Metabolic models of mixed culture fermentation diagnose bottlenecks and predict strategies for improved medium-chain fatty acid production

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ABSTRACT

Multi-species microbial communities determine the fate of materials in the environment and can be harnessed to produce beneficial products from renewable resources. In a recent example, fermentations by mixed microbial communities have produced medium-chain fatty acids (MCFA). Tools to predict, assess, and improve the performance of these communities, however, are limited. To provide such tools, we constructed two metabolic models of a MCFA-producing community fed a byproduct from a biofuel fermentation. The first model is a unicellular model (iFerment156) that contains a diverse set of fermentation pathways, including reverse β-oxidation for MCFA production while the second model (iFermGuilds564) separates fermentation activities into functional guilds. Both models predicted an energetic advantage for this community to produce octanoic acid as a major fermentation product. Simulations with iFermGuilds564 predicted that 6- and 8-carbon MCFA were largely produced by a sugar-consuming guild, while short-chain fatty acids were mainly produced by a guild consuming lactate. These models represent novel tools for exploring the role of mixed microbial communities on carbon recycling in the environment, as well as on beneficial reuse of organic residues.

INTRODUCTION

Mixed microbial fermentations have benefited humanity throughout history. Since the mid-18th century, mixed culture fermentations have produced food, valuable chemicals, and recovered energy from wastes. Many mixed microbial fermentations involve the synergistic activity of several functional groups to convert organic substrates into valuable products. In anaerobic digestion, fermentation products like acetic acid and H₂ are used to support microbial production of methane, thus enabling the recovery of a large fraction of the chemical energy originally present in the complex organic substrates. While anaerobic digestion technologies are
well established, other strategies to expand the range of potential products of mixed culture fermentations are only beginning to emerge, such as the carboxylate platform, which produces medium-chain fatty acids (MCFA) as a potentially valuable set of bio-based products.\textsuperscript{3,4}

MCFA are an attractive carboxylate platform product because of their many industrial uses.\textsuperscript{5} Octanoic acid (C8), an 8-carbon linear monocarboxylic acid, is a particularly valuable MCFA because of its numerous uses, high market value, and relative ease of recovery. Further, when C8 is reduced to its corresponding alkane, it can substitute for octane in liquid transportation fuels. Recent research has demonstrated the feasibility of using mixed culture fermentations to transform renewable resources into MCFA.\textsuperscript{6-8} While C8 is produced in some self-assembled microbial communities that develop in MCFA-producing bioreactors, hexanoic acid (C6) is often the primary MCFA, along with acetic and butyric (C4) acids.\textsuperscript{6-13}

Mathematical models have been established for the design and optimization of methane production via anaerobic digestion.\textsuperscript{14} These models describe the kinetics and stoichiometry of reactions attributed to the individual members of the community that are responsible for hydrolysis of complex organics, the fermentation of hydrolysis products, the transformation of fermentation products to acetic acid and H\textsubscript{2}, and finally, the transformation of these intermediates to methane.\textsuperscript{14} The metabolic networks in these models are well defined, with interspecies H\textsubscript{2} transfer known to regulate critical metabolic steps, and therefore, anaerobic digestion models are predictive of the behavior of self-assembled methane-producing microbiomes.

In contrast, the microbial metabolic networks involved in converting complex organic substrates to MCFA are not fully elucidated. Consequently, there is a lack of knowledge on how to obtain a carboxylate platform microbiome that produces C8 as the main MCFA, or how to minimize the accumulation of shorter-chain fatty acids. Initial models of mixed culture
fermentations that do not include methanogenesis have focused on simulating metabolic networks for production of C4, propionic acid, acetic acid, ethanol, lactic acid and H₂ from glucose. Subsequent models took into account that multiple electron carriers (e.g., ferredoxin, NADH) play different roles in these metabolic networks, enabling the simulation of energy-conserving electron-bifurcating reactions during production of C4 by reverse β-oxidation. While a recent model considered the role of homoacetogenesis as a H₂-consuming member of mixed culture fermentation, it was limited to producing C4 as the major MCFA.

In addition, existing models of carboxylate platform communities do not test the potential role of recently discovered energy-conserving mechanisms in anaerobic microbes that may shape the metabolic networks of these mixed culture fermentations. Therefore, one goal of this study was to test the ability of different models that considered all the known metabolic networks and energy-conserving mechanisms to predict bottlenecks and strategies to improve MCFA production. To do this, we constructed a single unit model (iFerment156) and a guild-based microbial community model (iFermGuilds564) with expanded capability compared to existing models, and tested their predictive ability using experimental data from a MCFA-producing bioreactor. We used the models to diagnose bottlenecks for improved MCFA production, with particular emphasis on predicting factors and specific enzymes that could alter the amount and type of MCFA produced by the mixed microbial community.

MATERIALS AND METHODS

A metabolic network of a mixed culture fermentation was assembled that contains reactions that occur during anaerobic metabolism of a previously described lignocellulsoic biorefinery residue. The first network, iFerment156, contains 156 reactions and 105 metabolites, models a mixed culture fermentation as a single unit, and expands the metabolic pathways and
substrate range of previously published mixed culture fermentation networks.\textsuperscript{15-17} iFerment156 is the most comprehensive metabolic model of mixed culture fermentation to date, describing metabolism of exogenous substrates including glucans, glucose, xylans, xylose, and glycerol, along with lactate, ethanol, acetate, CO\textsubscript{2} and H\textsubscript{2}, which are potential intermediates in this mixed culture fermentation (Fig. 1).

