Conversion of L-lactate into *n*-caproate by a continuously fed reactor microbiome

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ABSTRACT

Conversion of lactate to *n*-caproate had been described for the type strain *Megasphaera elsdenii* in batch systems. Recently, investigators have also described production of *n*-caproate from endogenous or exogenous lactate with batch-fed reactor microbiome systems. However, no reports exist of lactate to *n*-caproate conversion within a continuously fed bioreactor. Since continuously fed systems are advantageous for biotechnology production platforms, our objective was to develop such a system. Here, we demonstrated continuous lactate to *n*-caproate conversion for more than 165 days. The volumetric *n*-caproate production rate (productivity) was improved when we decreased the operating pH from 5.5 to 5.0, and was again improved when we utilized in-line product recovery *via* pertraction (membrane-based liquid-liquid extraction). We observed a maximum *n*-caproate productivity of 6.9 g COD/L-d for a period of 17 days at an L-lactate loading rate of 9.1 g COD/L-d, representing the highest sustained lactate to *n*-caproate conversion rate ever reported. We had to manage two competing lactate conversion pathways: 1) the reverse β -oxidation pathway to *n*-caproate; and 2) the acrylate pathway to propionate. We found that maintaining a low residual lactate concentration in the bioreactor broth was necessary to direct lactate conversion towards *n*-caproate instead of propionate. These findings provide a foundation for the development of new resource recovery processes to produce higher-value liquid products (*e.g.*, *n*-caproate) from carbon-rich wastewaters containing lactate or lactate precursors (*e.g.*, carbohydrates).

Keywords

Lactate; *n*-caproate; carboxylate platform; reactor microbiome; acrylate pathway; reverse β -oxidation pathway

Abbreviations

CoA: Coenzyme A; COD: chemical oxygen demand; D-LDH: NAD-dependent D-lactate dehydrogenase; GC: gas chromatography; HPLC: high-performance liquid chromatography; HRT: hydraulic retention time; iD-LDH: NAD-independent D-lactate dehydrogenase; MCC: medium-chain carboxylate; OTU: operational taxonomic unit; PC: principal coordinate; PCoA: principal coordinates analysis; PCR: polymerase chain reaction; RGD: reduction gas detector; SCC: short-chain carboxylate; TOPO: tri-*n*-octylphosphine oxide

1. INTRODUCTION

To sustain healthy communities and ecosystems, a new paradigm of resource recovery from wastewater is required (Guest et al. 2009). Carbon-rich wastewaters with high chemical oxygen demand (COD) pose ecological risks if left untreated, but these wastes can be converted into fuels and chemicals *via* the carboxylate platform (Agler et al. 2011, Angenent et al. 2004). In the carboxylate platform, anaerobic reactor microbiomes (open cultures of microbial consortia) are used to convert complex wastes into intermediate short-chain carboxylates (SCCs, ranging from two to five carbons) (Agler et al. 2012b). Throughout this text, mixtures of both dissociated carboxylates and their corresponding undissociated carboxylic acids are referred to as carboxylates. These intermediate SCCs can be converted into methane-rich biogas *via* anaerobic digestion, which is a carboxylate platform technology. Using similar reactor microbiomes, however, intermediate SCCs can also be chain-elongated to medium-chain carboxylates (MCCs, ranging from six to twelve carbons) (Xu et al. 2015) *via* the reverse β -oxidation pathway (Spirito et al. 2014) to create higher-value liquid fuels and chemicals (Agler et al. 2011).

The classically known reverse β -oxidation pathway occurs with ethanol as the source of carbon, reducing equivalents, and energy toward chain elongation of SCCs to *n*-caproate (Spirito et al. 2014). *n*-Caproate is a six-carbon MCC that has a variety of uses, including use as an antimicrobial to replace antibiotics in agriculture (Desbois 2012). *n*-Caproate also has emerging applications as a chemical precursor for products ranging from flavors (Kenealy et al. 1995) to aviation fuels (Harvey and Meylemans 2014). Besides ethanol (Grootscholten et al. 2013), other reduced substrates have also been utilized for production of *n*-caproate, including: carbohydrates (Gómez et al. 2009); polyols (Jeon et al. 2013); and lactate (Elsden et al. 1956).

Lactate is an important intermediate in the anaerobic breakdown of carbohydrates (Kleerebezem and van Loosdrecht 2007). Carbohydrates comprise ~18% of the COD in municipal wastewaters (Raunkjær et al. 1994) and up to ~70% of the COD in some food processing wastewaters (Arslan et al. 2013, Gómez et al. 2009). Lactate to *n*-caproate conversion has been proposed to proceed similarly to the reverse β -oxidation pathway used for the conversion of ethanol to *n*-caproate: lactate (a three-carbon SCC) is converted to acetyl-CoA, which then enters the reverse β -oxidation cycle (Spirito et al. 2014, Zhu et al. 2015). Until recently, *n*-caproate production from exogenous lactate had only been clearly demonstrated in pure-culture studies using *Megasphaera elsdenii* in batch systems (Elsden et al. 1956, Ladd 1959, Marounek et al. 1989, Weimer and Moen 2013). *M. elsdenii* is a bacterium isolated from the rumen that was found to be uniquely tolerant of pH values below 5.5, metabolizing DL-lactate even at pH values as low as 4.7 (Counotte and Prins 1981).

Recently, Zhu et al. (2015) produced *n*-caproate from exogenous lactate with a reactor microbiome for the first time. *Clostridium IV*, within the family of the Ruminococcaceae, dominated this microbiome, while *M. elsdenii* was not observed. The inoculum was collected from a pit mud that was used to convert carbohydrate-rich feedstocks (*e.g.*, wheat, sorghum, and maize) into the fermented precursor “yellow water” for Chinese strong-flavored liquor production. In batch bioreactors operated between pH 6.0 and 6.5, exogenous lactate was added as the sole carbon and energy source. From these batch periods of less than four days, a maximum volumetric *n*-caproate production rate (productivity) of 6.5 g COD/L-d was reported. Others had already described that the conversion of lactate to *n*-caproate in reactor microbiomes was likely after finding endogenous lactate consumption and simultaneous *n*-caproate accumulation in batch-fed systems with reactor microbiomes (Agler et al. 2012b, Sträuber et al. 2012, Sträuber et al. 2015). However, proof was missing because other intermediates from the hydrolysis and acidification of the complex organic substrates (*e.g.*, maize fiber hydrolysate, maize silage) were present in addition to lactate. Similarly to Zhu et al. (2015), Sträuber et al. (2015) observed an abundance of *Clostridium IV* and the absence of *M. elsdenii* in periods when endogenous lactate was consumed and *n*-caproate was simultaneously produced.

