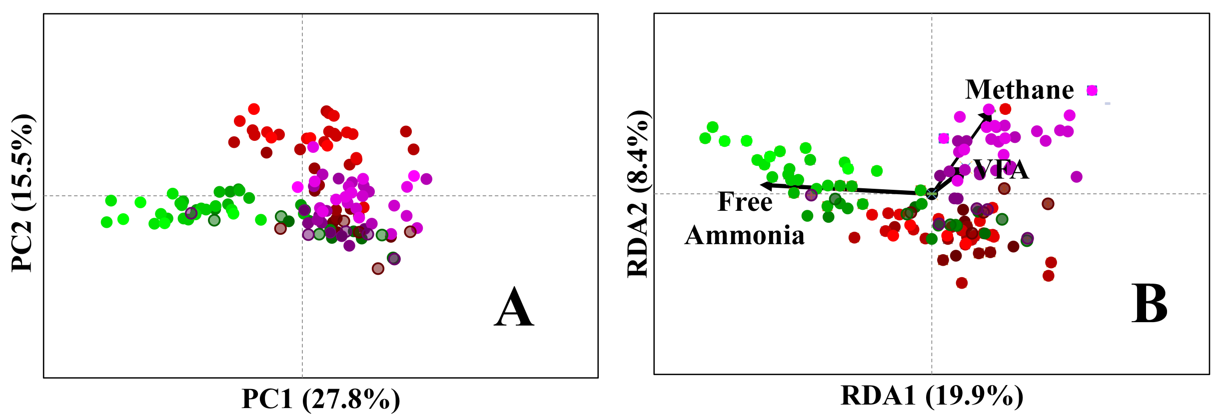


Figure 2: Two pathways with a different ATP production outcome for reverse β oxidation (chain elongation) for a ratio of ethanol and acetate that is similar but not exactly the same, which we reviewed in a to-be-submitted manuscript: **A.** at a low hydrogen partial pressure and substrate concentration, additional ATP can be generated *via* the Rnf complex, while re-forming oxidative power; and **B.** at a high hydrogen partial pressure and substrate concentration, re-forming oxidation power can occur with a different enzyme complex at a less negative thermodynamic threshold, but no additional ATP besides from ethanol oxidation to acetic acid is formed.

The reactor conditions were chosen based on a now published paper in *Energy and Environmental Science* with an Impact Factor of 25 [3]. By keeping a high ratio of ethanol to acetate (10:1), we observed that primarily *n*-caprylic acid was produced rather than *n*-caproic acid. This is an important breakthrough since *n*-caprylic acid has a price that is double per weight. The reason that we even tried a higher molar ratio for ethanol *vs*. acetic acid was an earlier paper we published that showed that syngas fermentation effluent with a high ethanol to acetic acid ratio can be used to produced medium-chain carboxylic acids, but that product extraction is needed [4]. We also tried to combine the syngas fermentation and chain elongation in one bioreactor with a binary culture (all other studies used open cultures). This was a success and was recently published in the open-access journal *Frontiers in Microbiology* [5].

Approach 2:

For this approach we had setup a cloud-based biocomputing system to monitor the microbiomes in the three bioreactors. The student spent considerable more time to apply multivariate statistical tools (*i.e.*, unconstrained and constrained ordination) to analyze existing bioinformatic datasets. The graduate student has completed and published a research paper in *Water Research* [6]. An exemplary **Figure 3** is shown for which environmental factors, such as ammonia concentration, explained why the community structure in an anaerobic digester (reactor microbiome) changed during the operating period.



PERIOD	CT-I	CT-II	CT-III	CT-IV	CT-V	CT-VI	CT-VII
Study Days	371 – 450	451 – 525	526 – 602	603 – 726	727 – 806	807 – 882	883 – 935
Manure							
Food Waste							
Glycerol*							

* The glycerol reactor was stopped on Day 895

Figure 3. Reactor microbiome succession and its correlation to environmental conditions, such as free ammonia concentration. **A.** 2D PCoA of the major principal coordinates based on the pairwise weighted UniFrac distances matrix (each dot represents a sample with an entire microbiome). **B.** Constrained Distance-Based Redundancy Analysis (db-RDA) showing the environmental parameters that best explain the variation seen in microbial community structure based on weighted UniFrac distances. Red circles represent data from control manure-only digestion (Manure); green circles represent data from food waste co-digestion (Food waste); and violet circles represent data from crude glycerol co-digestion (Glycerol).