The second network, iFermGuilds564, represents activities of different guilds (i.e., functional classes of organisms) within the microbial community. The iFermGuilds564 network describes the same reactions and metabolites as in the iFerment156 model but these are separated into six functional guilds with each guild representing a subset of compartmentalized reactions. iFermGuilds564 also includes additional transport reactions that simulate metabolite exchange among guilds. Combining the six guilds, the iFermGuilds564 network includes 564 reactions and 390 metabolites (Fig. 2). The guilds in the iFermGuilds564 model represent previously described organisms that perform fatty acyl chain elongation from sugars (SEOs), sugar fermenting organisms that produce mostly or only lactate, acetate, and ethanol (SFOs) as fermentation products, hydrogenic sugar fermenters (HSFs), organisms that perform fatty acyl chain elongation from lactate (LEOs) or ethanol (EEOs), and homoacetogenic organisms (HAOs). SEOs include organisms that utilize sugars and elongate intermediate products through reverse $\beta$-oxidation, such as Megasphaera and Caproicproduens.\textsuperscript{20, 21} SFOs include genera such as Lactobacillus and Bifidobacterium, which can ferment hexoses and pentoses to lactate, acetate and ethanol.\textsuperscript{22, 23} HSFs include organisms that generate H\textsubscript{2} while producing fermentation products, such as members of the Coriobacteriaceae family.\textsuperscript{24} LEOs include organisms that perform reverse $\beta$-oxidation with lactate, such as Pseudoramibacter\textsuperscript{25} and Ruminococcaceae bacterium CPB6.\textsuperscript{9} EEOs, such as Clostridium kluyveri,\textsuperscript{26} perform reverse $\beta$-oxidation with ethanol. HAOs produce acetate from H\textsubscript{2}
and CO₂ and include species within the _Clostridium_, _Acetobacterium_, _Eubacterium_, and _Blautia_.

When constructing each metabolic network, reactions were added to capture the exchange of substrates and products with the reactor (extracellular space). Where possible, standard metabolite and reaction abbreviations from the Biochemical, Genetic, and Genomic (BiGG) knowledgebase were used. All reactions were checked for mass and charge balance assuming an intracellular pH of 7.0 with the dominant ionic form of all metabolites obtained from the BiGG database. Methods for including transport constraints are discussed in Supplementary Text 1. Both metabolic models (iFerment156 and iFermGuilds564) are available as Supplementary File 1 and Supplementary File 2, respectively. Instructions for using the models are available at https://github.com/mscarbor/Mixed-Culture-Fermentation-Models.

The production capabilities of both metabolic networks were simulated using constraint-based methods. These methods predict the steady-state flux distribution through a metabolic network that is subject to physiochemical constraints. In this work, we used flux variability analysis (FVA) and parsimonious flux balance analysis (pFBA) to predict flux distributions through metabolic networks of mixed culture fermentation. When modeling bioreactors with the presence of solute gradients across the cell membrane, we considered the ATP required for transport of solutes as described in Supplementary Text 1. The objective function for all modeling scenarios was to maximize ATP production. When simulating the iFermGuilds564 network for a steady-state condition, we required all individual guilds to achieve the same ATP hydrolysis flux as a proxy for having the same growth rate, as the models do not include biomass equations. All simulations were performed in Python 3.5 using the cobrapy package (v 0.13.4).
Throughout this work, we present results in terms of electron equivalents (eeq), which report the number of electrons potentially transferred to an acceptor if the reduced chemical were completely oxidized (using factors provided in Table S1 to convert from molar concentrations to eeq concentrations). For each scenario, we calculated the overall reaction thermodynamics using the predicted stoichiometry of the model and the standard free energy of formation (\(\Delta G^\circ\)) of metabolites (Supplementary File 3) obtained from KBase.\(^{36}\) We ensured that at least 50 kJ was available per ATP produced under standard conditions at neutral pH.\(^{37, 38}\)

RESULTS

Simulation of mixed culture fermentations with iFerment156

By constraining the iFerment156 model to produce defined products, we initially tested its predictive ability with well-established fermentation conditions (See Supplementary Text 2; Table S2). We also investigated predictions derived from an unconstrained iFerment156 model (i.e., all pathways are on) under conditions where multiple substrates are simultaneously produced and consumed within this unit. This modeling analysis is a simplified representation of fermentation in a mixed microbial community where interspecies transfer of metabolites is assumed to have no energetic penalty, and is similar to previously published models of mixed culture fermentations.\(^{15-17}\) For this analysis, we required that all possible solutions released \(\geq50\) kJ per mol ATP produced as a condition that simulated microbial growth.\(^{37}\) Using FVA with this condition, iFerment156 provided a range of solutions that maximized ATP production depending on the substrates provided to the simulation (Fig. 3). For instance, when we examined the utilization of glucose as the sole substrate, iFerment156 predicted multiple conditions that would allow ATP production at 4.5 mol ATP per mol of glucose metabolized. One of these solutions predicts that 61.1\% of the eeq in glucose could be converted to C8 (with 22.2\% and 16.7\% of the
eeq converted to acetate and formate, respectively) (Fig. 3; Table S3). Another solution predicts 66.6% of the eeq converted to C6, with 16.6% eeq converted to acetate and 16.6% to formate (Table S3). Homofermentation to acetate is yet another solution that yields the maximum ATP production. Another prediction from this analysis is that fermentations that result in ethanol or lactate production, although feasible and known to occur in nature, do not result in the maximum possible ATP yields (Fig. 3). This prediction of iFerment156 agrees with the practice of using pure cultures (with limited metabolisms) for industrial ethanologenic fermentations, since mixed cultures would likely produce other unwanted fermentation products that help maximize ATP production by the microbial community.

