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Review

Fermentation of Organic Residues to Beneficial Chemicals: A Review of Medium-Chain Fatty Acid Production

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Abstract: Medium-chain fatty acids (MCFAs) have a variety of uses in the production of industrial chemicals, food, and personal care products. These compounds are often produced through palm refining, but recent work has demonstrated that MCFAs can also be produced through the fermentation of complex organic substrates, including organic waste streams. While “chain elongation” offers a renewable platform for producing MCFAs, there are several limitations that need to be addressed before full-scale implementation becomes widespread. Here, we review the history of work on MCFA production by both pure and mixed cultures of fermenting organisms, and the unique metabolic features that lead to MCFA production. We also offer approaches to address the remaining challenges and increase MCFA production from renewable feedstocks.

Keywords: chain elongation; carboxylate platform; medium-chain fatty acids; carboxylic acids; mixed culture fermentation

1. Introduction

Microbial fermentation processes play an important role in food production, nutrient cycling, and pollution remediation. For more than 9000 years, humans have relied on microbial fermentation to produce and protect food [1], and fermentation processes have been used to convert wastes into valuable products since at least the 13th century [2]. One wide-spread application of fermentation processes to recover valuable products from wastes is anaerobic digestion. Anaerobic digestion is used to produce biogas (a mixture of carbon dioxide, methane, moisture, and other trace gasses) from organic wastes, including sewage sludge, agricultural and food production residues, and high-strength industrial wastes. There are estimated to be more than 20,000 large-scale anaerobic digestion facilities across the globe [3]. These facilities largely combust biogas to generate heat and electrical power, but biogas can also be converted to renewable natural gas to augment fossil-fuel derived natural gas. Anaerobic digestion to produce biogas is a well-studied process and has been widely implemented, but there is interest in producing valuable chemicals other than biogas from these renewable feedstocks.

Medium-chain fatty acids (MCFAs) are monocarboxylic acids containing 6 to 12 carbon atoms (Table 1). Currently, MCFAs are largely produced as a byproduct of palm refining. Palm kernels contain a relatively high content of octanoic acid (aka, caprylic acid, C8), decanoic acid (aka, capric acid, C10) and dodecanoic acid (aka, lauric acid, C12) [4]. MCFAs have also gained attention as dietary supplements, especially as medium-chain triglycerides (MCTs; three MCFAs attached to a glycerol backbone) [5]. MCTs are typically produced from the hydrolysis and separation of fatty acids from kernel or palm oil and re-esterification to a glycerol backbone, and contain 50–80% C8 [6].

MCTs are used as a supplement in livestock feed [7–9] and are marketed under many tradenames for human consumption (e.g., MCT Oil, Brain Octane Oil). In addition to their role as a nutritional supplement, MCFAs are also used for the production of personal care products, pharmaceuticals, dyes, and antimicrobials [10]. MCFAs have also been proposed as a precursor for liquid transportation fuels [11,12].

Table 1. Properties of short- and medium-chain fatty acids.

| Compound Name | Common Synonyms | Solubility ¹ (mg L ⁻¹) | Electron Density ² (g COD g ⁻¹) | pK _a |
|-----------------|---------------------|---|--|-----------------|
| Acetic acid | Ethanoic acid, C2 | Miscible | 1.07 | 4.76 |
| Propionic acid | Propanoic acid, C3 | Miscible | 1.51 | 4.88 |
| Butyric acid | Butanoic acid, C4 | 60,000 | 1.82 | 4.82 |
| Pentanoic acid | Valeric acid, C5 | 24,000 | 2.04 | 4.84 |
| Hexanoic acid | Caproic acid, C6 | 10,300 | 2.20 | 4.80 |
| Heptanoic acid | Enanthic acid, C7 | 2820 | 2.34 | 4.80 |
| Octanoic acid | Caprylic acid, C8 | 735 ³ | 2.44 | 4.89 |
| Nonanoic acid | Pelargonic acid, C9 | 284 ⁴ | 2.53 | 4.95 |
| Decanoic acid | Capric acid, C10 | 62 | 2.60 | 4.90 |
| Undecanoic acid | Undecylic acid, C11 | 52 | 2.66 | 4.95 |
| Dodecanoic acid | Lauric acid, C12 | 4.8 | 2.72 | 5.30 |

¹ Solubility in water obtained from PubChem [13] at 25 °C unless otherwise noted. ² Electron density is presented as the chemical oxygen demand (COD). This value is the theoretical mass of O₂ required to completely oxidize a compound. ³ Solubility for octanoic acid was obtained by averaging the solubilities at 20 and 30 °C. ⁴ Solubility for nonanoic acid is reported for 20 °C.

MCFA production has been demonstrated by pure-cultures and mixed microbial communities (microbiomes) sourced from a variety of inocula. In bioreactor experiments, with either pure cultures or mixed communities, a variety of end products are typically formed, including short-chain fatty acids (SCFAs) and MCFAs. Studies of pure cultures provide valuable insights into MCFA production, and many novel MCFA-producing organisms have been described in recent years. Microbial communities are believed to be advantageous for MCFA production from complex feedstocks due to their ability to consume a wide range of substrates and to respond to perturbations due to changes in feedstock characteristics. Since the mid 2010's, the targeted production of MCFAs over short-chain fatty acids (SCFAs) has been termed “chain elongation,” and the technology has previously been reviewed by Angenent et al. [10] and others [14,15]. However, there have been many recent developments in MCFA production that warrant further review. Therefore, this review summarizes the current body of knowledge using both pure cultures and mixed microbial communities. Metabolic features, energetics, and thermodynamics that may drive MCFA production are then considered. Lastly, future work is proposed to improve our understanding of MCFA production and to elucidate tools to improve MCFA production from complex feedstocks.

2. Medium-Chain Fatty Acid Production by Bacterial Isolates

MCFAs have been known as a potential fermentation product for over 100 years. Work with bacterial isolates producing MCFAs has provided insights into the metabolism of MCFA production and the phylogenetic diversity of MCFA-producing organisms. Here, we summarize the chronological body of work on MCFA production leading to the 13 isolated bacterial strains currently known to produce MCFAs. We also discuss the variety of substrates that can be used and the natural environments from which these bacteria were isolated. Characteristics of the known MCFA-producing bacterial isolates are summarized in Table 2.

Rhodospirillum rubrum [16] was the first MCFA-producing bacterium to be isolated (originally named *Spirillum rubrum* in 1887 [17,18]). During dark fermentation, *R. rubrum* consumes glucose, fructose, sucrose and carbon monoxide, and produces C6 [10,19]. In the 1940s, *Clostridium kluyveri* was isolated from canal mud. *C. kluyveri* is an anaerobic, non-saccharolytic bacterium that produces C6 and C8, using ethanol, n-propanol and pyruvate as electron donors, and C2, C3 and C4 as electron

acceptors [14,20–22]. *C. kluyveri* is one of the most well-studied MCFA-producing species and a complete genome for this organism became available in 2008 [23]. The proposed metabolism of MCFA production is largely based on experiments performed with *C. kluyveri*, and supporting genomic evidence [22–26] and is discussed later in this review. Shortly after the isolation of *C. kluyveri* in 1938, *Eubacterium limosum* was isolated from pea-blanching wastes, and converted methanol and C2 to C4 and C6 [27,28].