In ethanol-fed systems, even-numbered carboxylates (*e.g.*, *n*-butyrate, *n*-caproate) are the predominant products produced *via* the reverse β -oxidation pathway (Spirito et al. 2014). In lactate-fed systems, the even-numbered reverse β -oxidation products are accompanied by odd-numbered SCCs (*e.g.*, propionate, *n*-valerate) (Weimer and Moen 2013), which are derived from the competing acrylate pathway that converts lactate into propionate (Hino and Kuroda 1993). In one of the few bioreactor studies in which DL-lactate was fed continuously to a pure culture of

the type strain *M. elsdenii*, the presence of these competing metabolic pathways was confirmed, but no *n*-caproate was produced (Prabhu et al. 2012). Others had already found that anaerobic conversion of lactate typically leads to propionate accumulation when lactate is abundant (Sträuber et al. 2015). This is in stark contrast to the recent pit mud reactor microbiome study (Zhu et al. 2015) in which no propionate accumulation was reported, indicating that the competing acrylate pathway was not active.

n-Caproate production from ethanol has been demonstrated using both batch-fed (Agler et al. 2012a, Steinbusch et al. 2011) and continuously fed bioreactors (Grootscholten et al. 2013). However, we found no reports of *n*-caproate production in bioreactors that were continuously fed exogenous lactate as the sole carbon and energy source. The most successful biotechnology production platforms for anaerobic treatment are continuously fed systems such as upflow anaerobic sludge blanket reactors (Lettinga 1995). To translate chain elongation with lactate into real anaerobic treatment systems, we had several experimental objectives: 1) to provide the first demonstration of sustained conversion of lactate to *n*-caproate in a continuously fed reactor microbiome system; 2) to evaluate the effects of lactate loading rates and residual lactate concentrations on the production of *n*-caproate and other carboxylates *via* the competing reverse β -oxidation and acrylate pathways; and 3) to characterize microbial community changes within the reactor microbiome corresponding to periods of increased *n*-caproate productivity.

2. MATERIALS & METHODS

2.1 Growth medium and inoculum

The basal medium was based on a previous batch study in which lactate was converted to *n*-caproate (Weimer and Moen 2013), and it contained nutrients and yeast extract (1.25 g/L, 1.6 g COD/L) with modifications as described previously (Vasudevan et al. 2014). L-Lactate and *n*-butyrate were added at varied substrate concentrations to achieve desired loading rates. The medium pH was adjusted with 5 M sodium hydroxide to the operating pH of the bioreactor. The inoculum was derived from a well-characterized reactor microbiome that was fed ethanol-rich yeast fermentation beer (Agler et al. 2012a). This reactor microbiome had been batch-fed semi-continuously (once every two days) throughout an operating period of more than three years at the time of inoculation (Ge et al. 2015). The inoculum was triple-washed in basal media, and approximately 100 mL of this inoculum was added to the bioreactor.

2.2 Bioreactor system

We employed an upflow anaerobic filter with constant bioreactor broth recycling through an in-line pertraction system (membrane-based liquid-liquid extraction) for product extraction. The bioreactor was constructed as a vertically oriented cylinder, which was made of Plexiglas[®], with an inner diameter of 6 cm (Fig. 1). The total volume was 0.70 L, but Kaldnes K1 packing material (Evolution Aqua, Wigan, United Kingdom) was added, resulting in a working volume of 0.55 L. The bioreactor was wrapped with tubing in which hot water from a heating bath (VWR Scientific Model 1104, Radnor, PA, USA) was recirculated for temperature control, resulting in a constant temperature of 34±1°C inside the bioreactor. A pH probe (Mettler 405-DPAS SC K85, Columbus, OH, USA) was mounted at the top of the bioreactor. Automated pH

control of the bioreactor broth was maintained with a controller (Eutech Instruments alpha-pH800, Vernon Hills, IL, USA) and a corresponding acid addition pump (Mityflex 913, Bradenton, FL, USA). Hydrochloric acid (0.5 M) was added to the well-mixed feed and recycle inlet at the base of the bioreactor. Fresh media containing L-lactate and *n*-butyrate was continuously fed from a refrigerated vessel (4°C) into the base of the bioreactor using a peristaltic feed pump (Cole Parmer L/S Digital Economy Drive, Vernon Hills, IL, USA) at an average rate of approximately 0.37 L/d and a hydraulic retention time (HRT) of 1.5 d (Fig. 1). The effluent continuously exited the bioreactor *via* an overflow line connected to the top of the bioreactor. The exit of the overflow line was submerged within a secondary effluent reservoir. An inverted funnel was used to collect the produced gas within the bioreactor and was connected to a flow meter (Ritter MGC-1, Bochum, Germany) (Fig. 1). In addition, a gas-sample septum and a bubbler were placed in the gas collection system. A sampling port for biomass samples was placed halfway up the vertically oriented bioreactor.

2.3 Pertraction system

Product recovery was accomplished with a pertraction system (Fig. 1) similar to those used in previous reports (Agler et al. 2012a, Ge et al. 2015). One forward and one backward membrane contactor (1.4 m² each, Membrana Liqui-Cel 2.5x8, X50 membrane, Charlotte, NC, USA) were used. The hydrophobic solvent was circulated continuously in the lumen of the hydrophobic hollow-fiber membrane modules; the solvent consisted of mineral oil with 30 g/L tri-*n*-octylphosphine oxide (TOPO) (Sigma Aldrich, St. Louis, MO, USA). The stirred alkaline extraction solution was initially buffered with 0.3 M sodium borate and was maintained at pH 9 with automated addition of 5 M sodium hydroxide using a controller (Eutech Instruments alpha-

pH800, Vernon Hills, IL, USA) and a corresponding base pump (Mityflex 913, Bradenton, FL, USA). A constant bioreactor broth recycle flow of 60 L/d was maintained using a peristaltic pump (ColeParmer 7553-30, Vernon Hills, IL, USA). To prevent fouling or solids accumulation in the forward membrane contactor, bioreactor broth was drawn from the top of the anaerobic filter and was then pumped through a custom-built, 1.6-mm stainless-steel strainer (Danco 88886, Shorewood, IL, USA), a 65- μ m filter (McMaster-Carr 44205K21, Elmhurst, IL, USA), and a subsequent 5- μ m filter (Pentek GS-6 SED/5, Upper Saddle River, NJ, USA). A peristaltic pump (ColeParmer 7553-30, Vernon Hills, IL, USA) equipped with two pump heads provided continuous recycle flows of 10 and 58 L/d for the mineral oil solvent and alkaline extraction solution, respectively.