The iFerment156 model predicts similar results when xylose is provided as the sole carbon source (Fig. 3, Table S3), albeit with a lower ATP production compared to glucose (3.75 mol ATP per mol substrate). We found that providing glycerol as a sole carbon source to iFerment156 yields multiple solutions that maximize ATP production (Fig. 3). On the other hand, a single solution that maximizes ATP production is predicted by iFerment156 if either lactate or ethanol are sole carbon sources. When providing lactate, this solution predicts stoichiometric conversion of lactate to C8, while with ethanol, 92% of the eeq would be converted to C8 and 8% to H2.

These results predict that microbial communities would energetically benefit from simultaneously producing multiple fermentation products, which is consistent with experimental observations of fermentation of carbohydrate-rich feedstocks.6, 8, 39 Consequently, we used iFerment156 to evaluate whether having multiple electron donors simultaneously available would change the predictions of maximum ATP production. In particular, since acetate and H2 are ubiquitous fermentation products in mixed microbial fermentations, we used iFerment156 to simulate co-utilization of either acetate or H2 with the other substrates analyzed (Fig. 3). With
acetate as a co-substrate with glucose, xylose, or glycerol, iFerment156 predicted the same multiple solutions that maximized ATP production in the absence of acetate (Table S3). This observation predicts, that from the point of view of maximizing microbial growth, acetate is a final fermentation product. In contrast, co-utilization of lactate and acetate is predicted by iFerment156 to increase the ATP yield and favor reverse β-oxidation with solutions that maximize ATP production including butyrate, C6 or C8 production (Fig. 3; Table S3). This analysis also found that acetate co-utilization with ethanol is predicted by iFerment156 to double the ATP yield compared to ethanol as the sole carbon source (Table S3). In this case, solutions for optimal ATP yields were obtained when butyrate, C6, or C8 were the main fermentation product, consistent with the ability of known carboxylate platform systems to produce a variety of short- and medium-chain products. Overall, these iFerment156 simulations suggest that acetate co-utilization can increase the range of accumulated products, but that this may be detrimental to processes targeting production of a single or small set of compounds. Further, these results predict that acetate may be a dead-end product in mixed culture fermentations unless other compounds, such as lactate or ethanol, are either present in the growth substrate or accumulated as intermediates.

We also tested the impact of H2 metabolism on mixed culture fermentation, since this has previously been proposed as either a fermentation intermediate or endogenous reductant to drive MCFA production. During utilization of sugars and glycerol, iFerment156 predicted that the presence of H2 favors higher ATP yields if C8 is the only fermentation product produced (Fig. 3). In the presence of ethanol, H2 is not predicted to be consumed by iFerment156 since redox balance cannot be maintained under these conditions. In the presence of H2 and acetate, or H2 and CO2, C8 production is favored for maximizing ATP production. Thus, iFerment156 predicts that H2
oxidation should improve C8 production because it would maximize the ATP production of the overall microbial community.

In sum, iFerment156 can predict substrates that favor accumulation of different products when a large set of biochemical reactions are available in a mixed culture fermentation (Fig. 1). When sugars and glycerol are the main substrates, mixed culture fermentations could give rise to a wide range of products since multiple sets of fermentation reactions yield the same maximum ATP production for the community. When lactate or ethanol are the main substrates, a narrower product spectrum may be expected. In addition, H₂ co-utilization may result in accumulation of a narrower range of products from sugars and glycerol as H₂ utilization would increase overall ATP yields. In a large-scale bioreactor, production of fewer products is often considered advantageous for downstream processing and to improve overall process economics.

Simulation of bioreactor performance with a single-unit model (iFerment156)

The iFerment156 model predicts that multiple substrates could lead to C8 production (Fig. 3), but that ATP could be maximized by co-production of other fermentation products. We wanted to assess if this single-unit model could simulate the observed product yields of a bioreactor fed lignocellulosic biorefinery residues, a complex organic substrate. The microbial community in the bioreactor (derived from wastewater sludge) was fed lignocellulosic biorefinery residues for 96 days, at which time bioreactor performance was evaluated using metagenomic and metatranscriptomic analyses along with end-product sampling. At this time, the bioreactor contained 11.7 g L⁻¹ of volatile suspended solids, which was assumed to represent microbial biomass. To represent organic substrate metabolism, the substrate uptake fluxes were set according to the eeq consumed for compounds in the feedstock (Table S4). We constrained iFerment156 to only contain reactions predicted by transcriptomic analyses to be active in the bioreactor microbial community.
community\textsuperscript{19} and performed FVA to identify the range of end-products that would support maximum ATP yields. Under these conditions, iFerment156 predicted that 90% of the eeq would be converted to C8 (Fig. 4A), which is much higher than the 3% conversion to C8 observed in the bioreactor. The total predicted ATP production rate is 1.37 mmol hr\textsuperscript{-1}. While this simulation of iFerment156 did not correctly predict the range of products (butyrate, C6 and C8) measured in the bioreactor, it did indicate that C8 production could be advantageous for maximizing ATP production.