Shortly after the isolation of *C. kluyveri* and *E. limosum*, Prevot and Taffanel isolated *Ramibacterium alactolyticum* [29]. In 1967, this organism was shown to produce carboxylic acid products, including C6 and C8, from sugars and lactate [30]. In 1973, the name was changed to *Eubacterium alactolyticum* [31], and 26 years later, the organism was renamed again as *Pseudoramibacter alactolyticus* [29]. The complete genome of *P. alactolyticus* was sequenced in 2011 as part of the Human Microbiome Project [32]. In 1951, a third MCFA-producing microbe was isolated from the rumen of a sheep [33]. *Rumen organism LC*, as it was initially named, used sugars and lactate to produce even- and odd-chain carboxylic acids. In 1958, the organism was renamed *Peptostreptococcus elsdenii* [34], and in 1971 Rogosa reclassified the isolate as the genus *Megasphaera* [35] and named the type species *Megasphaera elsdenii*. *M. elsdenii* is a well-studied organism that has been used for the production of MCFAs from a variety of complex and simple substrates [36–43]. The complete genome of the *M. elsdenii* type strain was made available in 2011 [44], and three additional strain genomes have been sequenced since then [45,46]. In 2020, a metabolic reconstruction and model of *M. elsdenii* was published [47].

Several additional MCFA-producing strains have been described since the early 2000s. In 2003, *Eubacterium pyruvativorans* was isolated from a sheep rumen [48]. *E. pyruvativorans* converts C3 and C4 to C5 and C6, using methanol and pyruvate as electron donors [48]. *Ruminococcaceae* bacterium CPB6, isolated from a fermentation pit in 2015, produces C6 from lactate and has been used to convert wastes from liquor-making to MCFAs [49,50]. The genome of the *Ruminococcaceae* bacterium CPB6 was published in 2017 [51]. *Caproiciproducens galactitolivorans* (originally *Clostridium* sp. BS-1 [52,53]) was isolated from an anaerobic digester and described in 2015. *C. galactitolivorans* produces C6 from galactitol and its genome was sequenced in 2019 [54,55]. In 2017 a new species of *Megasphaera* was described. *Megasphaera hexanoica* converts sugars, including fructose and mannose, to even- and odd-chain fatty acids, including C6. *M. hexanoica* was isolated from a cow rumen, and follow-up work has investigated conditions to increase MCFA production [56,57].

In the first nine months of 2020, five additional MCFA-producing bacteria have been isolated. *Caproicibacter fermentans* is a C6-producing bacterium isolated from a methanogenic bioreactor [58]. *Clostridium* sp. BL-3, BL-4 and BL-6 convert lactate to C6. One of the isolates, BL-4, also produces iso-butyrate. [59,60]. *Caproiciproducens* sp. 7D4C2 was recently isolated from an MCFA-producing bioreactor and produces C6 from sugars [61]. Further, though not isolated, two *Candidatus* species of bacteria, *Candidatus Weimeria bifida* and *Candidatus Pseudoramibacter fermentans*, were described in 2020 and metabolic reconstructions were proposed based on metagenomic and metatranscriptomic analyses [62].

MCFA-producing isolates have been derived from a variety of natural sources, from canal mud to rumens to the human body. This wide range of isolation sources suggests that MCFA production may have yet undiscovered roles within natural ecosystems. With the exception of *R. rubrum*, all of the MCFA-producing isolates belong to the Firmicutes phylum, and there are currently six genera of MCFA-producing bacteria within the Firmicutes: *Clostridium*, *Pseudoramibacter*, *Megasphaera*, *Eubacterium*, *Caproiciproducens*, and *Caproicibacter*. It is expected that many more MCFA-producing bacteria will be isolated in coming years, and studies with these organisms and their genomes are expected to provide new insights into MCFA production.

Table 2. Isolated bacterial strains known to produce MCFAs.

| Name | Isolation Source | Substrates | Products | Reference |
|---|---|---|---|------------|
| <i>Clostridium kluyveri</i> | Canal mud | Glucose, Fructose, Sorbitol, Mannitol, Lactate, Pyruvate, Serine, Formate | C4, C5, Ethyl crotonate, C6, C7, C8, CO ₂ and H ₂ | [14,20–23] |
| <i>Megasphaera elsdenii</i> | Sheep rumen | Glucose, Fructose, Maltose, Mannitol, Sorbitol, Lactate | C2, C3, C4, C5, C6, CO ₂ and H ₂ | [14,33–35] |
| <i>Ruminococcaceae</i> bacterium CPB6 | Fermentation pit | Lactate, Ethanol, Glucose | C4, C6 | [14,49,50] |
| <i>Caproiciproducens galactitolivorans</i> BS-1 | Wastewater treatment plant | Galactitol, C2 | Ethanol, Butanol, C2, C4, C6, H ₂ | [52,53] |
| <i>Eubacterium limosum</i> | Pea-blanching wastes | H ₂ and CO ₂ | C2, C4, C5, C6 | [14,27,28] |
| <i>Eubacterium pyruvativorans</i> | Sheep rumen | Formate, Acetate, Propionate, iso-butyrate, C4, Lactate | C4, C5, C6 | [48] |
| <i>Rhodospirillum rubrum</i> | Dead mouse | Glucose, Fructose, Sucrose, CO | C6 | [10,16–19] |
| <i>Pseudoramibacter alactolyticus</i> | Lung abscess | Glucose | Formate, C2, C4, C5, C6, C8 | [29–31,63] |
| <i>Megasphaera hexanoica</i> | Cow rumen | Fructose, Mannose | C2, iso-butyrate, iso-valerate, C6, H ₂ , CO ₂ , H ₂ S | [56,57] |
| <i>Clostridium</i> sp. BL-3 | Anaerobic bioreactor | Lactate, Ethanol, Xylose, Fructose | C2, C4, iso-butyrate, C6 | [59,60] |
| <i>Clostridium</i> sp. BL-4 | Anaerobic bioreactor | Lactate, Ethanol, Xylose, Fructose | C2, C4, iso-butyrate, C6 | [59,60] |
| <i>Clostridium</i> sp. BL-6 | Anaerobic bioreactor | Lactate, Ethanol, Xylose, Fructose | C2, C4, iso-butyrate, C6 | [59,60] |
| <i>Caproiciproducens</i> sp. 7D4C2 | Open-culture, chain-elongating bioreactor | Glucose, Fructose | Lactate, C2, C4, C6, H ₂ , CO ₂ | [61] |

3. MCFA Production from Complex Feedstocks with Microbial Communities

While microbial isolates are important for understanding MCFA production, the application of large-scale MCFA production is expected to rely on microbial communities using “open culture” processes [10]. Several renewable substrates have been tested for MCFA production, including the organic fraction of municipal solid waste (OFMSW), food waste (FW), lignocellulosic biomass and residues of lignocellulosic biomass refining (LCB), waste from grain ethanol production, brewing distilling, and wine making (EBDWW), dairy processing wastes (DPW) and manure. In some processes, pretreatments have been used to condition the feedstock, including homogenization and microbial pre-treatment (Figure 1). Homogenization typically includes the addition of water to convert high-solid feedstocks into pumpable liquids. Microbial hydrolysis and fermentation (a.k.a, acid digestion) rely on anaerobic microbes to break down complex organic matter and to ferment a portion of the resulting hydrolysis products to acids and alcohols. Both liquid- and solid-state processes (generating an acid-rich leachate) have been used. Alternatively, feedstocks can be fed directly to a single reactor that performs hydrolysis, fermentation, and MCFA production. Sometimes, a supplemental electron donor (such as ethanol) is added. The MCFA-production step has been tested using both suspended-growth and attached-growth processes. Further, up-flow granular sludge processes have been used. The advantage of attached growth and granular processes is that they decouple the solids retention time from the hydraulic retention time, allowing the growth of slow-growing microbes without the need for high hydraulic retention times. If included, the extraction of MCFAs takes place either subsequent to MCFA production or as part of the same reactor system. When implemented on a reactor recirculation

stream this process is referred to as pertraction. An advantage of pertraction is that it prevents the accumulation of MCFAs within the fermentation broth. While most tested extraction processes rely on liquid–liquid separation with organic solvents, electrolytic extraction has also been proposed [64]. Typical processes for MCFA production are depicted in Figure 1. The selected processes largely depend on the complexity of the feedstock and the availability of electron donors to drive MCFA production. Here, we provide a review of past research on converting complex organic substrates to MCFAs based on the type of feedstock (Table 3). Past studies have investigated different feedstocks, reactor configurations, hydraulic retention times (HRTs), and inoculum sources. We summarize these studies based on net conversion of chemical oxygen demand (COD) provided to the reactor, including both COD in the feedstock and COD added as a supplemental electron donor.

