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COMMUNICATION

Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates†

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Ethanol distillation in the biofuel industry is energetically expensive because ethanol is completely miscible in water. Upgrading ethanol into a hydrophobic chemical that is easier to separate would circumvent current fossil-fuel consumption for distillation. Here, we shaped a reactor microbiome to sequentially elongate carboxylic acids with 2-carbon units from dilute ethanol in yeast-fermentation beer. Our continuous bioprocess produced *n*-caproic acid, a 6-carbon-chain carboxylic acid that is more valuable than ethanol. No antimicrobials to inhibit methanogens were necessary. In-line product extraction achieved an *n*-caproic acid production rate exceeding 2 grams per liter of reactor volume per day, which is comparable to established bioenergy systems with microbiomes. Incorporation of other organics found in beer increased the mass of carbon in *n*-caproic acid by 10% compared to ethanol.

The United States produced ~50 billion liters of ethanol from corn grain in 2011 and mandated another ~60 billion liters of ethanol or ethanol-equivalent fuel by 2020 from cellulosic feedstock.[1] Ethanol is completely miscible in water due to its short 2-carbon chain and hydrogen-bonding interactions with water, which explains why 20–25% of the energetic value of the product is used during distillation for corn and cellulosic ethanol.[2,3] To circumvent fossil-fuel consumption for distillation, ethanol could be upgraded to a product that is more easily separated from water. Here, we describe the biological conversion of undistilled ethanol and unfermented substrate into the hydrophobic chemical *n*-caproic acid. This chemical has twice the value of ethanol per carbon atom and is not only a fuel precursor, but also a valuable industrial commodity. Our objective was to selectively generate *n*-caproic acid *via* bio-processing within the carboxylate platform. Many other products, such as liquid fuels, can be produced from *n*-caproic acid product with established chemical or electrochemical downstream processes.[4,5]

Anaerobic pure cultures of *Clostridium kluyveri* can produce *n*-caproic acid *via* chain elongation of carboxylic acids through a reversed β-oxidation pathway.[6–8] This bacterium uses ethanol as a source of carbon, energy, and reducing equivalents to sequentially elongate the carbon chain of carboxylic acids in two-carbon steps (*e.g.*, acetic acid to *n*-butyric acid to *n*-caproic acid *etc.*). For every five molecules of ethanol that are used for elongation by *C. kluyveri*, one molecule of ethanol is oxidized to acetic acid for metabolic energy.[6] Open microbial communities (hereafter microbiomes) can also elongate carboxylic acids as shown in past studies.[5,9–12] Our microbiome utilized undistilled, dilute ethanol in yeast-fermentation beer (*i.e.*, unprocessed fermentation broth) to elongate short-chain carboxylic acids at industrial rates. The short-chain carboxylic acids originated from: (i) the microbial conversion of yeast, sugars, and

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Broader context

A lack of mechanistic understanding has lead to a general perception that engineered systems with complex reactor microbiomes are somewhat inefficient and unpredictable. This resulted in the absence of wide-spread attempts to develop engineered systems that can produce carboxylic acids, which are precursors for liquid fuels and chemicals, even though microbiomes in anaerobic digesters have been producing gaseous methane successfully at industrial scales with stable, predictable, and functionally redundant community structures. Here, we show that the medium-chain carboxylic acid *n*-caproic acid can be produced at high efficiencies and rates for long periods of time with reactor microbiomes by feeding ethanol as a reduced feedstock to perform chain elongation of carboxylic acid backbones. This was performed in lab-scale bioreactors at a constant pH of 5.5 with in-line extraction of *n*-caproic acid and methane production from hydrogen and carbon dioxide. We also show that the reactor microbiome was shaped nonrandomly during a period of improving *n*-caproic acid production rates. We used real fermentation beer from the corn-to-ethanol industry with long-term goals to: (1) produce a higher value product than ethanol; and (2) circumvent energy-intensive distillation.

left-over corn grain biomass present in beer; and (ii) the oxidized ethanol (acetic acid).

We inoculated a nonsterile, 5 L bioreactor with natural microbiomes and fed beer from the corn ethanol industry for months, while controlling the pH at 5.5 and the temperature at 30 °C (Fig. 1A). We achieved an *n*-caproic acid production rate of 76.5 mmol C L^{-1} per day (1.5 g L^{-1} per day), which is similar to methane production with complex substrates in anaerobic digesters (an established bioenergy system with reactor microbiomes), and a product specificity of 79% (carbon in *n*-caproic acid compared to all fermentation products) (Fig. 1B). This performance was achieved by continuously removing *n*-caproic acid with liquid–liquid extraction, and was limited by extraction rates; an extraction system failure immediately decreased production, but the system recovered following repair of the extraction module (Fig. S1†). Further, by increasing the extraction rate (after increasing the membrane surface area), we sustained a higher maximum *n*-caproic acid production rate of 108.3 mmol C L^{-1} per day (2.1 g L^{-1} per day) (Fig. S1†).