Because C6 and C8 are known biocides,\textsuperscript{43, 44} it is possible that their production is limited by their accumulation. To test this hypothesis, we performed simulations constraining this version of iFerment156 to produce C6 and C8 at the rates measured in the bioreactor (Table S3). Using these parameters, iFerment156 predicted an ATP production rate of 1.32 mmol hr\textsuperscript{-1} (Fig. 4B), corresponding to 96% of the maximum ATP production rate predicted when C6 and C8 production were not constrained. Under these conditions, iFerment156 predicted that 50% of the eeq were converted to butyrate, approximately 1.5-fold higher than was observed in the bioreactor (34% of the eeq, Fig. 4B). Although the discrepancies between predicted and measured MCFA levels indicate limitations in the ability of iFerment156 to predict performance of this mixed microbial community, the similar ATP yield predictions when the model was constrained to the observed C6 and C8 concentrations illustrates the difficulty of simulating the sum of all metabolic processes with a single-unit model. However, energetic losses associated with interspecies transfer of substrates may influence the predicted ATP yields and the resulting levels of fermentation products. To investigate this, a functional guild fermentation model was developed, based on the reactions present in iFerment156, as described in the next section.
Simulation of mixed culture fermentations with a functional guild model (iFermGuilds564)

In iFermGuilds564, the metabolic activities of a microbial community are partitioned among a number of functional guilds. This biological division of labor negates the free exchange of reducing equivalents (e.g., NADH, ferredoxin) and of many pathway intermediates (e.g., acetyl-CoA) between community members, ultimately constraining how individual organisms maintain redox balance, which can potentially impact ATP and product yields. For instance, the single unit model (iFerment156) cannot predict the production of lactate via a NADH-dependent dehydrogenase and re-consumption of lactate via an electron-confurcating dehydrogenase, activities that may happen in a microbial community that includes lactate producers and lactate consumers. Thus, to better represent the compartmentalized nature of microbial activities within microbiomes we developed a functional guild model (iFermGuilds564) to simulate mixed culture fermentations where community members exchange extracellular intermediates. The iFermGuilds564 model contains the fermentation pathways in six guilds (Fig. 2), with each guild including predictions on transport reactions to simulate uptake and release of intermediate metabolites.

Initial predictions with iFermGuilds564 were focused at evaluating the role of each community member on the performance of this mixed culture fermentation. First, we used iFermGuilds564 to predict the fermentation of glucose by each of the guilds individually (Table 1) using an approach similar to that used with the iFerment156 model. That is, we required a release of ≥50 kJ per mol ATP produced as a condition that simulated microbial growth. As expected, only the guilds with reactions needed to degrade carbohydrates were predicted to produce ATP under these conditions. Specifically, iFermGuilds564 predicted that sugar-elongating organisms (SEOs) are predicted to have the highest ATP yield and produce C8, CO₂,
and H₂ (Table 1). In addition, sugar-fermenting organisms (SFOs) are predicted by iFermGuilds564 to produce both acetate and lactate, but not CO₂. In contrast, iFermGuilds564 predicts that HSFs have a higher ATP yield than SFOs and produce acetate, ethanol CO₂, and H₂, while no growth was predicted for several guilds that represent other major members of the mixed microbial community (Table 1). In total, simulations of these individual guilds show that they vary in the amount of ATP that can be produced from the same substrate. In addition, these simulations predict that all of the guilds produce less ATP than a single organism containing all of these reactions (iFerment156, 4.5 mol ATP mol⁻¹ glucose, Fig. 3).

Table 1. iFermGuilds564 predicted overall chemical reactions, ATP yields, thermodynamics, and carbon capture by individual guilds fed glucose.

| Functional Guild | Predicted Reaction | ATP Yield | ΔG° mol⁻¹ ATP | Carbon Capture |
|------------------|--------------------|-----------|---------------|---------------|
| SEOs             | C₆H₁₂O₆ → 0.5 C₃H₅O₂⁻ +H₂O + H₂ + 0.5 H⁺ + 2 CO₂ | 4.0       | -69.3         | 66.7%         |
| SFOs             | C₆H₁₂O₆ → C₃H₅O₂⁻ + 1.5 C₂H₄O₂⁻ + 2.5 H⁺ | 2.5       | -74.1         | 100%          |
| HSFs             | C₆H₁₂O₆ + 0.5H₂O → 2C₂H₆O₃⁻ + 0.5C₂H₆O + H₂ +2H⁺ + CO₂ | 3.0       | -87.9         | 83.3%         |
| LEOs             | No Growth Predicted |           |               |               |
| EEOs             | No Growth Predicted |           |               |               |
| HAOs             | No Growth Predicted |           |               |               |

To explore whether iFermGuilds564 would better simulate the experimental observations in the MCFA-producing bioreactor, compared to iFerment156 (Fig. 4), we used a version of iFermGuild564 with only four guilds, which represented the most abundant community members in the bioreactor as determined by metagenome sequencing,¹⁹ which were a Lachnospiraceae (SEO guild), Eubacteriaceae (LEO guild), Lactobacillus (SFO guild), and Coriobacteriaceae (HSF guild). Performance of these guilds were simulated with substrate constraints that were based
on a combination of experimental observations and predictions from previous metabolic network reconstructions\textsuperscript{19}: SEOs were constrained to use glucose, xylose and glycerol. SFOs could consume glucose, xylose, xylans, glucans, and glycerol whereas HSFs were constrained to only consume glucans, glucose and glycerol, and LEOs were restricted to consuming only lactate and glycerol (Table S5).