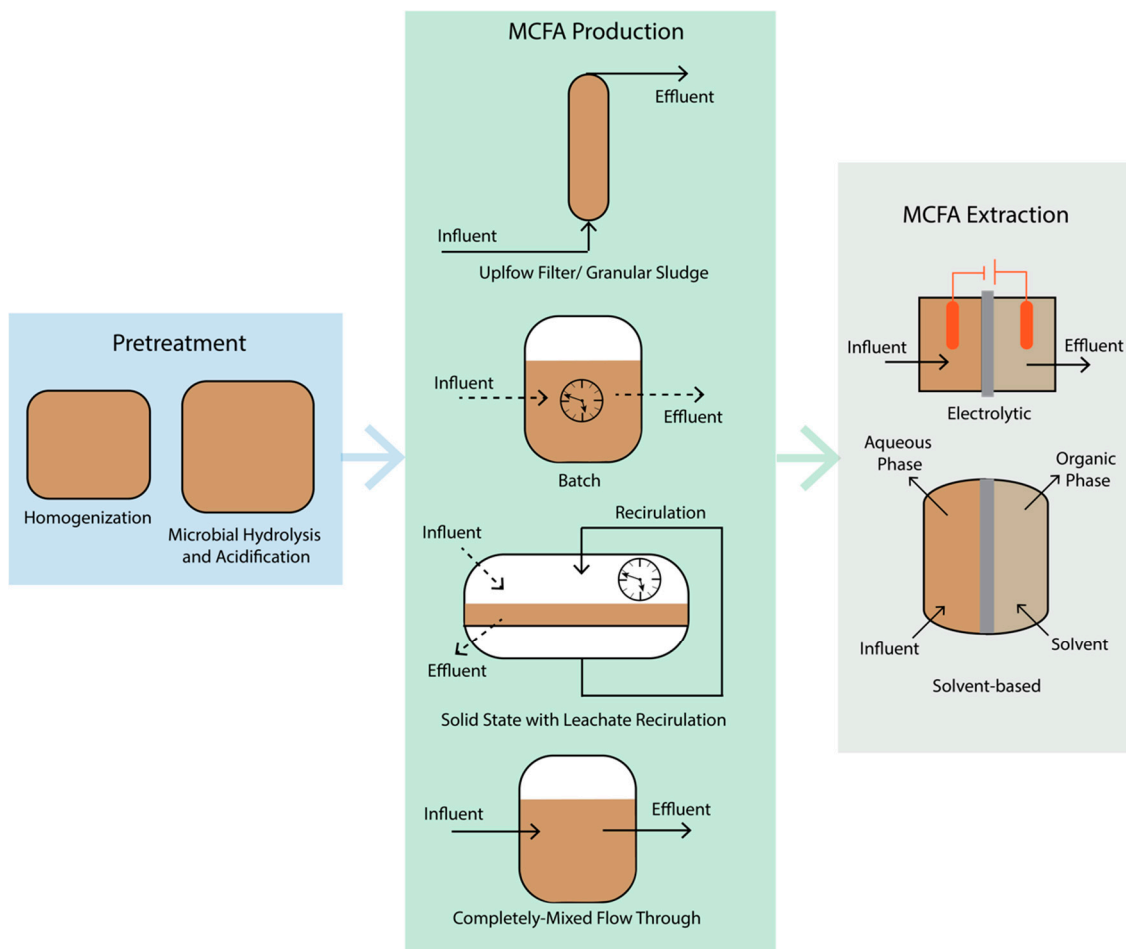


Figure 1. Processes for converting organic wastes to MCFAs. Several processes have been implemented for converting organic waste streams to MCFAs.

Table 3. A summary of studies investigating MCFA production from high-strength organic feedstocks.

| Substrate Type | Specific Substrates | Supplemental Electron Donor | Reactor Type | HRT (d) | Pre-Treatment | Inoculum | COD Conversion | Productivity (g COD L ⁻¹ d ⁻¹) | | | Other Products | Ref. |
|----------------|---|-----------------------------|---|---------|--|--|----------------|---|--------|--------|---------------------|------|
| | | | | | | | | C6 | C7 | C8 | | |
| OFMSW | Influent waste from a full-scale organic waste treatment facility | Ethanol | Up-flow fixed bed | 0.46 | Microbial hydrolysis and acidification | Acetate and ethanol consuming consortium | 19% | 61 | 4.6 | 2.1 | C2, C3, C4, C5 | [65] |
| OFMSW | Approximately 90% yard waste and 10% food waste | None | Batch leach bed with leachate recirculation | 35 | Homogenization | OFMSW | 5.6% | 0.17 | 0.10 | 0.035 | C2, C3, C4, C5 | [66] |
| OFMSW | Approximately 90% yard waste and 10% food waste | Ethanol | Batch leach bed with leachate recirculation | 28 | Homogenization | OFMSW | 2.3% | 0.13 | 0.033 | 0 | C2, C3, C4, C5 | [66] |
| FW | Restaurant wastes | None | Leach bed with leachate recirculation | 7 | Homogenization | Full-scale granular anaerobic digester sludge | 19% | 7.3 | NR | NR | C2, C3, C4 | [67] |
| FW | Outdated food scraps | Ethanol | Continuously stirred flow-through | 4 | Microbial hydrolysis and acidification | Lab-scale bioreactor | 28% | 13 | 0.35 | 0.37 | C2, C4 | [68] |
| FW | Outdated food scraps | Ethanol | Continuously stirred flow-through | 1 | Microbial hydrolysis and acidification | Lab-scale bioreactor | 8.2% | 16 | 0.058 | 0.061 | C2, C4 | [68] |
| FW | Food waste from homes | Ethanol | Continuously mixed batch | 51 | Microbial hydrolysis and acidification | Undescribed anaerobic bacteria | 4.1% | 0.12 | 0.0043 | 0.0048 | C2, C3, C4, C5, C10 | [69] |
| FW | Food waste from homes | Ethanol | Continuously mixed batch | 28 | Microbial hydrolysis and acidification | Undescribed anaerobic bacteria, <i>C. kluyveri</i> | 12% | 0.64 | 0.01 | 0.01 | C2, C3, C4, C5, C10 | [69] |

Table 3. Cont.