We used a pH gradient (5.5–9.0) as the driving force to specifically extract acidic product by diffusion through membranes. This is a low-energy extraction process, requiring energy only to pump bioreactor, solvent, and extraction solutions (Fig. 1A; ESI†). The solvent solution was 3% tri-*n*-octylphosphineoxide in mineral oil, which preferentially extracted hydrophobic molecules, resulting in extraction efficiencies of 83–93% for medium-chain carboxylic acids and 5–31% for short-chain carboxylic acids (Fig. 1C). The continuous extraction resulted in high specificity for recovering and concentrating *n*-caproate as a product (97%; Fig. 1D), representing 10% more carbon than was provided as ethanol. Further improvements in extraction technology are anticipated to increase the rates considerably.

Anaerobic microbiomes at near-neutral pH values are known for pervasive methane production because it maximizes their free energy yield from substrate oxidation.[13] To guarantee satisfactory *n*-caproic acid yields, research in the past had indicated the need to completely inhibit methanogenesis to prevent diversion of carbon and electrons, including from acetic acid and *n*-caproic acid (the product), to methane.[4,10–12] At a bioreactor pH of 7.0, Steinbusch *et al.*[10] resorted to adding an expensive, nonspecific methanogen inhibitor. Our breakthrough occurred due to utilization of in-line extraction of *n*-caproic acid and controlling the bioreactor pH at 5.5 – we discovered that simultaneous chain elongation and methanogenesis is feasible when methane is produced only from hydrogen and carbon dioxide and not from acetic acid (Fig. S2†). Continuous extraction of the unionized *n*-caproic acid was necessary due to its microbial toxicity at a pH of 5.5 (close to its pK_a of 4.88 at 30 °C). Carbon in the bioreactor off gas consisted of primarily methane (99.6%; 3% of fermentation product carbon); an advantageous co-product compared to carbon dioxide.

We have shown previously with studies of community structure–function relationships that diverse anaerobic microbiota are stable and resilient, that the community structure dictates performance, and that operating conditions affect structure.[14] These findings not only refute the claim that microbiomes are inefficient and unpredictable, but also suggest that they can be shaped for a specific function. We characterized membership and function of microbiome samples over time using the: (i) 454 titanium platform for 16S rRNA gene sequencing; and (ii) Illumina platform for shotgun metagenomic sequencing (ESI†). We generated an average of ~5800 high-quality 16S rRNA gene sequences per sample ($n = 7$) from which we picked 839 operational taxonomic units (OTUs; 97% ID) and assigned taxonomy (ESI†). We found five abundant OTUs that were significantly correlated ($r > 0.8$ and $p < 0.05$) with increasing *n*-caproic acid production rates (Fig. 2A and Table S1†). Relative abundance of *C. kluyveri* reached 4% of the microbiome on day 120 of the operating period when chain elongation rates were high (Fig. 1B and 2A), while other OTUs, including one from the family Ruminococcaceae,