To mimic steady-state bioreactor conditions, we constrained each iFermGuilds564 model so that specific ATP production rates (mmol ATP gDCW\textsuperscript{-1} hr\textsuperscript{-1}) for each guild were equal, assumed that the guilds were present according to their measured relative DNA abundance,\textsuperscript{19} and performed FVA optimizing for the overall ATP production rate of the community. Under these conditions, iFermGuilds564 had multiple solutions that predicted a maximum ATP rate of 0.58 mmol ATP hr\textsuperscript{-1}. The solutions derived from these iFermGuilds564 simulations predict that a range of products could be produced while maintaining the maximum ATP yield (Fig. 4C). This is different from the predictions derived from the iFerment156 model, where single solutions were found that maximized ATP yield (Fig. 4A). However, both iFerment156 and iFermGuilds564 predict that maximum ATP yields would be attained if C8 is produced from organics in conversion residue and they overpredict the percent of eeq that is required to be converted to C8 when compared to experimental data (30-90%, Fig. 4C).

Constraining the iFermGuilds564 simulation of the above four guilds to produce C6 and C8 based on their observed production in the bioreactor (Fig. 4D), improved its predictions, with only a minor reduction in the predicted rate of ATP production (i.e., 0.57 mol ATP hr\textsuperscript{-1}, ~99% of the maximum ATP yield predicted when C6 and C8 production was not constrained). This finding supports the notion that toxicity of individual MFCA\textsubscript{S} may impact the observed product composition and that the energetic cost of reduced C8 production is minimal. In these simulations,
the predicted levels of other end-products using iFermGuilds564 were better aligned with those observed in a bioreactor that is fed with the lignocellulosic biorefinery residues (Fig. 4D).

The predicted ATP production rates of 1.32-1.37 mmol ATP hr\(^{-1}\) with iFerment156 (Figs. 4A and 4B) are about two-fold higher than the 0.57-0.58 mmol ATP hr\(^{-1}\) predicted in iFermGuilds564 when the reaction sets are compartmentalized in functional guilds. This suggests that compartmentalization of reaction sets into individual guilds (microbes) greatly influences ATP production by mixed culture fermentation communities. Therefore, our analysis predicts that functional guild metabolic models have advantages over unicellular models for assessing the performance of mixed microbial communities like those in a carboxylate platform bioreactor.

**Assessing interspecies metabolite flux using iFermGuilds546**

Our previous data predict that the individual microbes within the MCFA-producing microbial community perform different functions, compete for resources, and participate in the interspecies transfer of metabolites that ultimately allows the lignocellulosic biorefinery residues to be converted to short- and medium-chain fatty acids. Based on this, we sought to use iFermGuilds564 to test our ability to model the predicted function of each guild in the transformation of substrates as well as the production and transfer of intermediate metabolites between microbes. For this, we constrained iFermGuilds564 to produce end-products at the rates observed in the MCFA-producing bioreactor (Table S3), set the substrates consumed by each guild as described above, and predicted fluxes using pFBA which calculates a single flux distribution when the model is constrained to the observed end-product production rates. Under these conditions, iFermGuilds564 predicted a production rate of 0.54 mmol ATP hr\(^{-1}\), which is 93% of the maximum ATP production rate derived from the FVA analysis (Fig. 4C). The agreement of
the predicted ATP production rates by these methods suggests that, as a whole, the bioreactor community is functioning near its optimal growth rate.

Under these conditions, iFermGuilds564 predicted that the glycerol would be consumed by SEOs, xylose consumption would be shared by SEOs and SFOs, glucose would exclusively be consumed by HSFs, glucans consumed by SFOs and HSFs, and xylans exclusively consumed by SFOs (Fig. 5). In addition, these iFermGuilds564 simulations predicted that SEOs would produce all the C8, most of the C6, and some H2; SFOs were predicted to perform homofermentative lactate production; HSFs were predicted to perform heterofementative production of lactate, acetate, ethanol, and H2; LEOs were the predicted lactate consumers who produced a combination of acetate, butyrate, C6 and H2. When we modeled how the H2 partial pressure might impact the predicted conversion routes, a thermodynamic analysis of each guild indicates that these predicted reactions are feasible at both high and low H2 partial pressures (Table 2). Thus, these iFermGuilds564 simulations indicated that, unlike conventional anaerobic digestion, MCFA production via mixed culture fermentation may proceed under a wide range of H2 partial pressures.

Table 2. Thermodynamics of the net chemical reactions for each functional guild. ΔG\textsuperscript{r} values are calculated at neutral pH at the noted H2 partial pressures assuming all other compound concentrations are present at 1 mM.

| Functional Guild | Reactor Population Name | Relative Abundance | Transport-associated ATP (mmol hr\textsuperscript{-1}) | Growth-associated ATP (mmol hr\textsuperscript{-1}) | Total ATP Production (mmol hr\textsuperscript{-1}) | ΔG\textsuperscript{r} mol\textsuperscript{-1} ATP |
|------------------|-------------------------|-------------------|-----------------------------|-----------------------------|---------------------------------|-----------------------------|
| SEO Lachnospiraceae | 45%                      | 0.00766           | 0.243                       | 0.250                       | -67.5                           | -75.5                       |
| SFO Lactobacillus | 40%                      | 0.00              | 0.216                       | 0.216                       | -73.8                           | -73.8                       |
| HSF Coriobacteriaceae | 10%                     | 0.00143           | 0.0539                      | 0.0554                      | -81.0                           | -83.0                       |
| LEO Eubacteriaceae | 5%                       | 0.0390            | 0.0270                      | 0.0660                      | -146                            | -163                        |

These iFermGuilds564 simulations also predicted that lactate was the only product that was re-consumed by the community. This prediction suggests that acetate and butyrate are
undesirable fermentation end-products if the goal is to maximize C6 or C8 production in the bioreactor.