| Substrate Type | Specific Substrates | Supplemental Electron Donor | Reactor Type | HRT (d) | Pre-Treatment | Inoculum | COD Conversion | Productivity (g COD L ⁻¹ d ⁻¹) | | | Other Products | Ref. |
|----------------|--|-----------------------------|-----------------------------------|---------|--|--|----------------|---|-------|--------|---------------------|------|
| | | | | | | | | C6 | C7 | C8 | | |
| FW | Outdated food scraps | None | Fed-batch | 45 | None | Lab-scale bioreactor | 14% | 4.20 | NR | trace | C2, C3, C4, C5 | [70] |
| LCB | Shredded paper and chicken manure | Ethanol | Continuously mixed batch | 27 | None | Marine bacteria | 14% | 0.81 | NR | NR | C2, C4 | [71] |
| LCB | Switchgrass ethanol production residues | None | Continuously stirred flow-through | 6 | None | Municipal wastewater acid digester sludge | 22% | 3.1 | trace | 0.40 | C2,C4,C5 | [72] |
| LCB | Milled switchgrass | None | Unmixed batch | 3 | None | Rumen fluid | 0.33% | 0.034 | NR | NR | C2, C3, C4, C5 | [73] |
| LCB | Milled switchgrass | Ethanol | Unmixed batch | 3 | None | Rumen fluid | 0.17% | 0.017 | NR | NR | C2, C3, C4, C5 | [73] |
| LCB | Milled switchgrass | Ethanol | Unmixed batch | 3 | None | Rumen fluid, <i>C. kluyveri</i> | 35% | 3.6 | NR | NR | C2, C3, C4, C5 | [73] |
| LCB | Farmland grass | None | Continuously stirred flow-through | 2 | Microbial hydrolysis and acidification | Lab-scale bioreactor | 5.4% | 5.7 | NR | NR | C2, C3, C4, C5 | [74] |
| LCB | Corn silage | None | Batch | 13 | None | Digestate from full-scale silage digester | 3.8% | 0.10 | NR | 0.0077 | C2, C3, C4, C5 | [75] |
| LCB | Corn stover hydrolysate | None | Fed-batch | 9.6 | Dilution | <i>Megasphaera elsdenii</i> | 23% | 0.29 | NR | NR | C2, C4 | [37] |
| EBDWW | Stillage and beer from a wheat bioethanol facility | None | Continuously stirred flow-through | 7.5 | None | Lab-scale bioreactor | 18% | 3.0 | NR | trace | C2, C3, C4, C5, C10 | [53] |
| EBDWW | Corn beer | None | Sequencing batch reactor | 15 | None | Digestate from first phase of full-scale silage digester | 58% | 1.7 | 0.056 | 2.70 | C2, C3, C4, C6 | [75] |

Table 3. Cont.

| Substrate Type | Specific Substrates | Supplemental Electron Donor | Reactor Type | HRT (d) | Pre-Treatment | Inoculum | COD Conversion | Productivity (g COD L ⁻¹ d ⁻¹) | | | Other Products | Ref. |
|----------------|--|-----------------------------|-----------------------------------|---------|---|---|----------------|---|-------|-------|--------------------|------|
| | | | | | | | | C6 | C7 | C8 | | |
| EBDWW | Solids-free thin stillage | None | Up-flow reactor, granular sludge | 0.5 | None | Reactor fermenting solid-free thin stillage | 29% | 1.5 | 0.046 | 0.037 | C2, C3, C4, C5 | [76] |
| EBDWW | Thin stillage from bioethanol production | None | Continuously stirred flow-through | 6 | None | Thin stillage | 0.11% | 0.060 | NR | NR | C2, C3, C4, C5 | [64] |
| EBDWW | Chinese liquor-making wastewater | None | Expanded granular sludge bed | 8 | Dilution | Pit mud and activated sludge | 77% | 0.55 | 0.057 | 0.081 | C2, C3, C4, C5 | [77] |
| EBDWW | Lactate-rich liquor-brewing wastewater | None | Fed-batch | 3 | Dilution | <i>Ruminococcaceae</i> bacterium strain CPB6, Municipal wastewater sludge | 51% | 11.6 | NR | NR | C2, C4 | [50] |
| EBDWW | Wine lees with 11% ethanol by volume | None | Continuously stirred flow-through | 9.5 | Dilution | Lab-scale bioreactor | 67% | 2.0 | NR | 2.0 | C2, C3, C4, C5 | [78] |
| DPW | Yogurt acid whey | None | Up-flow anaerobic filter | 2.1 | Microbial hydrolysis and acidification (thermophilic) | Lab-scale food waste fermentation | 61% | 21 | 4.3 | 4.9 | C2, C3, C4, C5, C9 | [79] |
| DPW | Quark acid whey | None | Up-flow anaerobic sludge blanket | 2.5 | None | Full-scale anaerobic digester sludge | 32% | 0.24 | NR | trace | C2, C3, C4, C5 | [80] |
| DPW | Cheese whey | None | Up-flow packed-bed reactor | 6 | None | Lab-scale acidogenic reactor | 22% | 1.5 | NR | NR | C2, C3, C4, C5 | [81] |
| Manure | Swine manure | Ethanol | Batch | 76 | Microbial hydrolysis and acidification | Full-scale anaerobic digester sludge | 28% | 0.11 | 0.084 | 0.034 | C2, C3, C4, C5 | [82] |

3.1. Organic Fraction of Municipal Solid Waste (OFMSW)

Organic fraction of municipal solid waste (OFMSW) consists of yard waste, food waste, and other organic waste materials. As such, it can contain a mixture of plant- and animal-based products that vary widely in composition. High fractions of yard waste can increase the cellulose, hemicellulose, and lignin content, while high amounts of meat wastes can increase the protein content. OFMSW is collected as a solid material and may require dilution and homogenization prior to processing, especially if the food waste is treated in liquid-based reactors. An alternative leach bed-based approach has also been proposed. In these configurations, leachate is circulated through the solid waste to stimulate fermentation and capture fermentation products [66]. The highest productivities and COD conversion were obtained in work using an up-flow fixed-bed reactor supplemented with ethanol, relying on a two-stage process in which OFMSW was pre-fermented prior to MCFA-production [65]. The lower productivity of MCFAs in other OFMSW experiments [66] may be partly contributed to by a high fraction of yard waste in the feedstock. Cellulose, hemicellulose, and lignin polymers in yard waste are particularly recalcitrant [83]. While OFMSW is an abundant feedstock, it contains recalcitrant materials that may necessitate pre-treatment (e.g., hydrolysis) to achieve high conversion efficiencies of COD to MCFAs.

3.2. Food Waste (FW)

Food waste (FW) (a component of OFMSW) is another abundant organic waste that has been tested as a feedstock for MCFA production. As with OFMSW, FW characteristics can also vary significantly, with vegetables and starch-based products providing carbohydrates of various monomeric and polymeric compositions and meats providing high quantities of protein and fat. The pre-processing of food waste can include homogenization and pre-fermentation. De-packaging of food waste is an additional concern [84]. Work published in 2018 by Nzeteu et al. [67] demonstrated the use of a leach bed reactor with liquid recirculation, and achieved a maximum conversion of 19% of the influent COD in food waste to C6. Based on the characterization of reactor influent and effluent, they showed that 90% of hemicellulose, 60% of proteins, 50% of cellulose, and 20% of fats were degraded. In addition to the sustained demonstration with the leachate reactor, Nzeteu and colleagues tested the ability to produce additional MCFAs from the leachate bed effluent through the addition of either ethanol, H₂, or both ethanol and H₂ as supplemental electron donors in batch experiments. They found that lactate in the leachate reactor effluent was converted to C6 when H₂ or a combination of H₂ and ethanol was added. Another process for processing food waste is a two-stage configuration in which food waste is first pre-treated in a hydrolysis and fermentation bioreactor. Roghair and colleagues [68] used a batch hydrolysis and fermentation reactor operated at a pH of 5.5 and retention time of 18 days to acidify food waste prior to feeding to an MCFA-producing bioreactor. In total, 28% of the COD was converted to MCFAs with a 4 day retention time, but decreasing the retention time to 1 day reduced MCFA production [68]. Reddy et al. performed batch tests with and without bioaugmentation with *C. kluyveri* and found that the augmentation of *C. kluyveri* improved MCFA production [69]. Contreras-Davila et al. enriched a bioreactor that produced lactate as an intermediate to MCFAs, and the microbial community was dominated by species related to the genera *Lactobacillus* and *Caproicproducingens* [70]. While lacking the high lignocellulosic content of OFMSW, the production of MCFAs from food waste still has challenges, including the de-packaging of abundant food wastes (such as those obtained from a grocery store). High protein content is also a concern due to potential ammonia toxicity as amino acids are degraded.