2.4 Operating strategy and calculations

This bioreactor experiment was divided into four phases: I) batch phase; II) start-up phase with continuous feeding (with the pertraction system off or on); III) high *n*-caproate productivity phase (with continuous feeding and the pertraction system on); and IV) post-lactate overloading phase (with continuous feeding and the pertraction system on). Each phase was then divided into several distinct operating periods. For each period, several operating parameters were held constant, including the: organic loading rate (OLR); HRT; bioreactor pH; and operation with or without pertraction. Each operating period was operated for at least five HRT periods, and average bioreactor loading rates and concentrations were reported.

Carboxylate productivities were also calculated as average values for each operating period. Herein, the average bioreactor effluent production rate (g COD/d) *plus* the average

transfer rate *via* pertraction (g COD/d) were summed to yield the total production rate (g COD/d). Effluent production rates were calculated as the average bioreactor broth concentration divided by the average HRT for each period. Average transfer rates were calculated by first plotting the increasing amounts of individual carboxylates in the alkaline extraction solution against time. Least squares methods were then used to determine the slope and the sample standard deviation (LINEST function, Microsoft Excel). Production rates were divided by the working bioreactor volume to determine the total volumetric production rates (productivities) (g COD/L-d). All concentrations, rates, and yields were converted to a g COD basis. Feed flow rates were determined volumetrically; effluent rates were determined gravimetrically. Uncertainty was represented by 95% confidence intervals: the standard error was first calculated as the quotient of the sample standard deviation divided by the square root of the number of samples; then, the standard error was multiplied by a t-value corresponding to the degrees of freedom (based on the number of samples). Uncertainty was propagated through calculations, and 95% confidence intervals were included with reported data (*e.g.*, productivities).

2.5 Liquid and gas analysis

Liquid samples (1.5 mL) were collected from the bioreactor and the alkaline extraction solution every other day or daily. Bioreactor broth samples were collected from the broth recycle line between the 5- μ m filter and the forward membrane contactor. Alkaline extraction solution samples were collected from the well-mixed reservoir ($\sim$ 3 L). Concentrations of most carboxylates were determined with gas chromatography (GC) (Usack and Angenent 2015). Concentrations of lactate were quantified with high performance liquid chromatography (HPLC). Lactate samples were filtered through a sterile Acrodisc 0.22- μ m pore size polyvinylidene

fluoride membrane syringe filter (Pall Life Sciences, Port Washington, NY, USA) prior to analysis to remove possible biological and particulate contaminants. Lactate was separated from other compounds on an Aminex HPX-87H 300 mm x 7.8 mm cation-exchange column for carboxylate analysis (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 60°C with 5 mM sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min. HPLC analysis was conducted with the Waters 600E system controller, 717Plus autosampler, and the 410 refractive index detector (Waters Corp, Milford, MA, USA). Data were recorded and analyzed with the PeakSimple Chromatography Data System SRI Model 302, and PeakSimple software v4.44 (Schemback, Germany). The concentrations of methane, carbon dioxide, and hydrogen gases (>2000 ppm) were measured using a GC system (Usack and Angenent 2015). Furthermore, the concentration of hydrogen gas (<2000 ppm) was determined using a reduction gas detector (RGD) (Trace Analytical RGD, Menlo Park, CA, USA). The RGD inlet was connected to a packed column (Restek, ShinCarbon ST 80/100, Bellefonte, PA, USA) for peak separation, which was installed in a GC system (Gow Mac 580, Bethlehem, PA, USA).

2.6 Microbial community analysis: biomass samples, DNA extraction, PCR, sequencing, and data analysis

We collected biomass samples from the bioreactor broth at 18 time points throughout the experiment, as well as one sample from the inoculum. The bioreactor broth was thoroughly mixed by quickly withdrawing and refilling a 60 mL syringe ten times. During this sampling, settled flocculent biomass was resuspended. The sample was collected in 2-mL Eppendorf tubes. These 2-mL samples were then centrifuged at 16,873 x g and the supernatants were discarded.

Concentrations of wet solids in these pelleted biomass samples ranged from 23 to 76 mg/L. These pelleted biomass samples were stored at -80°C until further processing.

Genomic DNA was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA). Modifications to the protocol include utilization of custom bead tubes containing a mixture of 300 mg of 0.1-mm diameter and 100 mg of 0.5-mm diameter silica/zirconia beads (Hospodsky et al. 2010), and physical cell lysis with bead-beating at 3450 oscillations/min for 45 s. The DNA amplification protocol was described previously (Regueiro et al. 2015) with the following exceptions: 1) we used Mag-Bind RxnPure Plus magnetic beads solution (Omega Biotek, Norcross, GA, USA) instead of Mag-Bind E-Z Pure; 2) we pooled only 20 ng DNA per sample instead of 100 ng. We used 515 forward and Golay-barcoded 806 reverse primers targeting the hypervariable V4 region of the 16S rRNA gene. We used QIITA (qiita.microbio.me) for initial processing of the sequencing data. The sortmerna method was used to bin sequences into operational taxonomic units (OTUs) at 97% identity. Taxonomy was assigned for representative sequences selected for each OTU using the Greengenes v13.8 database from August 2013 (McDonald et al. 2012). The remaining analyses were performed in QIIME v1.9 (Caporaso et al. 2010). Singleton OTUs were removed from the dataset.

Community analysis, including beta diversity and unconstrained ordination, was performed as described previously (Regueiro et al. 2015) with the following exceptions: 1) the alpha diversity was calculated using the Shannon diversity index (Shannon 1948) rather than Chao1; 2) the Pearson correlation coefficient was calculated for samples collected during Phase III with the functions cor and cor.test in the R stats package (Team 2014). At a significance level

of $p < 0.05$ and $n = 11$, the relative abundance of an OTU would be positively correlated with *n*-caproate productivity if the Pearson r was greater than 0.602. Heatmaps were created to represent OTU relative abundances during bioreactor operation *via* the *gplots* package in R (Warnes et al. 2015).