An additional prediction of iFermGuilds564 is that LEOs, which only represented 5% of the microbial community, were predicted to produce 72% of the H₂, 87% of the acetate, 100% of the butyrate and no significant quantities of C6 and C8 (Fig. 5A). This predicted flux of lactate to end-products that do not contribute to MCFA production suggests that LEOs could be a potential bottleneck for high levels of MCFA production. This prediction is in apparent contradiction with those from the iFerment156 simulations (Fig. 3) that suggested maximization of ATP production would occur if C8 is the final product of lactate utilization. One possible explanation for this discrepancy is that LEOs are growing at sub-optimal conditions in the bioreactor given the imposed restrictions on growth rate (see below).

**Predicted metabolic efficiency of guilds in a MCFA-producing bioreactor**

The previous simulations with iFermGuilds564 assumed that the specific ATP production rates (mmol ATP gDCW⁻¹ hr⁻¹) for each guild were equal. This constraint was imposed to simulate observed steady-state bioreactor conditions where the relative concentration of each guild remains constant over time. However, the previous iFermGuilds564 simulations did not consider potential variations in maintenance energy requirements across the different guilds. Since we lack pure cultures of the organisms in the bioreactor, we used the metabolic models to estimate the efficiency of ATP production for each guild within the reactor community. To do this, we constrained the individual guild models to consume the substrates that they are predicted to metabolize in the mixed culture community (Fig 5). Under these conditions, and in the absence of competition for substrate or restrictions of needing to have a common specific ATP production rate for all the guilds, we allowed the iFermGuilds564 simulation to predict the maximum yield of ATP used for
growth (Table 3). An ATP production efficiency was then calculated based on comparing the predicted growth-associated ATP production in the iFermGuilds564 model and the maximum ATP yield in the single unit iFerment156 simulation (Table 3). The predicted ATP production efficiencies of guilds vary widely from SEOs (96.4%) and SFOs (95.6%) to LEOs (14.1%) (Table 3). This result predicts that LEOs grow at sub-optimal conditions in the bioreactor where there is no selective pressure for them to produce MCFA over shorter chain organic acids or other products.

**Table 3. Predicted ATP production efficiency of each guild modeled in the bioreactor.**

| Guild | Predicted Growth ATP with iFermGuilds564 (mmol hr\(^{-1}\)) | Maximum predicted Growth ATP (mmol hr\(^{-1}\)) | Efficiency (%) |
|-------|---------------------------------------------------------|---------------------------------|---------------|
| SEOs  | 0.244                                                   | 0.253                           | 96.4%         |
| SFOs  | 0.217                                                   | 0.227                           | 95.6%         |
| HSFs  | 0.054                                                   | 0.067                           | 80.6%         |
| LEOs  | 0.027                                                   | 0.191                           | 14.1%         |
| LEOs\(^1\) | 0.027                                                   | 0.064                           | 42.4%         |

\(^1\)LEOs constrained to use electron confurcating lactate dehydrogenase exclusively

Another explanation for these findings is that the metabolic networks used in the simulation of LEOs are not correct, causing the model to overpredict ATP production by this guild. In order to test this hypothesis, we evaluated an alternative route for lactate metabolism in LEOs. That is, LEOs were initially modeled as using two different lactate dehydrogenases for the transformation of lactate to pyruvate, a NADH-dependent lactate dehydrogenase (LDH) and an electron confurcating lactate dehydrogenase (ECLDH), in which lactate transformation is coupled to ferredoxin oxidation as described for the anaerobic lactate-consuming Acetobacterium woodii.\(^{45}\) With these assumptions, the iFermGuilds564 simulation of the LEOs indicated preferential use of LDH over ECLDH (Fig. S1A). Thus, we also ran simulations in which the LEOs exclusively use ECLDH (Fig. S1B). We found that, if LEOs are forced to use ECLDH, their maximum predicted ATP production drops from 0.191 to 0.0637 mmol ATP hr\(^{-1}\), predicting an efficiency of 42.4% in the bioreactor, rather than 14.1% (Table 3). While this scenario results in an improvement in the
prediction, it suggests that use of ECLDH alone does not fully explain the inefficiencies of LEOs. In scenarios where the LEOs exclusive using ECLDH for lactate metabolism, the simulations predict that the overall flow of carbon through the community is nearly identical to those in other simulations (Supplementary File 4). In addition, while the overall ATP production rate for the community remains at 0.54 mmol ATP hr\(^{-1}\) when lactate is metabolized by ECLDH but the predicted energy metabolism of LEOs changes in several ways (Supplementary Text 3).

In sum, simulating the bioreactor community with iFermGuilds564 suggests that SEOs are responsible for producing C8 in the bioreactor. While the model of LEOs contain all of the reactions necessary for producing C8, these microbes are predicted to produce most of the acetate, butyrate and H\(_2\) in the bioreactor. LEOs are predicted to have low growth efficiencies and may require large amounts of ATP for processes other than growth and transport. While the inclusion of ECLDH results in a higher overall energetic efficiency for LEOs (42.4%), this is still lower than the predicted efficiencies of the other guilds in the mixed microbial community (Table 3). Combined these predictions suggest that LEOs use ECLDH and may have other ATP-consuming processes not currently captured by the metabolic model. The iFermGuilds564 predictions also suggest that SEOs are a better source for MCFA production than LEOs when fed a carbohydrate-rich substrate. Further, H\(_2\) and acetate were always predicted to be metabolic end-products rather than intermediates in MCFA production, suggesting that acetate is not a productive intermediate in MCFA production by this community and that electron transfer between species could be improved to alter the suite of desired products.