3.3. Lignocellulosic Biomass (LCB)

LCB feedstocks include the non-grain parts of crops (such as corn-stover), non-food plants (such as switchgrass), and paper. Lomkar, Fu and Holtzapple showed the production of C6 from a mixture of chicken manure and shredded paper by adding supplemental ethanol in batch experiments [71]. Scarborough, et al. demonstrated the production of C6 and C8 from the stillage (the material remaining

after distillation) of a switchgrass biorefining process [72]. The lignocellulosic stillage was rich in xylose and complex carbohydrates, and lactate accumulated as an intermediate upon spike-feeding the reactor with stillage. Subsequent multi-omic studies suggested that two organisms, *Ca. Weimeria bifida* and *Ca. Pseudoramibacter fermentans*, expressed genes for producing MCFAs [62,85]. Metabolic modeling, however, suggested that all of the MCFAs were produced by *Ca. Weimeria bifida* from xylose, while *Ca. Pseudoramibacter bifida* was predicted to produce C4 [86]. Other studies have explored the use of grass directly [73,74]. Weimer, Nerdahl and Brandl achieved a high conversion of COD (35%) with milled grass and ethanol using a microbiome from cow rumen fluid augmented with *C. kluyveri* [73]. Khor et al. used a two-stage process in which grass was first fermented to lactate using a leach bed reactor [74]. Leach bed fermenter effluent was converted to C6 in a microbial community enriched in *Clostridium*-related organisms. MCFAs were extracted via electrolytic extraction and Kolbe electrolysis was used to convert the enriched caproate stream into dodecane, which can be used as a liquid transportation fuel [74]. MCFA production from corn silage (partly fermented corn stover commonly used as animal feed) has also been demonstrated [75]. Corn stover hydrolysate (produced from the chemical depolymerization and enzymatic hydrolysis of corn stover) has also been converted to MCFAs using a pure culture of *M. elsdenii* [37]. The high COD and carbon content of LCB feedstocks make them attractive for MCFA production. Further, integrating MCFA production into next generation biorefineries may provide additional sources of revenue.

3.4. Ethanol, Brewery, Distillery, and Winery Waste (EBDWW)

EBDWW includes high-strength organic streams containing stillage (material remaining after distillation), yeast biomass (trub, dregs, or lees), and spent grains. EBDWW can contain high amounts of ethanol, which is a well-studied electron donor for MCFA production [10]. Multiple studies have investigated MCFA production from beer and/or stillage from grain-based ethanol biorefineries [53,64,75,76]. Andersen et al. produced C6 and C8 from beer and stillage derived from wheat-based ethanol production. The production of C10 was also reported, and the microbial community was enriched in *Lactobacillus* and *Clostridium*-related organisms [53]. While ethanol was provided in the feedstock (targeting an influent concentration of 6 g/L under steady-state conditions), lactate was also predicted to be an important electron donor produced as an intermediate from carbohydrates [56]. Urban et al. converted 58% of the COD in corn beer to MCFAs, extracted the MCFAs with pertraction, and converted MCFAs to alkanes using Kolbe electrolysis [75]. Similar to work by Khor et al. [74], Urban et al. demonstrated the complete conversion of a complex organic material to alkanes with MCFAs as an intermediate. Carvajal-Arroyo et al. implemented a granular sludge process to convert 29% of COD in stillage to MCFAs [76]. The resulting granules were enriched in *Ruminococcaceae*-related organisms. Andersen et al. converted stillage to a mixture of VFAs—including a minor amount of C6—in a bioreactor integrated with electrolytic extraction. Electrolytic extraction has the advantage of removing the MCFA products while also supplying H₂ as an electron donor and consuming H⁺ to reduce or eliminate the need for the addition of pH control chemicals [64]. Wu et al. used an up-flow granular sludge reactor to convert 77% of the COD in liquor-making wastewater to MCFAs (77%). Zhu et al. used a recently isolated MCFA-producing strain (*Ruminococcaceae* CPB6) and converted 51% of COD in lactate-rich liquor-making wastewater to MCFAs [50]. Kucek et al. also achieved high conversion efficiencies from wine lees (a waste stream from wine production containing yeast biomass) using a bioreactor equipped with a pertraction system. In summary, EBDWW feedstocks are attractive due to their high organic content and availability of electron donors, including ethanol and lactate, which can drive MCFA production from the carbohydrates remaining in the waste stream.

3.5. Dairy Processing Wastewater (DPW)

Cheese, milk and yogurt processing produce low-value streams that are rich in organic materials. DPW can be rich in simple substrates, such as lactose, that have the potential for many biorefining applications. Whey, a dairy-derived protein, is also abundant. Three known studies have investigated

the use of DPW for MCFA production. Using acid whey from yogurt production, Xu et al. used a two-stage, temperature-phased process to produce MCFAs from acid whey. The MCFA production reactor was coupled to a pertraction system, and a sample from the first stage fermentation was diluted to maintain target organic loading rates. This system achieved a 61% conversion of COD and the highest production rates of MCFAs reported in the literature thus far [79]. The high production rates are likely due to an easily-degradable substrate, pre-fermentation to produce lactic acid, the use of an up-flow filtration reactor to decouple solids retention time from the hydraulic retention time, and the removal of MCFAs with pertraction to prevent product inhibition. In another study, acid whey from the production of quark (a curd cheese product) was used as a feedstock to produce C6 with an up-flow sludge-blanket reactor [80]. As with other studies, the microbial community was enriched in *Ruminococcaceae*-related organisms [80]. In a third study, the feedstock was created by mixing cheese whey with water [81]. This study provided a robust analysis of different operational parameters, including the HRT and the organic loading rate. The biofilm generated in the up-flow reactor was enriched in microbial communities, including organisms related to *Lactobacillus*, *Clostridium*, and *Ruminococcaceae* [81]. DPW are an attractive substrate due to their high content of simple substrates, including sugars (such as lactose) and lactate.

3.6. Manure

To our knowledge, there has been only one published study investigating manure as a substrate for MCFA production. Zhang et al. used a two-stage batch system to convert swine manure to MCFAs [82]. The swine manure contained 37% solids, and the solids were comprised of 25% hemicellulose, 20% protein, 18% cellulose, and 6.5% fat. Diluted manure was used as the feedstock, and ethanol was added as a supplemental electron donor. A COD conversion of 28% was achieved, but this required a long duration of 76 days. Still, this study demonstrates that manure may be a suitable substrate for MCFA production.

3.7. Ideal Feedstocks and Processes for MCFA Production

Past work shows that MCFAs can be produced from a wide variety of feedstocks (Table 3). Supplemental electron donors, such as ethanol, can be added to potentially increase MCFA yield, but the addition of ethanol results in significant economic and environmental costs [87]. Feedstocks containing easily degradable materials—such as DPW, containing high amounts of sugar—may not require supplementation with an electron donor. Further, some feedstocks—such as EBDWW—may be rich in electron donor materials, such as lactate or ethanol. A variety of reactor configurations have also been tested. Pretreatment with microbial hydrolysis and acidification may improve MCFA yields for some substrates, and the real-time removal of products via pertraction can greatly increase production rates. However, there is still much work required to optimize reactor systems, based on cost and productivity, for MCFA production.