RESULTS AND DISCUSSION

3.1 Conversion of L-lactate into n-caproate was demonstrated in a continuously fed bioreactor for the first time

In an upflow anaerobic filter, which was continuously fed L-lactate, the maximum *n*-caproate productivity achieved was 6.9 g COD/L-d (Fig. 2). This maximum productivity was sustained for 17 days (Table S1) at an HRT of 1.9 d (Table S2) (Phase III, Period 12, Days 176-193). The corresponding L-lactate loading rate was 9.1 g COD/L-d, and the total organic loading rate (OLR) was 17.7 g COD/L-d (Table S2). We fed both L-lactate and *n*-butyrate to the bioreactor, since we had anticipated that *n*-butyrate would be chain-elongated to *n*-caproate by the L-lactate-derived acetyl-CoA. However, addition of *n*-butyrate was unnecessary for *n*-caproate production: a net positive production of *n*-butyrate was observed (Table S2) from the chain elongation of endogenous acetate (0.4-2.0 g COD/L, 6-31 mM) with lactate (Fig. 3).

We observed continuous *n*-caproate production for over 165 days in this study (Fig. 3). Importantly, in-line pertraction was used throughout Phase III, and more than 95% of the *n*-caproate produced was recovered *via* pertraction. To our knowledge, this is the first demonstration of lactate to *n*-caproate conversion in a continuously fed bioreactor, while it is the second demonstration of *n*-caproate production with exogenous lactate in a reactor microbiome

(Zhu et al. 2015). In a very early batch study with *M. elsdenii*, the reported productivity of *n*-caproate from lactate was 8.5 g COD/L-d, but the duration of fermentation for which this productivity was calculated to be only 45 min (Ladd 1959). Therefore, our maximum *n*-caproate productivity of 6.9 g COD/L-d represents the highest sustained conversion rate of lactate to *n*-caproate. However, this maximum rate remains lower than the *n*-caproate productivities (123.1 g COD/L-d) from bioreactors that were continuously fed ethanol (Grootscholten et al. 2013).

3.2 L-Lactate was converted to n-caproate during startup, but n-caproate productivity was low

Before reaching the maximum *n*-caproate productivity in Phase III (Period 12), the bioreactor was operated in a sequence of operating phases and periods. In Phase I, the bioreactor was batch-fed and pertraction was not used. The initial L-lactate concentrations for the batches in Phase I (Periods 1 and 2) were 19.7 and 6.1 g COD/L (205 and 64 mM), respectively, and the operating pH was maintained at 5.5 (Fig. 3A). The second of these two batch periods (Phase I, Period 2, Days 22-30) led to some *n*-caproate accumulation (0.2 g COD/L, 1 mM) (Fig. 3B). This result indicated that microbial conversion of L-lactate to *n*-caproate was active; therefore, we initiated continuous feeding of media containing L-lactate and *n*-butyrate during Phase II.

The initial continuous feeding rate was high for the first four days of Phase II, corresponding to a short HRT of 0.7 d (Phase II, Period 3, Days 30-33) (Table S2). Soon thereafter, the media feed rate was decreased to provide a longer HRT of 1.4 d (Phase II, Period 4, Days 33-59), and the HRT was maintained at an average of 1.5 ± 0.1 d throughout the remainder of the experiment. Consequently, to vary the L-lactate loading rate and the total OLR without varying the HRT, the concentrations of L-lactate (3-24 g COD/L, 30-250 mM) and *n*-

butyrate (1-20 g COD/L, 8-125 mM) in the basal media were adjusted (Table S2). The concentration of yeast extract in the media was consistently 1.6 g COD/L (1.25 g/L), corresponding to an average yeast extract loading rate of 1.1 g COD/L-d. This yeast extract loading was fed in excess. For commercial applications, optimization to determine the minimum required yeast extract loading rate (or replacement with nutrients contained in complex waste substrates) would be necessary.

Pertraction was initiated on Day 33 of Period 4 (Phase II) in a first attempt to continuously recover *n*-caproic acid. The L-lactate loading rate was 2.3 g COD/L-d (Phase II, Period 4, Days 33-59) (Table S2), and the residual lactate was consumed to levels below the detection limit (<0.02 g COD/L, <0.2 mM) (Fig. 3A). Undissociated *n*-butyric acid and other hydrophobic carboxylic acids were transferred to the alkaline extraction solution. Consequently, the residual concentrations of carboxylates in the bioreactor broth declined, consisting primarily of acetate and *n*-butyrate (Fig. 3B). However, Period 4 was marked by failure to produce *n*-caproate: the concentration of *n*-caproate in the bioreactor broth diminished to below the detection limit (<0.02 g COD/L, <0.1 mM), but the rate of recovery of *n*-caproate *via* pertraction also declined to zero.

Due to the lack of *n*-caproate production in Period 4, the pertraction system was turned off on Day 59 to start Period 5. During Period 5, the L-lactate and total organic loading rates were maintained at similar levels as those in Period 4, and residual lactate concentrations in the bioreactor increased slightly above the detection limit to 0.03 g COD/L (0.3 mM) (Fig. 3A). The concentrations of all carboxylates in the bioreactor broth increased once pertraction was turned off, and *n*-butyrate was the dominant carboxylate observed (Fig. 3B). However, some *n*-caproate

was also observed in the bioreactor broth in Period 5, corresponding to an *n*-caproate productivity of 0.1 g COD/L-d.

3.3 Lowering the pH led to increased n-caproate productivities

The *n*-caproate productivity increased considerably once the operating pH was decreased from 5.5 to 5.0 (Fig. 3) (Phase II, Period 6, Days 76-84). The impetus for this change was based on the low productivities we had reached, thus far, and the rationale for decreasing the pH from 5.5 to 5.0 was based on observations from the literature. For equivalent total (undissociated plus dissociated) carboxylate concentrations, this decrease in pH by 0.5 would lead to a ~2x increase in undissociated hydrophobic carboxylic acids. These hydrophobic acids are inhibitory due to their ability to permeate hydrophobic cellular membranes; dissociate into carboxylates and protons; and exhaust cellular efforts of expelling excess protons to maintain a neutral intracellular pH.