**Improved MCFA production by a mixed microbial community**

The predictions derived from metabolic models like iFermGuilds564 could also help guide the engineering of natural or synthetic communities that convert complex organic residues to a
desired set of products. While a reactor containing only SEOs is predicted by iFermGuilds564 to favor C8 production (Supplementary File 4), 26% of the carbon from these organisms is predicted to be lost as CO₂. To test if a synthetic microbial community could convert 100% of the carbon and eeq to C8 when fed lignocellulosic biorefinery residues, we used iFermGuilds564 to assemble an *in silico* community to convert all of the carbon in this feedstock to C8. To limit carbon loss as CO₂ by the SEOs, we included HAOs in this synthetic community and simulated the addition of an external electron donor, H₂, to drive homoacetogenic activity to convert endogenous CO₂ to acetate that could undergo chain elongation. We tested the performance of this synthetic community, under various conditions, including different ratios of each microbe, partial pressures of H₂ and others.

One synthetic community that was predicted to convert all of the eeq in lignocellulosic biorefinery residues and exogenous H₂ to C8 contained 90% SEOs and 10% HAOs, both of which were predicted to both use exogenous H₂ (Fig. 6). In this modeled synthetic community, HAOs were predicted to use H₂ to fix CO₂ produced by SEOs, and acetate produced by the HAOs was a major source for chain elongation by the SEOs. Formate produced by SEOs would also be used by the HAOs as a source of carbon and reducing power. Analyzing the scenarios predicted for each guild indicate that a H₂ partial pressure ≤ 10⁻⁶ atm would make the predicted ATP produced by HAOs thermodynamically infeasible. However, at H₂ partial pressures ≥ 10⁻³ atm, the transformations and predicted ATP yields of the two-guild mixed culture fermentation were sufficient to support C8 production (Table 4). Further, metabolic modeling of this synthetic community predicts that increased C8 production could be achieved if HAOs could more readily utilize H₂ that is produced or provided to the reactor.
Table 4. Predicted thermodynamics of a synthetic community of SEOs and HAOs converting conversion residue and H₂ to C8.

| Functional Guild | Relative Abundance | Predicted ATP Production (mol hr⁻¹) | ΔG' mol⁻¹ ATP | P_H₂ = 1 atm | P_H₂ = 10⁻³ atm |
|------------------|--------------------|-------------------------------------|----------------|-------------|-----------------|
| SEO              | 90%                | 0.728                               | -100           | -87.6       |                 |
| HAO              | 10%                | 0.081                               | -160           | -86.8       |                 |
| Total            |                    | 0.809                               | -106           | -87.6       |                 |

DISCUSSION

Under anaerobic conditions, a diverse set of mixed microbial communities catalyze the transformation of organic substrates to a variety of products. Some fermentation metabolisms are well understood, using genetically tractable pure cultures that can be analyzed, modeled and improved for use in industrial applications. For over a century, anaerobic microbial communities have been harnessed to recover energy, in the form of methane-rich biogas, from complex organic matter in anaerobic digestion. The ability to harness the metabolic activity of mixed microbial communities to produce alternative fermentation products is limited since metabolic and kinetic models that accurately predict the distribution of fermentation products are only starting to emerge. To address this knowledge gap, we constructed metabolic models of mixed culture fermentations with the objective of gaining greater insights into how to increase MCFA production by mixed microbial communities. We focused on producing C6 and C8, linear medium-length MCFA of industrial value that can be produced from a variety of organic residues.

Since many microbes in self-assembled fermentation communities remain uncultured, our knowledge and ability to predict important metabolic networks of these mixed cultures relies largely on information from metagenomics, metatranscriptomics and metabolomics analyses to
build the functional activities in each guild\cite{19, 48}. We show in this work that metabolic modeling offers additional insight that is not gained by these analyses, but that is critical in their evaluation and in developing hypothesis of how to direct metabolism towards a specific set of fermentation products. For instance, while iFermGuilds564 included three guilds capable of MCFA production (SEOs, LEOs, and EEOs; Fig. 2), only SEOs and LEOs were included in many simulations since reactor performance did not indicate ethanol as a fermentation product\cite{19}. From prior analyses\cite{19, 49} we anticipated that both SEOs and LEOs participated in MCFA production, but the iFermGuilds564 simulation of interspecies metabolite flux (Fig. 5) predicted that, to maximize the overall ATP production in the community, C8 was largely produced by SEOs, while LEOs were responsible for production of butyrate and acetate as fermentation end-products that were not converted to MCFA. This finding makes the unexpected prediction that minimizing the abundance or activity of LEOs would maximize MCFA production by this community.