4. Metabolic Features of Medium-Chain Fatty Acid Production

The primary route for MCFA production is believed to be reverse β -oxidation. β -oxidation is a route of fatty acid catabolism, and metabolic engineering to reverse this pathway in model organisms has been suggested [88]. Certain fermenting organisms, however, have long been known to use reverse β -oxidation as a strategy for redox balancing. Here, we focus on the natural metabolism of MCFA-producing bacteria described through various phenotypic and genomic studies.

4.1. Reverse β -Oxidation

MCFAs are proposed to be produced by reverse β -oxidation (Figure 2) [10,89]. The cycle is initiated by an acyl-CoA C-acyl transferase (E.C. 2.3.1.16, E.C. 2.3.1.9) that combines acetyl-CoA with an acyl-CoA (e.g., acetyl-CoA, propionyl-CoA, butyryl-CoA, valeryl-CoA, hexanoyl-CoA). In the second step, the 3-hydroxyacyl-CoA is reduced via 3-hydroxyacyl-CoA dehydrogenase (E.C. 1.1.1.157, E.C. 1.1.1.35) with electrons from NADH. In the third step, enoyl-CoA hydratase (aka, crotonase; E.C. 4.2.1.55, E.C. 4.2.1.17) creates a double bond between the second and third carbons creating

an enoyl-CoA. In the fourth step, an electron-bifurcating acyl-CoA dehydrogenase (E.C. 1.3.1.109) oxidizes two molecules of NADH, with one electron pair being transferred to the enoyl-CoA and one being transferred to ferredoxin, producing reduced ferredoxin (Fd_{RED}) and an acyl-CoA [90]. The electron-bifurcating acyl-CoA dehydrogenase complex relies on two electron transfer flavoproteins (EtfA and EtfB) to mediate the transfer of electrons [91]. In total, reverse β -oxidation increases the length of an acyl-CoA by two carbons, while oxidizing three molecules of NADH to NAD^+ and reducing one molecule of oxidized ferredoxin (Fd_{OX}) to Fd_{RED} .

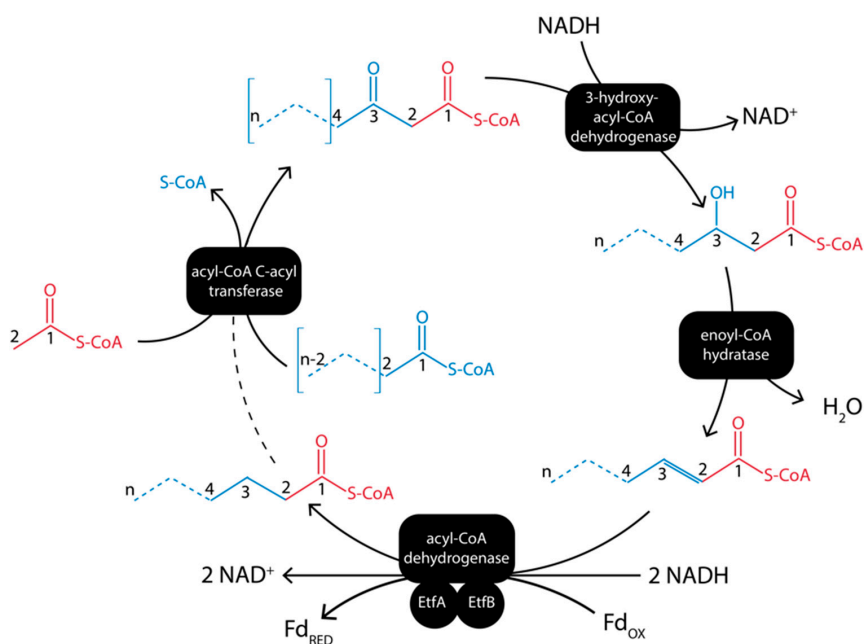


Figure 2. Reverse β -oxidation with an electron-bifurcating acyl-CoA dehydrogenase.

4.2. Terminating Reverse β -Oxidation

Reverse β -oxidation produces a CoA-ligated carboxylate (Figure 2). The terminal step of reverse β -oxidation is the cleaving of the carboxylate from CoA. Acyl-CoA hydrolases (E.C. 3.1.2.20) use water to cleave the CoA from the acid (Figure 3). A CoA transferase (E.C. 2.8.3.8) transfers a CoA from one acid to another. The replacement of the CoA with phosphate acyl transferases (e.g., phosphate butyryl transferase, E.C. 2.3.1.19) and subsequent ADP phosphorylation via a kinase (e.g., butyrate kinase, E.C. 2.7.2.7) have also been proposed [92].

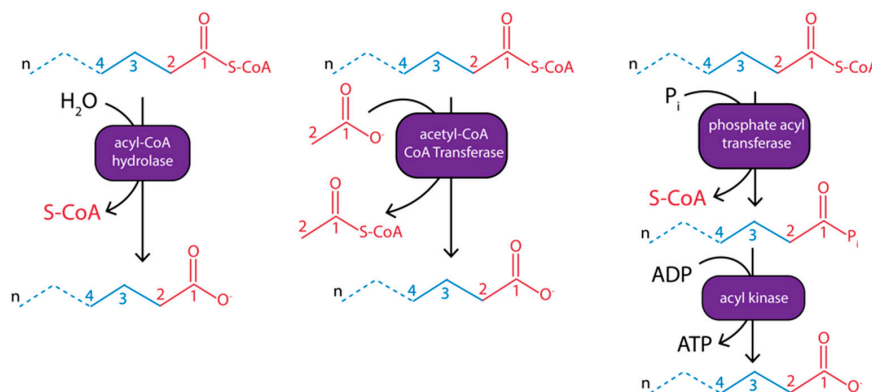


Figure 3. Terminal enzymes for hydrolyzing the CoA-ligated products of reverse β -oxidation to release a carboxylate and CoA.

4.3. Energy-Conservation Coupled with Reverse β -Oxidation

Reverse β -oxidation does not directly produce ATP. Instead, organisms are predicted to use one or more enzymes to couple highly exergonic redox reactions to the generation of an ion motive force (IMF). The IMF can be used to generate ATP via a membrane-bound ATP synthase. One example is the *Rhodobacter* nitrogen fixation (RNF) complex (aka, ion-translocating ferredoxin NAD⁺ oxidoreductase, E.C. 7.2.1.2) (Figure 4). The RNF complex couples the reduction of NAD⁺ by Fd_{RED} with the translocation of proton or sodium ions across the cell membrane to generate IMF. This enzyme complex has been found in many fermenting bacteria, as well as methanogenic archaea [93–96]. The catalyzed reaction is reversible and the RNF complex plays a role in maintaining ratios of intracellular NADH and Fd_{RED} [97]. Another example is a membrane-bound energy-conserving hydrogenase (ECH) that couples the reduction of protons by Fd_{RED} with IMF generation. Regardless of the route of IMF generation, Fd_{RED} (a highly electronegative electron carrier) is needed to generate IMF. Therefore, the electron-bifurcating acyl-CoA dehydrogenase (Figure 2) is key to producing energy from reverse β -oxidation.

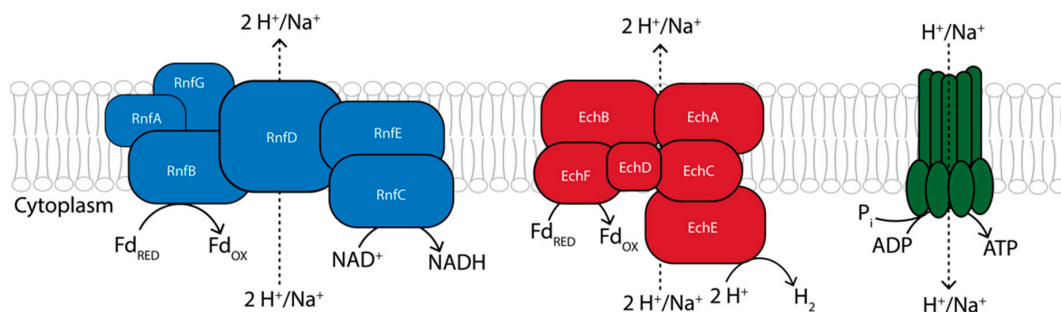


Figure 4. Membrane-bound enzymes for energy conservation in MCFA-producing bacteria. The RNF complex couples the reduction of NAD⁺ with Fd_{RED} with ion translocation to generate an ion motive force. Similarly, energy-conserving hydrogenase couples the reduction of protons with Fd_{RED} to ion translocation. The ion motive force is consumed by ATP synthase to produce ATP.