Acidic conditions provide an environmental pressure to select for acid-tolerant microbes. In a previous study of rumen bacteria, only *M. elsdenii* was able to grow and continue to utilize lactate at pH values below 5.5, growing even at pH values as low as 4.7 (Counotte and Prins 1981). The capacity to chain elongate SCCs (*e.g.*, acetate) *via* reverse β -oxidation to *n*-butyrate and *n*-caproate is one method to relieve excess reducing equivalents, and *M. elsdenii* was the only microbe that was known to be capable of converting lactate into *n*-caproate at the time of our experiment. Moreover, in another study of *M. elsdenii*, the absence of *n*-caproate production was reported for a bioreactor operated at pH 6.5 and continuously fed DL-lactate at a lactate loading rate of 10.1 g COD/L-d (Prabhu et al. 2012). We hypothesized that if we lowered the pH

to 5.0, perhaps bacteria such as *M. elsdenii* would be able to outcompete other lactate-utilizing microbes and direct its metabolism to increase *n*-caproate productivity.

When we decreased the pH from 5.5 to 5.0 (Fig. 3A), the average *n*-caproate productivity increased from 0.1 to 0.6 g COD/L-d (Fig. 3B) (Phase II, Period 6, Days 76-84). The net productivities of other carboxylates (*e.g.*, *n*-butyrate, propionate) remained consistent with the rates achieved at similar OLRs during Period 5, and the residual lactate concentrations (0.04 g COD/L, 0.4 mM) were also similar. The marginal differences in loading rates were insufficient to explain this 6x increase in *n*-caproate productivity. The *n*-caproate concentration in the bioreactor broth accumulated to ~2 g COD/L (8 mM), corresponding to an undissociated *n*-caproic acid concentration of less than ~1 g COD/L (3 mM). This concentration of undissociated *n*-caproic acid was lower than concentrations previously hypothesized to induce product inhibition (1.8 g COD/L, 7 mM) (Ge et al. 2015). To prevent *n*-caproic acid concentrations from subsequently increasing to inhibitory levels, we turned the pertraction system on to start Phase III.

3.4 Use of pertraction increased n-caproate productivity

Before using pertraction, *n*-caproate productivity was observed at rates up to 0.6 g COD/L-d (Fig. 3B, Phase II, Period 6, Days 76-84). When the pertraction system was turned on, the *n*-caproate productivity increased from 0.6 to 1.0 g COD/L-d at an equivalent L-lactate loading rate (2.6 g COD/L-d) (Phase III, Period 7, Days 84-112). This indicates that, although *n*-caproate production was possible without pertraction, the use of pertraction led to considerably improved productivities. In previous work, this in-line product recovery system had decreased

the impacts of product toxicity (Agler et al. 2012a), especially at operating pH values at and below 5.5, and had consistently improved *n*-caproate productivity (Ge et al. 2015).

3.5 Increased L-lactate loading rates led to linear increases in n-caproate productivities

During Phase III, when the L-lactate loading rate was steadily increased from 2.6 g COD/L-d to 9.1 g COD/L-d, the *n*-caproate productivity increased linearly (Fig. 2). Overall, steady operating conditions were experienced for most of Phase III. One key exception was due to a large disturbance on Day 166 (Fig. 3) (Phase III, Period 11). This anomaly was caused by an air intrusion upstream of the bioreactor recycle pump, which evacuated the bioreactor and pertraction system of liquid and largely filled the system with air. The perturbations led to reduced bioreactor volumes and temporarily increased concentrations of lactate (Fig. 3A) and other carboxylates (Fig. 3B). The bioreactor pH was upset to a temporary value of ~3.9, but the system recovered to steady operating conditions within one HRT period.

During steady operating conditions, we observed gas production, and the concentrations of carbon dioxide and methane increased steadily with increasing loading rates (Fig. S1). In the mechanism proposed in the literature of lactate to *n*-caproate conversion, the intermediate three-carbon pyruvate is converted to the two-carbon acetyl-CoA, releasing an equimolar amount of carbon dioxide (Fig. 4). The carbon dioxide observed was probably from the added lactate (62 to 505 mM/d) instead of from the sodium carbonate added (0.2 mM/d). The hydrogen gas concentration in the headspace (typically below 10 ppm) was likely maintained at low levels by hydrogenotrophic methanogens. The pH was maintained near 5.0 for most of this study, which would have inhibited acetoclastic methanogens. Finally, unsubstantial production of *n*-

heptanoate and iso-carboxylates was observed. We did not detect any *n*-caprylate or longer-chain carboxylate production. Some *n*-valerate, which is a chain elongation product from propionate, was produced, especially during periods when propionate production was high and when residual lactate concentrations were non-zero (Fig 3).

3.6 Residual lactate concentrations led to propionate production, but not n-caproate production

The highest *n*-caproate productivity (6.9 g COD/L-d) was observed when the L-lactate loading rate was 9.1 g COD/L-d. When the L-lactate loading rate was subsequently increased to 16.2 g COD/L-d, the resulting *n*-caproate productivity decreased to 3.0 g COD/L-d (Fig. 2) (Phase III, Period 13, Days 193-204). To recover from this overloading event, we lowered the L-lactate loading rate from 16.2 to 10.1 g COD/L-d (Phase IV, Period 14, Days 204-213), but the *n*-caproate productivity never recovered (Fig. 3B). While *n*-caproate productivity decreased following this overloading event, the corresponding propionate productivity reached its highest rate (5.5 g COD/L-d) in Period 13, concluding a linear trend of steady increases with increased L-lactate loading rates throughout Phase III. Propionate productivity increased when *n*-caproate productivity declined due to one key factor: the residual concentration of lactate in the bioreactor broth had increased. These residual lactate concentration began to increase steadily (Fig. 3A) from below detection (Phase III, Period 12, Days 176-193) to 0.2 g COD/L (2 mM) (Phase III, Period 13, Days 193-204).

3.6.1 Mechanism: reverse β -oxidation pathway

Other studies concluded that a low residual concentration of lactate would be necessary to prevent production of propionate *via* the acrylate pathway (Prabhu et al. 2012) (Fig. 4).

Accordingly, L-lactate can then be converted to *n*-caproate *via* the reverse β -oxidation pathway through three steps: 1) L-lactate is first isomerized to D-lactate *via* lactate racemase, which is a reversible and lactate-induced enzyme (Fig. 4, Step 1); 2) D-lactate is then converted to pyruvate *via* the NAD-independent D-lactate dehydrogenase (iD-LDH), which is a favorable and irreversible reaction as long as pyruvate is consumed in subsequent reactions (Fig. 4, Step 2) (Hino and Kuroda 1993); and 3) pyruvate is converted to acetyl-CoA (Fig. 4, Step 3), which enters the reverse β -oxidation pathway to chain elongate available SCCs (*e.g.*, acetate) to yield even-numbered chain elongation products such as *n*-butyrate and *n*-caproate (Spirito et al. 2014).