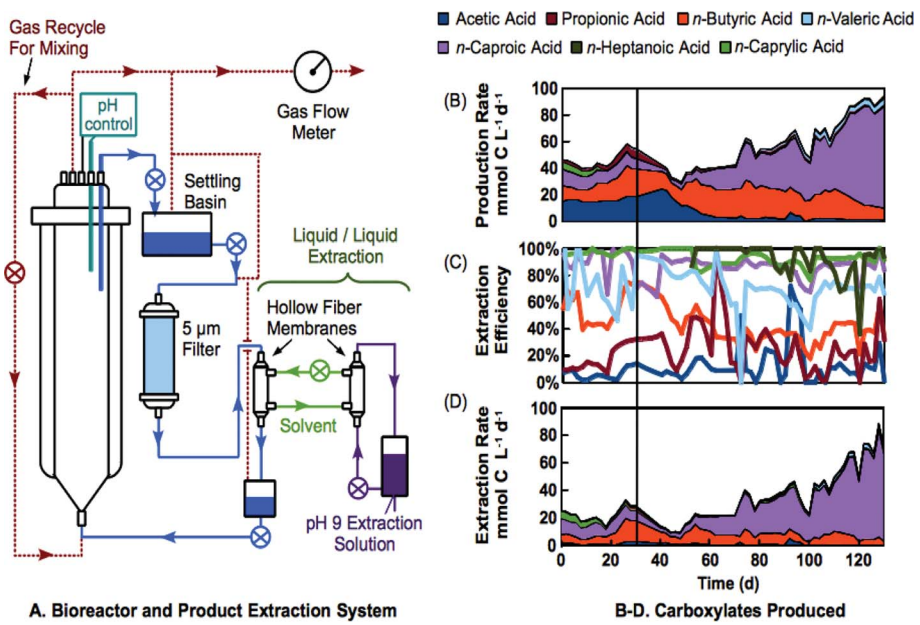

Fig. 1 System configuration and performance: (A) bioreactor setup with in-line extraction; (B) production rate of C2–C8 carboxylic acids over time; (C) extraction efficiency of C2–C8 carboxylic acids as the percentage of produced acid that was extracted over time; and (D) extraction rate of C2–C8 carboxylic acids over time. The vertical line in (B–D) represents the switch to real yeast-fermentation beer as substrate and an increase in extraction membrane surface area from 1 m^2 to 2 m^2.

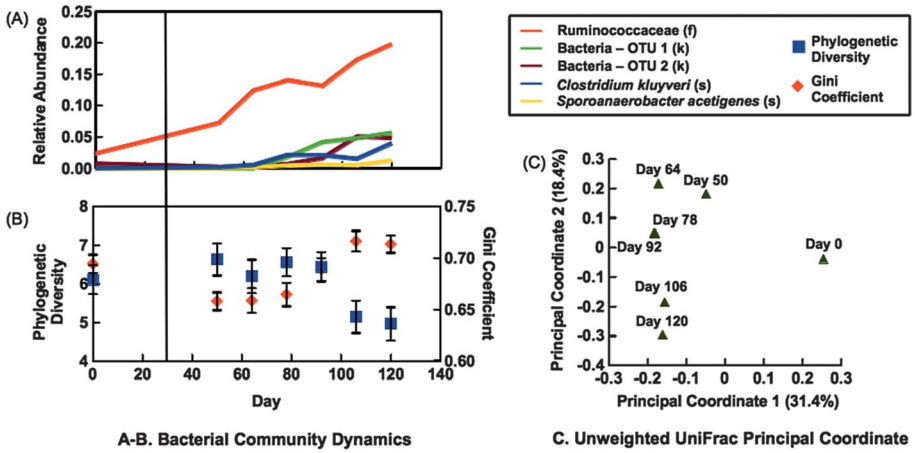

Fig. 2 Time-series analysis of the microbiome: (A) relative abundance *vs.* time of OTUs that were significantly ($r > 0.8$) correlated with the rate of *n*-caproic acid production. OTU taxonomy was assigned to the most specific level possible with (k): kingdom, (f): family, and (s): species; (B) α diversity and evenness of samples (100 rarefactions of 500 reads per sample). Gini coefficient is a measure of community evenness where 0 is a perfectly even community with OTU abundance distributed and 1 is a perfectly uneven community for which only one OTU dominates; and (C) β diversity for which unweighted UniFrac principal coordinates represent as much of the between-sample community phylogenetic variation as possible in two coordinates (49.8%). The line in (a and b) represents a switch to real yeast-fermentation beer and an increase in extraction membrane surface area.

became important as well. A lower community richness (α diversity) and more uneven distribution of taxa within the microbiome (Fig. 2B) coincided with improvements in reactor performance.

Our shotgun metagenomic analysis (~2.4 billion bp per sample [$n = 10$]) suggested that more than 50% of all assigned reads were from *Clostridium* spp., and that this genus dominated many of the major metabolic pathways (Fig. S3†). Other genera, including *Ethanoligenens* (fam. Ruminococcaceae), *Bifidobacterium*, and *Desulfitobacterium*, represented important pools of genes for hydrolysis and ethanol oxidation (Fig. S3 and S4†). This explains why a Ruminococcaceae OTU was correlated with *n*-caproic acid production (Fig. 2A). *Clostridium* spp. dominated the chain-elongation gene pool (Fig. S3†); however, *C. kluyveri* may not have been the only *Clostridium* spp. involved in carboxylic acid elongation because other phylogenetically diverse rumen isolates produce *n*-caproic acid from complex substrates.[15]

The differentiation between microbiomes (β diversity) showed a clear time-series path during which production was elevated (Fig. 2C). We identify this nonrandom behavior of the microbiome as shaping, resulting from adaptation to: (i) a pH of 5.5; (ii) a temperature of 30 °C; (iii) feeding a reduced compound (ethanol [Fig. S5†]) together with organic compounds; and (iv) extracting the product continuously and selectively. In other words, we have the tools to control the reactor microbiome to generate primarily the products we want. The chain-elongation rate of our shaped microbiome is comparable with pure cultures of *C. kluyveri* and a metabolically engineered *Escherichia coli*, which both utilize the reversed β-oxidation pathway.[16,17] The advantages of using microbiomes compared to pure cultures lies in their ability to: (i) incorporate carbon from complex streams due to a broad-substrate spectrum; (ii) maintain functionality even with nonsterile input; and (iii) be resilient in response to disturbances.