4.4. Additional Electron Bifurcating Enzymes for Energy Conservation and Redox Management

Other enzymes are proposed to conserve energy by producing or conserving Fd_{RED} to use for IMF generation [98]. One example is NAD(P)⁺ transhydrogenase (Nfn) (Figure 5), which transfers electrons from NADH and Fd_{RED} to NADP⁺ and can be used in reverse to produce Fd_{RED} and NADH from NADPH [26]. Another example is HydABC (Figure 5), which produces H₂ through the bifurcation of electrons from Fd_{RED} and NADH [99]. While Nfn produces Fd_{RED} directly, HydABC decreases the amount of Fd_{RED} needed for H⁺ reduction compared to ferredoxin hydrogenase (Hyd1, E.C. 1.12.7.2) (Figure 5). Alternatively, HydABC may be used in reverse for the production of Fd_{RED} and NADH from H₂.

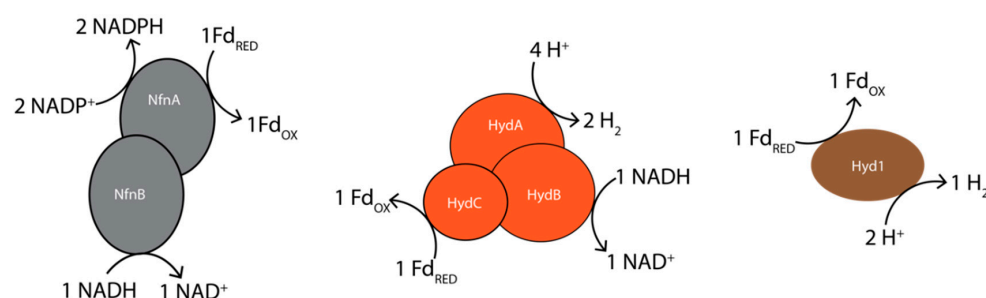


Figure 5. Additional enzymes for redox balancing in MCFA-producing bacteria. NfnAB couples NADH and Fd_{RED} oxidation with NADP⁺ reduction. HydABC relies on electrons from both NADH and Fd_{RED} to reduce protons. A ferredoxin hydrogenase uses only Fd_{RED} for hydrogen production.

4.5. Hypothesized Routes for MCFA Production from Ethanol with and without Acetate as an Electron Acceptor

To illustrate how bacteria tie MCFA production to energy conservation, we considered the well-studied example of ethanol and C2 conversion to MCFAs [23,89,98]. Ethanol conversion with reverse β -oxidation is well described in *C. kluyveri*, and the metabolism has been reconstructed for C4 and C6 production from the co-utilization of ethanol and acetate [10,23]. One example of this overall transformation is the conversion of 1 mol C2 and 2 mols ethanol to C6 (Figure 6). In our example, we assumed C2 is incorporated via acetyl-CoA CoA transferase (Figure 3). Under this condition, C6 production results in the production of 1 mol of ATP (0.5 mol ATP per mol ethanol). Under this condition, no H_2 is predicted to be produced. The ratio of ethanol-to-C2, however, is not fixed and H_2 is predicted as a product when the molar ratio of ethanol-to-C2 is greater than 2:1 (Figures S1–S13).

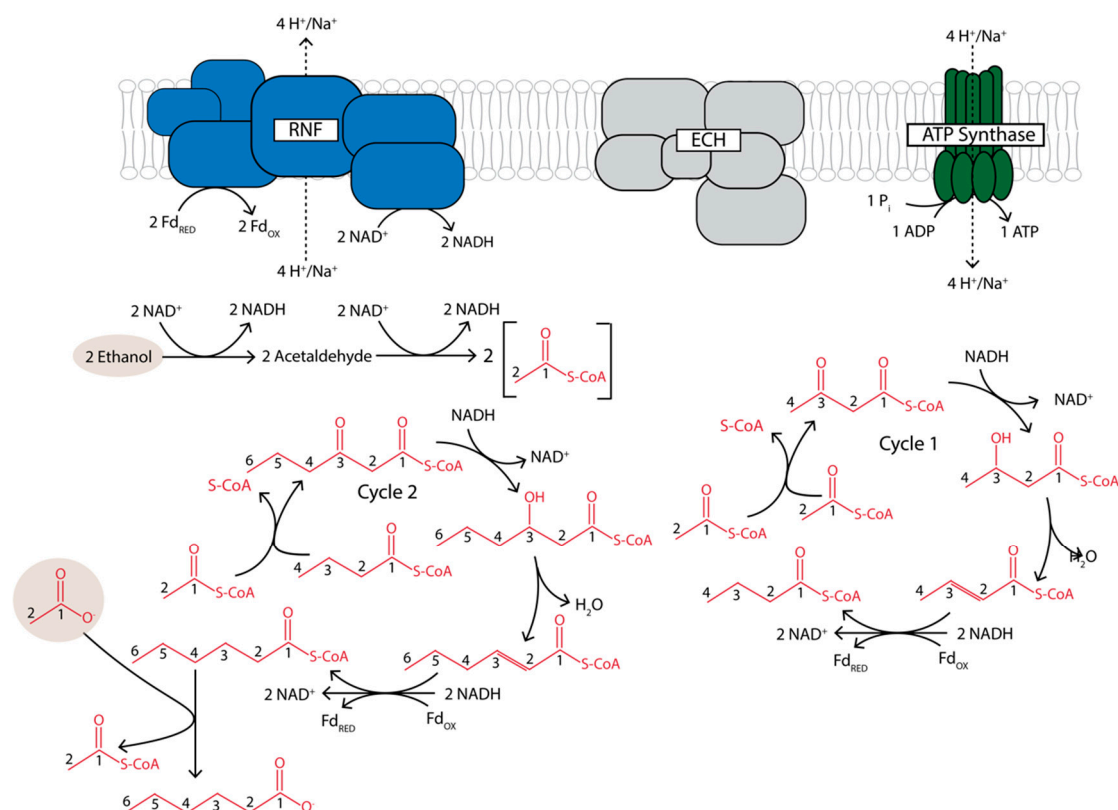


Figure 6. An example model of C6 production from ethanol (2 mols) and acetate (1 mol) without production of H_2 .

While ethanol and acetate can be used as co-substrates, acetate is not required as an electron acceptor for converting ethanol to MCFAs [100]. When 2 mols of ethanol are converted to acetyl-CoA, a total of 4 mols NADH are predicted to be produced (Figure 7). One cycle of reverse β -oxidation consumes 3 mols NADH, leaving 1 mol NADH and 1 mol Fd_{RED} . There are multiple potential ways to maintain redox balance, depending on the hydrogenase present. With ECH, Fd_{RED} could reduce H^+ to H_2 while producing IMF (Figure 7). IMF can then be consumed by the RNF complex to transfer electrons from the remaining NADH to Fd_{RED} , in order to recycle NAD^+ . Fd_{RED} can also be used via ECH to generate IMF that drives ATP synthase. In this model (Figure 7A), 0.5 mol ATP are predicted (0.25 mol ATP per mol of ethanol). If 3 moles of ethanol are consumed, then six NADH are produced (Figure 7B). Two cycles of reverse β -oxidation would consume all six NADH and produce 2 mols Fd_{RED} . Then, the 2 mols Fd_{RED} could be used to generate IMF with ECH, resulting in the net production of 1 mol ATP (0.333 mol ATP per mol of ethanol consumed). Thus, the production of C6 is predicted to increase the ATP yield by 33% per mol ethanol compared to C4 production. These two scenarios

illustrate how MCFA production can increase ATP production compared to the production of C4, but the overall ATP production is lower than when acetate is used as an electron acceptor (Figure 6).