3.6.2 Mechanism: acrylate pathway

In the competing acrylate pathway, L-lactate is converted to propionate (Fig. 4, Step 4). Under L-lactate-rich conditions, lactyl-CoA forms as an intermediate. When this occurs, a subsequent state to achieve redox balance perpetually generates propionyl-CoA (Prabhu et al. 2012). Consequently, available L-lactate would be continually directed toward propionate production (Prabhu et al. 2012). This explanation suggests that once residual L-lactate concentrations accumulate and induce lactyl-CoA formation, it may be difficult to direct lactate-carbon flux toward pyruvate and even-numbered chain elongation products (*e.g.*, *n*-caproate).

Indeed, we observed increased propionate productivity and decreased *n*-caproate productivity *during* and *after* our overloading event in which L-lactate accumulated and induced the acrylate pathway (Phase III, Period 13, Days 193-204). The results of Phase IV (post-overload), thus, confirm that once the acrylate pathway is activated, it is difficult to return to

reverse β -oxidation. In addition, our results explain why most batch studies with high initial lactate concentrations do not result in promising *n*-caproate productivities.

Surprisingly, in the first reactor microbiome study in which exogenous lactate was converted into *n*-caproate, no propionate production was reported (Zhu et al. 2015). Their batch-fed systems were operated with lactate constantly in excess, which we expected would induce the acrylate pathway. It is possible that microbes with the competing acrylate pathway had disappeared or were outcompeted based on the unique environmental selection process they employed, but we anticipate that the lack of propionate production is atypical for most lactate-fed reactor microbiome systems. For open-culture bioreactor systems (*e.g.*, wastewater treatment) in which substrate flow consistently introduces diverse bacteria to the reactor microbiome, our results suggest that the acrylate pathway would persist as an important competing pathway that would need to be carefully managed.

3.7 Reactor microbiome analyses revealed a surprising absence of M. elsdenii

We also investigated reactor microbiome dynamics throughout the experiment. Among all samples collected, we found 1,584 operational taxonomic units (OTUs) from high-quality sequence reads. The number of sequences per sample ranged from 35,367 to 139,674 sequences, with a median sequence count of 63,726. We found 49 OTUs that each comprised at least 1% of the relative OTU abundance for one or more samples (Fig. S2), and these comprised between 84.0 and 94.8% of the total high-quality sequence reads for each sample. The highest observed relative abundance of a single OTU was 63.3% for Rhodocyclaceae K82 spp. (Phase I, Period 2, Day 30). During the phase of high *n*-caproate productivity, *Acinetobacter* spp. was dominant at a

relative abundance of 62.9% (Phase III, Period 9, Day 140) (Fig. 5). While *M. elsdenii* was previously known as the only type strain for which lactate conversion to *n*-caproate had been demonstrated in pure culture, the highest relative abundance observed for *M. elsdenii* was less than 0.01% (Phase III, Period 12, Day 184). We detected several OTUs with phylogenetic similarities to *M. elsdenii*, including species of *Ruminococcus*, *Oscillospira*, *Clostridium*, and genera from the families Veillonellaceae, Ruminococcaceae, and Clostridiales (Fig. S2). Of the OTUs detected above 1% relative abundance, an unknown OTU from the family Veillonellaceae was most similar to *M. elsdenii*. Moreover, during the phase of high *n*-caproate productivity (Phase III), the abundance of this OTU was positively correlated with *n*-caproate productivity ($p = 0.005$) (Fig. S3). Two other OTUs were also positively correlated with *n*-caproate productivity: *Bacteroides* spp. ($p = 0.042$); and *Comamonas* spp. ($p = 0.040$). In the other reactor microbiome study with conversion of exogenous lactate into *n*-caproate, *M. elsdenii* was also scarce or not present (Zhu et al. 2015).

In our study, no clear trends in alpha diversity were apparent from the Shannon diversity index, suggesting that the richness and evenness of the microbial community at each sampling point remained similar (Fig. S4). The beta diversity, or dissimilarity of OTU composition between samples, showed that at the onset of overloading, the communities of the final three samples (darkest circles) were distinctly different from early community samples from the bioreactor (light circles) (Fig. S5). Constrained ordination did not find a correlation between microbial community composition and operating parameters, environmental conditions, or bioreactor performance at significant levels ($p < 0.05$).

3.8 Strategy and suggestions for future work

Based on our experimental results, we propose a model to explain how to direct a lactate-fed system toward improved *n*-caproate productivities (Fig. 6). First, it was necessary to maintain residual lactate concentrations near zero. Under these conditions, we observed robust *n*-caproate production and restrained propionate production (Fig. 3, Phase III, Periods 7-12, Days 84-193). Second, our results suggest that operating at pH values near 5.0 may be necessary for conversion of lactate into *n*-caproate in most reactor microbiomes. Specifically, when we decreased the operating pH from 5.5 to 5.0, the *n*-caproate productivity increased from approximately 0.1 to 0.6 g COD/L-d (Phase II, Period 6, Days 76-84). Finally, we observed that the use of pertraction increased the *n*-caproate productivity with all other parameters held constant. At longer HRTs, the importance of this pertraction system would be increasingly essential because of the lower dilution rates of the inhibiting undissociated carboxylic acids.

When L-lactate was fed continuously to a bioreactor, the L-lactate-derived carbon flux was primarily towards reverse β -oxidation products (*e.g.*, *n*-butyrate, *n*-caproate). We speculate that if we had fed D-lactate or DL-lactate at an equivalent lactate loading rate, then *n*-caproate productivity would have been at least equal, if not superior, to those observed with L-lactate. This is because the extra metabolic step from L-lactate to D-lactate *via* lactate racemase would not have been required, and also because lactate racemase has been described as potentially rate-limiting in the conversion of L-lactate to D-lactate (Hino and Kuroda 1993).

Finally, this research supports future efforts using real wastes as substrates. Specifically, wastes containing high lactate concentrations (*e.g.*, fermented food processing wastes) or with

high concentrations of precursors to lactate (*e.g.*, carbohydrate-rich food processing wastes) should be evaluated. The upflow anaerobic filter is designed primarily for low-solids substrates; initial efforts could focus on waste effluents with high loading from dissolved lactate or carbohydrates. Waste carbon can be directed towards *n*-caproate instead of lower-value products by applying the findings of our study. Consequently, new applications are now possible for chain elongation technologies within the carboxylate platform to promote effective resource recovery.