We further showed that a guilds-based fermentation model such as iFermGuilds564 can be used to evaluate scenarios for how to direct a mixed microbial community to produce specific fermentation products from more efficient utilization of complex organic substrates (Fig. 6). For example, iFerment156 predicted that H2 is both a product during MCFA production (Fig. 5) and a source of reducing power that could maximize MCFA production by simplifying the range of accumulated fermentation products (Fig. 3). In addition, the results of this simulation predict a previously unrealized role of HAOs, a guild that is not abundant in bioreactors transforming lignocellulosic biorefinery residues to MCFA\cite{6, 19}, but that could be enriched by an exogenous supply of H2 (Fig. 6). The use of metabolic models to assemble an ideal microbial community represents an important new step to bioprocess design that include synthetic or self-assembled communities optimized through the application of selective pressures such as H2 supply.
iFerment156 and iFermGuilds564 are examples of first-generation predictive models of mixed culture fermentations. Their utility can be improved by obtaining additional knowledge of metabolic pathways in individual microbes and genetic studies to test predictions of the metabolic potential of the community members that compliment multi-omic analyses. Further effort is needed to simulate poorly appreciated conditions that shape community structure, such as the toxicity of fermentation products on the specific guilds or the contribution(s) of low abundance microbes to the overall activity of the mixed culture fermentation. Additionally, ours and many models are limited to maximizing community ATP production as a proxy for microbial growth, so computational methods to predict the abundance of specific guilds, rather than relying on relative abundances as a model input, are needed. Efforts to assemble such models have begun for the human gut microbiome, and as these approaches are applied to mixed microbial fermentations, their benefits to the production of MCFA and other chemicals of societal importance will continue to improve.

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Figure 1. Summary of metabolic networks in iFerment156. Open hexagons indicate consumption of the ATP (orange), NADH (green), and reduced ferredoxin (red). Closed hexagons indicate production of these compounds and consumption of ADP (orange), NAD+ (green), and oxidized ferredoxin (red). For reversible reactions, larger arrowheads indicate the direction of the indicated compounds’ consumption or production. Dashed lines indicate multiple reactions. Key reactions are indicated according to the abbreviation used in iFerment156 (Supplementary File 3).

Pathways are identified by number: (1) upper glycolysis; (2) phosphoketolase metabolism of...
fructose; (3) pentose phosphate; (4) phosphoketolase metabolism of xylulose; (5) glycerol utilization; (6) lower glycolysis; (7) lactate metabolism via NADH-dependent lactate dehydrogenase; (8) lactate metabolism via electron-confurcating lactate dehydrogenase; (9) pyruvate metabolism via pyruvate dehydrogenase; (10) acetate metabolism; (11) ethanol metabolism; (12) pyruvate metabolism by flavodoxin oxidoreductase; (13) pyruvate metabolism by formate lyase; (14) propionate production via the acryloyl-CoA pathway; (15) propionate production via the methylmalonyl-CoA pathway; (16) even-chain reverse β-oxidation; (17) odd-chain reverse β-oxidation; (18) ferredoxin-dependent hydrogenase; (19) proton-translocating hydrogenase; (20) electron confurcating hydrogenase; (21) the RNF complex; (22) homoacetogenesis via the Wood Ljungdahl pathway. More information on the reactions and metabolites contained in iFerment156 is provided in Supplementary File 3.
Figure 2. Summary of metabolic networks for six functional guilds contained in iFermGuilds564. Refer to Fig. 1 for details on pathways included in the iFermGuilds564 guild models. Information on the reactions and metabolites contained in the iFermGuilds564 guild models is provided in Supplementary File 3.
Figure 3. Flux variability analysis results for iFerment156 maximizing ATP production from the indicated substrates. The maximum production of each end-product that can maintain the maximum ATP yield is indicated. For instance, when consuming glucose, iFerment156 predicts that production of formate, acetate, butyrate, hexanoate, octanoate, and H2 can each sustain maximum ATP yields. However, if lactate is the sole substrate, only octanoate production can maintain the maximum ATP yield. The color intensity represents the percent of eeq that is predicted by iFerment156 to be converted to the noted end-product while maintaining maximum ATP production. The chart above the heatmap indicates the mol ATP generated per mol of substrate utilized. For scenarios with two growth substrates, the ATP yield is relative to the substrate indicated in bold text. Example model solutions are provided in Table S3.
**Figure 4.** Predicted and observed product formation for a mixed culture fermentation bioreactor fed lignocellulosic biorefinery residues. Blue bars represent the range of products consistent with maximum ATP production by the microbial community. Products represented by black lines indicate there is a unique percent of eeq that must be converted to that compound. Closed black circles represent the observed level of product accumulated in a bioreactor fed lignocellulosic biorefinery residues (A) predictions with iFerment156; (B) predictions with iFerment156 with constrained hexanoate and octanoate production; (C) predictions with iFermGuilds564; (D) predictions with iFermGuilds564 with constrained hexanoate and octanoate production.
Figure 5. Predicted substrate flow through a mixed culture fermentation producing a mixture of MCFA from a complex feedstock using iFermGuilds564. Values were obtained using pFBA while constraining the products according to observed bioreactor performance. The relative abundance of each functional guild assumed based on reactor DNA abundance is given in parentheses. The relative amount of eeq in the substrates, products, and intermediates are also provided. Line width indicates the relative amount of each substrate consumed and product produced by each of the functional guilds. H₂ was not measured, so for these simulations it was assumed that the difference between eeq in the substrate and eeq in the measured soluble and insoluble products represented H₂ production. In this simulation, 64% of the eeq in conversion residue was predicted to be directed to lactate as an intermediate.
Figure 6. iFermGuilds564 predicted fluxes through a community of SEOs and HAOs converting lignocellulosic biorefinery residue and exogenous H₂ into octanoate. SEOs provide CO₂ and formate to the HAOs, while the HAOs provide acetate to SEOs.