We have already used our cloud-based system in several other papers for this project [3, 7, 8]. Now, we are using our system for a 16S rRNA and metagenomics analysis (the metagenomic sequences are recently made available to us) to answer the question whether the hydrogen partial pressure will have an effect on the microbiome (Approach 2). The answer will be made available in the thesis of Catherine Spirito and a future journal paper.

Approach 3:

Prior to initiating continuous experiments, batch bottle tests were conducted at three different temperatures (*i.e.*, 30°C, 37°C, 55°C) containing either lactic acid or ethanol as electron donors. Results from these tests showed that the greatest MCCA accumulation occurred at the lowest of these temperatures; therefore, all continuous experiments were conducted at 30°C. We had planned the temperature experiments with lactic acid as the electron donor since we had initially observed this reaction to occur at 55°C. We continued with the lactic acid study, but at the lower temperatures. A continuous upflow anaerobic filter and in-line pertraction system was designed and constructed to produce and accumulate medium-chain carboxylic acids (MCCAs) when fed a synthetic substrate containing lactic acid and butyric acid. The working volume of the bioreactor was 0.5 L, and the hydraulic retention time was 0.5 days.

The pertraction system consisted of two hollow-fiber membrane contactors with hydrophobic transfer areas of 8.1 m^2 each; the first transferred MCCAs from the bioreactor broth to a TOPO-enriched (3 % w/v) mineral oil solvent, and the second transferred MCCAs from the solvent to an alkaline stripping solution. The pH of the feed and the bioreactor were maintained at 5.5; the pH of the alkaline stripping solution was maintained at pH 9.0. That this extraction system is extremely important for the long-term operating conditions became clear in our recently published research paper in *Environmental Science & Technology* on the effect of this extraction system on the performance of a bioreactor [9]. In addition to the extraction system, we have also published a research paper for a separation system to phase separate *n*-caproic acid and *n*-caprylic acid after accumulating these acids in the high pH buffer of the extraction system. Because of using an electrochemical cell (*i.e.*, a membrane electrolysis cell), we used electric power rather than expensive chemicals to create the pH gradient across a membrane to induce phase separation. In the published paper in *ChemComm* about this separation system [10], we have shown that a continuously fed bioreactor can generate a phase-separated oil that consists mainly of *n*-caproic acid and *n*-caprylic acid (~95%). We refer to this oil as MCCA oil, which is produced by a microbiome under conditions of room temperatures and atmospheric pressures. A perspective in *Energy and Environmental Science* [11] to describe the system followed up this work.

With this extraction system (but without the separation system), we found that only lactic acid was converted into *n*-caproic acid, and that the *n*-butyric acid remained in the effluent. This study was a success, though, since we were the first to convert lactic acid into *n*-caproic acid with a continuously fed anaerobic bioreactor. We have now published the research paper in *Water Research* [7]. The production rates of *n*-caproic acid from lactic acid were a little below 10 g/L/day (**Figure 4**), which is already at the rates for methane production in anaerobic digesters.