6. CONCLUSIONS

- We demonstrated *n*-caproate production from lactate in a continuously fed bioreactor.
- The maximum *n*-caproate productivity of 6.9 g COD/L-d was achieved with a reactor microbiome.
- Low residual lactate concentrations in the bioreactor broth, a bioreactor broth pH of 5, and the use of pertraction were all key operating parameters for effective conversion of L-lactate to *n*-caproate.
- The reactor microbiome was diverse and showed *Acinetobacter* spp. to be abundant during periods of high *n*-caproate productivity while the type strain *M. elsdenii* was nearly absent.
- This study promotes consideration of real waste streams for use in the carboxylate platform to produce MCCs, including those rich in lactate or lactate-precursors (*e.g.*, carbohydrates).

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550

FIGURE CAPTIONS

Fig. 1: Bioreactor system schematic. An upflow anaerobic filter was continuously fed basal media containing L-lactate and *n*-butyrate. In-line pertraction (membrane-based liquid-liquid extraction) was used to continuously recover hydrophobic, undissociated medium-chain carboxylic acids (MCCAs) (*e.g.*, *n*-caproic acid) from a bioreactor broth recycle flow. After intermediary recovery in a mineral oil solvent, MCCAs were then transferred across a second membrane contactor to an alkaline extraction solution. Through automatic base addition to the alkaline extraction solution, the pH gradient was maintained, and these carboxylic acids accumulated in the alkaline extraction solution as medium-chain carboxylates (MCCs).

Fig. 2: *n*-Caproate and propionate productivities are functions of the L-lactate loading rate. *n*-Caproate productivities increased up to 6.9 g COD/L-d when L-lactate loading rates were increased to 9.1 g COD/L-d. After the L-lactate loading rate was increased to 16.2 g COD/L-d, the *n*-caproate productivity decreased to 3.0 g COD/L-d. At this maximum loading rate, the propionate productivity increased to 5.5 g COD/L-d. Error bars represent 95% confidence intervals of average productivities. Data shown are from Phase III only.

Fig. 3: Bioreactor broth concentrations. Concentrations of lactate (A) and other carboxylates (B) in the bioreactor broth were determined from samples collected every other day or daily. Bioreactor pH (A) and average productivities of *n*-caproate and propionate (B) were also determined. Operating phases, operating periods, and presence (+) or absence (-) of product

recovery *via* in-line pertraction are indicated. Detection limits were 0.02 g COD/L (0.2 mM) for lactate and approximately 0.02 g COD/L (~0.1 mM) for other carboxylates.

Fig. 4: Lactate conversion pathways. DL-Lactate can proceed to either *n*-caproate or propionate *via* the reverse β -oxidation pathway or the acrylate pathway, respectively. Based on Spirito et al. (2014) and Hino and Kuroda (1993).

Fig. 5: OTU relative abundances during the phase of high *n*-caproate productivity. Relative abundances of operational taxonomic units (OTUs) varied during the phase of high *n*-caproate productivity (Phase III). Each of the 27 OTUs listed comprised at least one percent of the relative abundance for one or more of the samples collected from this phase. OTUs were clustered hierarchically (average linkage) based on the Bray-Curtis dissimilarity index. OTUs were grouped together based on both the average relative abundance and abundance profile throughout the duration of Phase III. This resulted in the localization of OTUs with lower abundances in the top half, and OTUs with higher abundances in the bottom half. Within lower abundant OTUs, several OTUs increased in relative abundance over time, as indicated in the first cluster on top. However, within high abundant OTUs, relative abundance profiles did not indicate any significant trend over time. In Phase III, *Acinetobacter* spp. was dominant, comprising up to 62.9% of the relative abundance. The relative abundances of three OTUs (marked with asterisks) were correlated ($p < 0.05$) with *n*-caproate productivity. Data shown are from Phase III only.

Fig. 6: Lactate to *n*-caproate decision tree. A simplified model is presented to evaluate if operating conditions are favorable for conversion of lactate to *n*-caproate. For high-rate *n*-

596 caproate productivity, our results suggest the following conditions: 1) low residual lactate
597 concentrations (<0.02 g COD/L, <0.2 mM); 2) low bioreactor pH (<5); and 3) sufficient *n*-
598 caproate recovery ($>95\%$ recovery). Lactate-utilizing chain-elongating microbes must also be
599 functional and abundant.

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REFERENCES

- Agler, M.T., Wrenn, B.A., Zinder, S.H. and Angenent, L.T. (2011) Waste to bioproduct conversion with undefined mixed cultures: The carboxylate platform. *Trends in biotechnology* 29(2), 70-78.
- Agler, M.T., Spirito, C.M., Usack, J.G., Werner, J.J. and Angenent, L.T. (2012a) Chain elongation with reactor microbiomes: Upgrading dilute ethanol to medium-chain carboxylates. *Energy & Environmental Science* 5(8), 8189-8192.
- Agler, M.T., Werner, J.J., Iten, L.B., Dekker, A., Cotta, M.A., Dien, B.S. and Angenent, L.T. (2012b) Shaping reactor microbiomes to produce the fuel precursor *n*-butyrate from pretreated cellulosic hydrolysates. *Environmental science & technology* 46(18), 10229-10238.
- Angenent, L.T., Karim, K., Al-Dahhan, M.H., Wrenn, B.A. and Domínguez-Espinosa, R. (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends in biotechnology* 22(9), 477-485.
- Arslan, D., Steinbusch, K., Diels, L., De Wever, H., Hamelers, H. and Buisman, C. (2013) Selective carboxylate production by controlling hydrogen, carbon dioxide and substrate concentrations in mixed culture fermentation. *Bioresource technology* 136, 452-460.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J. and Knight, R. (2010) Qiime allows analysis of high-throughput community sequencing data. *Nature Methods* 7(5), 335-336.
- Counotte, G.H. and Prins, R. (1981) Regulation of lactate metabolism in the rumen. *Veterinary research communications* 5(1), 101-115.
- Desbois, A.P. (2012) Potential applications of antimicrobial fatty acids in medicine, agriculture and other industries. *Recent patents on anti-infective drug discovery* 7(2), 111-122.
- Elsden, S., Volcani, B., Gilchrist, F. and Lewis, D. (1956) Properties of a fatty acid forming organism isolated from the rumen of sheep. *Journal of bacteriology* 72(5), 681.
- Ge, S., Usack, J., Spirito, C.M. and Angenent, L.T. (2015) Long-term *n*-caproic acid production from yeast-fermentation beer in an anaerobic bioreactor with continuous product extraction. *Environmental science & technology* 49(13), 8012-8021.
- Gómez, X., Cuertos, M., Prieto, J. and Morán, A. (2009) Bio-hydrogen production from waste fermentation: Mixing and static conditions. *Renewable Energy* 34(4), 970-975.
- Grootscholten, T., Steinbusch, K., Hamelers, H. and Buisman, C. (2013) Improving medium chain fatty acid productivity using chain elongation by reducing the hydraulic retention time in an upflow anaerobic filter. *Bioresource technology* 136, 735-738.
- Guest, J.S., Skerlos, S.J., Barnard, J.L., Beck, M.B., Daigger, G.T., Hilger, H., Jackson, S.J., Karvazy, K., Kelly, L. and Macpherson, L. (2009) A new planning and design paradigm to achieve sustainable resource recovery from wastewater 1. *Environmental science & technology* 43(16), 6126-6130.
- Harvey, B.G. and Meylemans, H.A. (2014) 1-Hexene: A renewable C6 platform for full-performance jet and diesel fuels. *Green Chemistry* 16(2), 770-776.

