Development of a highly specific and productive process for n-caproic acid production: applying lessons from methanogenic microbiomes

SUPPLEMENTARY MATERIALS AND METHODS

Carbon Dioxide Reduction Activity Test

On day 400 of the operating period, we collected biomass that had accumulated in the unheated filtration unit that was part of the extraction method outside of the reactor (Figure 1B). We used the biomass to test whether activity had developed for oxidation of hydrogen and/or ethanol coupled to carbon dioxide reduction to methane. In short, we added 10 mL of biomass to 20 mL batch fermentation bottles in triplicate for each treatment. In each bottle, we flushed the headspace for 15 min with nitrogen gas to remove as much oxygen as possible. The control bottles contained only inoculum. To one experimental set, we added 75 kPa of 50/50 hydrogen/carbon dioxide to the headspace to test for hydrogenotrophic methanogen activity. To the next test set, we added only 10 mmol/L ethanol to test for pathways consuming ethanol without carbon dioxide as a sink for hydrogen as the electron acceptor. To the final set, we again added 10 mmol/L ethanol, but this time with 37.5 kPa carbon dioxide as an electron acceptor to test for reduction to methane. Each set was incubated at 30 °C for 93 h. Hydrogen, carbon dioxide, and methane levels were measured (Gow-Mac) in the headspace before and after the experiments. At the beginning and end of the experiment, a sample of the liquid fraction was taken, filtered through a 0.2-μm nitrocellulose filter, acidified, and analyzed (HP 5890 Series II) for acetic acid, n-butyric acid, and ethanol.
SUPPLEMENTARY RESULTS AND DISCUSSION

The Fate of Ethanol

We tested the catalytic activity of the biomass accumulated in the filtration unit and demonstrated that ethanol was indeed oxidized. In the first batch set with only hydrogen and carbon dioxide, methanogens reduced carbon dioxide to methane, with a final methane produced to carbon dioxide consumed ratio of ~2:3. It is possible that some carbon dioxide was consumed in other reactions that were not measured. When we added only ethanol to the bottles with a nitrogen headspace, there was a small amount of methane production that probably occurred due to ethanol oxidation and reduction of trace levels of carbon dioxide. The limited amount of

Supplementary Figure S4 | 93-h test of inoculum collected from the filtration unit of R on day 400 of the operating period to measure the activity of carbon dioxide reduction to methane with either hydrogen or ethanol as the electron donor. The change in metabolite levels over the course of the experiment are normalized to the amount of biomass (g VS), measured after the experiment. The error bars represent the standard deviation on the mean of the triplicate reactions. The four test conditions are: Set 1: control; Set 2: hydrogenotrophic methanogen activity; Set 3: ethanol consumption without carbon dioxide; and Set 4: ethanol consumption in the presence of carbon dioxide. The additions compared to the control are shown in each panel (also see supplementary materials and methods) (the full colour version of this figure is available in the online version of this paper, at http://www.iwaponline.com/wst/toc.htm).

Supplementary Figure S5 | A model for the role and fate of ethanol in the process for n-caproic acid production via chain elongation. The blue lines represent the flow of reducing equivalents (electrons) while the black lines represent the flow of carbon. The red, blue, and green boxes are reactions that we identified in our reactor biomass, while the grey box represents a potential process that did not occur. A. Some ethanol is directly oxidized to acetic acid with concurrent hydrogen production. B. Methane is produced from hydrogen and carbon dioxide, but limiting concentrations of carbon dioxide prevent removal of all hydrogen. C. The buildup of hydrogen always inhibits the possible oxidation of products, and reaches a high enough concentration to control further ethanol oxidation. The rest of the ethanol is then free to contribute to n-caproic acid production via chain elongation (the full colour version of this figure is available in the online version of this paper, at http://www.iwaponline.com/wst/toc.htm).
carbon dioxide must have prohibited further ethanol oxidation, because an increase in hydrogen was not detected. Ethanol did decrease further concomitantly with acetic acid in this experiment, however, coinciding with an increase in $n$-butyric acid. Therefore, although we were not testing directly for it, we found evidence of chain elongation activity (ethanol + acetic acid $\rightarrow$ $n$-butyric acid) in the biomass. Finally, in the third experimental set, we added ethanol along with extra carbon dioxide as electron acceptor. Again, we saw evidence of chain elongation because $n$-butyric acid increased the same amount as in the second test set. Acetic acid did not decrease this time, however, because much more ethanol could be oxidized. The further ethanol oxidation could occur because the extra carbon dioxide provided an electron sink for hydrogen-utilizing methanogens, so that ethanol oxidation was not thermodynamically inhibited by hydrogen buildup.