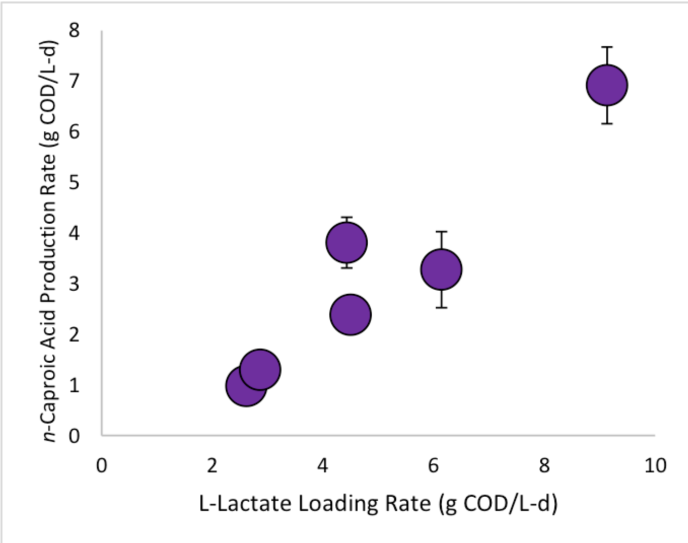


Figure 4. Production of *n*-caproic acid per L-lactic acid fed in a 0.5-L continuously fed bioreactor, which was only fed with L-lactic acid and *n*-butyric as a substrate.

<u>Bibliography for papers that were published during this project period:</u>

1. Angenent, L.T., H. Richter, W. Buckel, C.M. Spirito, K.J.J. Steinbusch, C.M. Plugge, D.P.B.T.B. Strik, T.I.M. Grootscholten, C.J.N. Buisman, and H.V.M. Hamelers, *Chain elongation with reactor microbiomes: open-culture biotechnology to produce biochemicals.* Environmental Science & Technology, 2016. **50**(6): p. 2796-2810.
2. Spirito, C.M., H. Richter, K. Rabaey, A.J.M. Stams, and L.T. Angenent, *Chain elongation with anaerobic reactor microbiomes to recover resources from waste.* Current Opinion in Biotechnol, 2014. **27**: p. 115-122.
3. Kucek, L.A., C.M. Spirito, and L.T. Angenent, *High n-caprylate productivities and specificities from dilute ethanol and acetate: chain elongation with microbiomes to upgrade products from syngas fermentation.* Energy & Environmental Science, 2016. **9**(11): p. 3482-3494.
4. Vasudevan, D., H. Richter, and L.T. Angenent, *Upgrading dilute ethanol from syngas fermentation to n-caproate with reactor microbiomes.* Bioresource Technology, 2014. **151**(1): p. 378-382.
5. Richter, H., B. Molitor, M. Diender, D.Z. Sousa, and L.T. Angenent, *A Narrow pH Range Supports Butanol, Hexanol, and Octanol Production from Syngas in a Continuous Co-culture of Clostridium ljungdahlii and Clostridium kluyveri with In-Line Product Extraction.* Frontiers in Microbiology, 2016. **7**(1773).
6. Regueiro, L., C.M. Spirito, J.G. Usack, D. Hospodsky, J.J. Werner, and L.T. Angenent, *Comparing the inhibitory thresholds of dairy manure co-digesters after prolonged acclimation periods: Part 2 – correlations between microbiomes and environment.* Water Research, 2015. **87**: p. 458-466.
7. Kucek, L.A., M. Nguyen, and L.T. Angenent, *Conversion of L-lactate into n-caproate by a continuously fed reactor microbiome.* Water Research, 2016. **93**: p. 163-171.
8. Kucek, L.A., J. Xu, M. Nguyen, and L.T. Angenent, *Waste conversion into n-caprylate and n-caproate: resource recovery from wine lees using anaerobic reactor microbiomes and in-line extraction.* Frontiers in Microbiology, 2016. **7**(1892).

9. Ge, S., J. Usack, C.M. Spirito, and L.T. Angenent, *Long-term n-caproic acid production from yeast-fermentation beer in an anaerobic bioreactor with continuous product extraction.* Environmental Science & Technology, 2015. **49**(13): p. 8012-8021.
10. Xu, J., J.J.L. Guzman, S.J. Andersen, K. Rabaey, and L.T. Angenent, *In-line and selective phase separation of medium-chain carboxylic acids using membrane electrolysis.* Chemical Communications, 2015. **51**(31): p. 6847-6850.
11. Schröder, U., F. Harnisch, and L.T. Angenent, *Microbial electrochemistry and technology: terminology and classification.* Energy & Environmental Science, 2015. **8**(2): p. 513-519.