- Hino, T. and Kuroda, S. (1993) Presence of lactate dehydrogenase and lactate racemase in *Megasphaera elsdenii* grown on glucose or lactate. Applied and environmental microbiology 59(1), 255-259.
- Hospodsky, D., Yamamoto, N. and Peccia, J. (2010) Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. Applied and Environmental Microbiology 76(21), 7004-7012.
- Jeon, B.S., Moon, C., Kim, B.-C., Kim, H., Um, Y. and Sang, B.-I. (2013) In situ extractive fermentation for the production of hexanoic acid from galactitol by *Clostridium* sp. BS-1. Enzyme and Microbial Technology 53(3), 143-151.
- Kenealy, W., Cao, Y. and Weimer, P. (1995) Production of caproic acid by cocultures of ruminal cellulolytic bacteria and *Clostridium kluyveri* grown on cellulose and ethanol. Applied microbiology and biotechnology 44(3-4), 507-513.
- Kleerebezem, R. and van Loosdrecht, M.C. (2007) Mixed culture biotechnology for bioenergy production. Current opinion in biotechnology 18(3), 207-212.
- Ladd, J. (1959) The fermentation of lactic acid by a gram-negative coccus. Biochemical Journal 71(1), 16.
- Lettinga, G. (1995) Anaerobic digestion and wastewater treatment systems. Antonie van Leeuwenhoek 67(1), 3-28.
- Marounek, M., Fliegrova, K. and Bartos, S. (1989) Metabolism and some characteristics of ruminal strains of *Megasphaera elsdenii*. Applied and environmental microbiology 55(6), 1570-1573.
- McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R. and Hugenholtz, P. (2012) An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of Bacteria and Archaea. The ISME Journal 6(3), 610-618.
- Prabhu, R., Altman, E. and Eiteman, M.A. (2012) Lactate and acrylate metabolism by *Megasphaera elsdenii* under batch and steady-state conditions. Applied and Environmental Microbiology 78(24), 8564-8570.
- Raunkjær, K., Hvitved-Jacobsen, T. and Nielsen, P.H. (1994) Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. Water Research 28(2), 251-262.
- Regueiro, L., Spirito, C.M., Usack, J.G., Hospodsky, D., Werner, J.J. and Angenent, L.T. (2015) Comparing the inhibitory thresholds of dairy manure co-digesters after prolonged acclimation periods: Part 2—correlations between microbiomes and environment. Water Research (In press).
- Shannon, C.E. (1948) A mathematical theory of communication. Bell System Technical Journal 27(3), 379-423.
- Spirito, C.M., Richter, H., Rabaey, K., Stams, A.J. and Angenent, L.T. (2014) Chain elongation in anaerobic reactor microbiomes to recover resources from waste. Current opinion in biotechnology 27, 115-122.
- Steinbusch, K.J., Hamelers, H.V., Plugge, C.M. and Buisman, C.J. (2011) Biological formation of caproate and caprylate from acetate: Fuel and chemical production from low grade biomass. Energy & Environmental Science 4(1), 216-224.
- Sträuber, H., Schröder, M. and Kleinsteuber, S. (2012) Metabolic and microbial community dynamics during the hydrolytic and acidogenic fermentation in a leach-bed process. Energy, Sustainability and Society 2(1), 1-10.

- Sträuber, H., Lucas, R. and Kleinstüber, S. (2015) Metabolic and microbial community dynamics during the anaerobic digestion of maize silage in a two-phase process. *Appl Microbiol Biotechnol.* (In Press).
- R Development Core Team (2014) R: A language and environment for statistical computing. Vienna, Austria: R foundation for statistical computing; 2012.
- Usack, J. and Angenent, L.T. (2015) Comparing the inhibitory thresholds of dairy manure co-digesters after prolonged acclimation periods: Part 1–performance and operating limits. *Water Research* (In press).
- Vasudevan, D., Richter, H. and Angenent, L.T. (2014) Upgrading dilute ethanol from syngas fermentation to *n*-caproate with reactor microbiomes. *Bioresource technology* 151, 378-382.
- Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A., Lumley, T., Maechler, M., Magnusson, A., Moeller, S., Schwartz, M. and Venables, B. (2015) Gplots: Various r programming tools for plotting data. R package version 2(17).
- Weimer, P. and Moen, G. (2013) Quantitative analysis of growth and volatile fatty acid production by the anaerobic ruminal bacterium *Megasphaera elsdenii* T81. *Applied microbiology and biotechnology* 97(9), 4075-4081.
- Xu, J., Guzman, J.J., Andersen, S.J., Rabaey, K. and Angenent, L.T. (2015) In-line and selective phase separation of medium-chain carboxylic acids using membrane electrolysis. *Chemical Communications* 51(31), 6847-6850.
- Zhu, X., Tao, Y., Liang, C., Li, X., Wei, N., Zhang, W., Zhou, Y., Yang, Y. and Bo, T. (2015) The synthesis of *n*-caproate from lactate: A new efficient process for medium-chain carboxylates production. *Scientific reports* 5.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6