Outlook

Chain elongation with a reactor microbiome to convert dilute ethanol into a more hydrophobic compound could circumvent distillation while producing a higher-value product. This would improve the energy balance in the ethanol industry considerably due to a large reduction in the use of fossil fuels at the biorefinery, and contribute to achieving energetic and economic goals for biofuels in the United States and abroad. Energy balance evaluations of current ethanol production in the United States have been variable, but many have agreed that the amount of energy that is required to produce 50 billion liters of ethanol annually is higher than desirable.[18] If the United States achieves the targeted ~110 billion liters of ethanol-equivalent fuel as ethanol in 2020, we estimate a $\sim 4.6 \times 10^{11}$ MJ (127.8 million MWh) energetic requirement for ethanol distillation at biorefineries (which, in comparison, is ~3.6% of the 2010 total US electricity generating capacity[19]). Reactor microbiome technology can be rapidly implemented at a large scale because it does not require sterile fermentation conditions. Replacing distillation with a low-energy *n*-caproic acid production and extraction process, would also affect process economics, because 10% more product carbon would be recovered in a higher-value product compared to dilute ethanol. In addition, it would diversify the biorefinery product market, because *n*-caproic acid is not only a precursor for liquid fuels (alcohols and alkanes), but is already a valuable commodity as, for example, animal feed additives and green antimicrobials.

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References

1 U. S. Congress, 110th Session edn, ch. Title II, Section 201, 2007.
2 P. W. Madson, in *The Alcohol Textbook*, ed. K. A. Jacques, T. P. Lyons and D. R. Kelsall, Nottingham University Press, Nottingham, UK, 2003, pp. 319–336.
3 H. Shapouri, P. W. Gallagher, W. Nefstead, R. Schwartz, S. Noe and R. Conway, *2008 Energy Balance for the Corn-Ethanol Industry*, USDA, Washington, DC, 2010.
4 M. T. Agler, B. A. Wrenn, S. H. Zinder and L. T. Angenent, *Trends Biotechnol.*, 2011, **29**, 70–78.
5 P. F. Levy, J. E. Sanderson, R. G. Kispert and D. L. Wise, *Enzyme Microb. Technol.*, 1981, **3**, 207–215.
6 H. Seedorf, W. F. Fricke, B. Veith, H. Bruggemann, H. Liesegang, A. Strittmatter, M. Miethke, W. Buckel, J. Hinderberger, F. Li, C. Hagemeier, R. K. Thauer and G. Gottschalk, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 2128–2133.
7 H. A. Barker, M. D. Kamen and B. T. Bornstein, *Proc. Natl. Acad. Sci. U. S. A.*, 1945, **31**, 373–381.
8 R. K. Thauer, K. Jungermann, H. Henninger, J. Wenning and K. Decker, *Eur. J. Biochem.*, 1968, **4**, 173–180.
9 H.-B. Ding, G.-Y. A. Tan and J.-Y. Wang, *Bioresour. Technol.*, 2010, **101**, 9550–9559.
10 K. J. J. Steinbusch, H. V. M. Hamelers, C. M. Plugge and C. J. N. Buisman, *Energy Environ. Sci.*, 2011, **4**, 216–224.
11 D. Smith and P. McCarty, *Biotechnol. Bioeng.*, 1989, **34**, 885–895.
12 D. P. Smith and P. L. McCarty, *Biotechnol. Bioeng.*, 1989, **34**, 39–54.
13 K. W. Hanselmann, *Cell. Mol. Life Sci.*, 1991, **47**, 645–687.
14 J. J. Werner, D. Knights, M. L. Garcia, N. B. Scalfone, S. Smith, K. Yarasheski, T. A. Cummings, A. R. Beers, R. Knight and L. T. Angenent, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 4158–4163.
15 B. Jeon, B.-C. Kim, Y. Um and B.-I. Sang, *Appl. Microbiol. Biotechnol.*, 2010, **88**, 1161–1167.
16 W. R. Kenealy, Y. Cao and P. J. Weimer, *Appl. Microbiol. Biotechnol.*, 1995, **44**, 507–513.
17 C. Dellomonaco, J. M. Clomburg, E. N. Miller and R. Gonzalez, *Nature*, 2011, **476**, 355–359.
18 J. Hill, E. Nelson, D. Tilman, S. Polasky and D. Tiffany, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 11206–11210.
19 U. S. E. I. Administration, *Electric Power Annual 2010*, U.S. Department of Energy, Washington, DC, 2011.