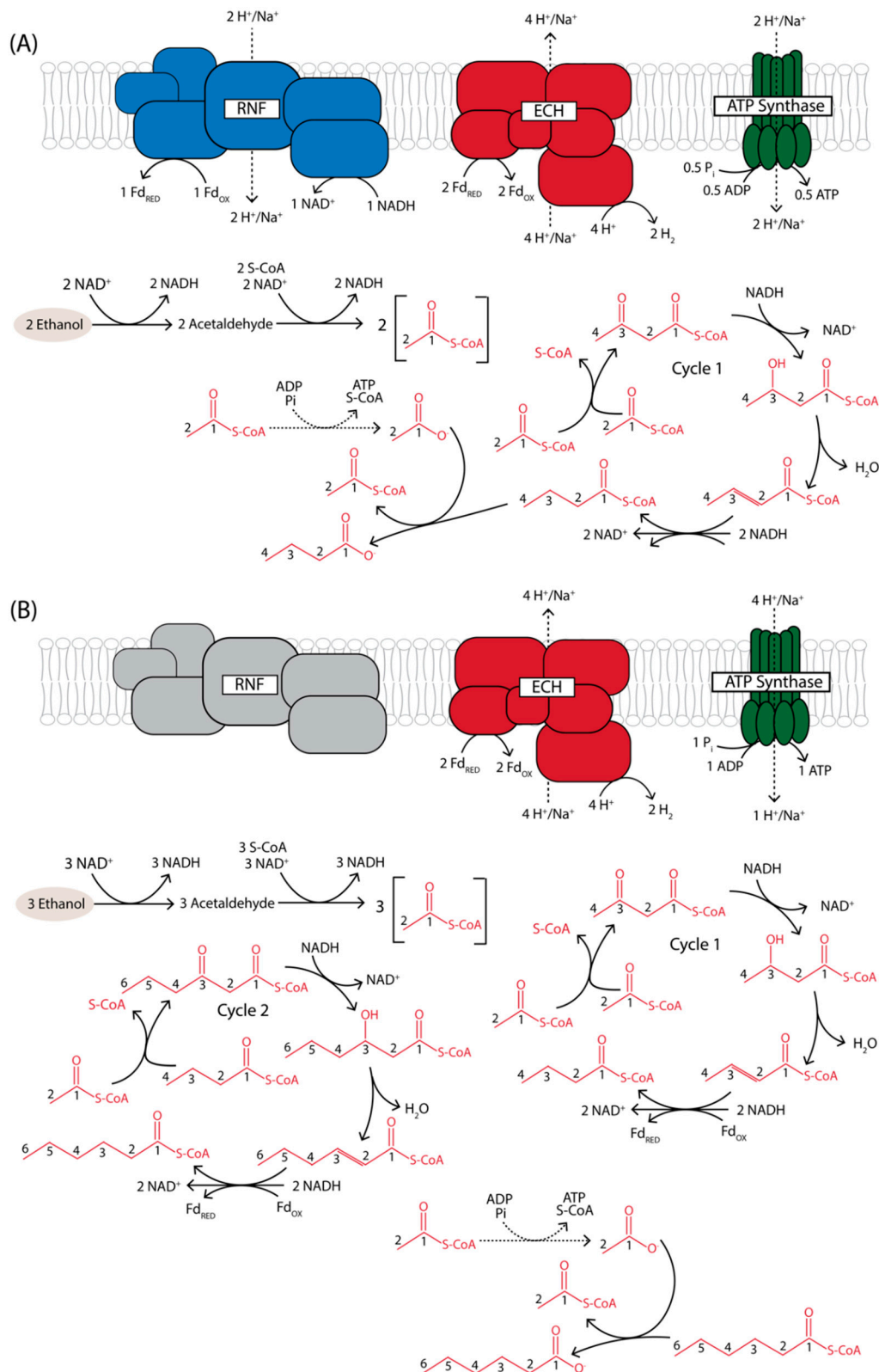
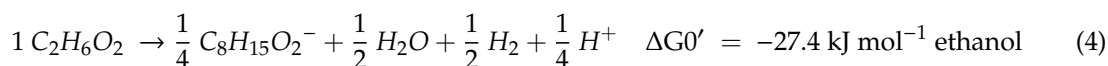
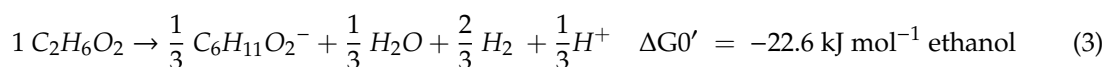


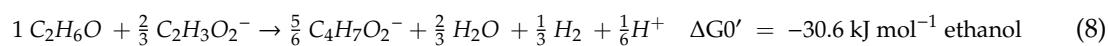
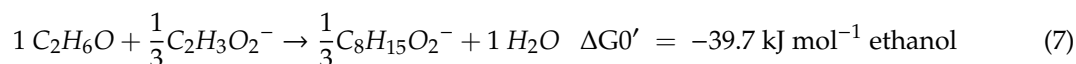
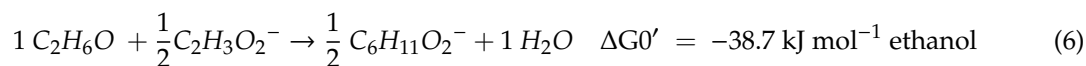
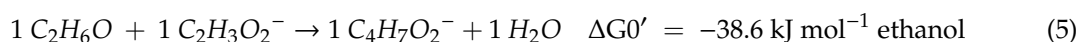
Figure 7. Example models of C4 production (A) and C6 production (B) when ethanol is used as a sole substrate without acetate as an electron acceptor. The conversion of C6 is predicted to increase the ATP yield per mol of ethanol by 33%.

4.6. Thermodynamic and Energetic Drivers of MCFA Production

The energetic drivers of MCFA production have been explored [101], but recent discoveries of novel energy-conserving mechanisms in anaerobic bacteria suggest that energetic drivers are not fully elucidated [90]. To illustrate the potential energetic drivers of MCFA-production, we considered the conversion of ethanol to MCFAs. All free energies of formation were assumed based on previously described methods [85]. When consuming ethanol as a sole substrate, H₂ is predicted to be produced for all scenarios, and it is shown that the amount of H₂ produced decreases with the length of the product (Equations (1)–(4)). The reaction depicted in Equation (3) was also described in Angenent et al. [10].



The co-consumption of ethanol and acetate reduces the need for H₂ production (Equations (5)–(8)) as acetate can accept electrons that would otherwise be used to produce H₂. When consuming 1 mol C₂ per mol of product (Equations (5)–(8)), no H₂ is predicted. The ratios of ethanol-to-C₂, however, are not fixed and the utilization of 3 mols ethanol to 2 mols acetate has been described previously for *C. kluyveri* [10]. Many ethanol-to-C₂ ratios have been tested, and high ratios of ethanol to acetate have been shown to favor MCFA production [100].



Metabolic reconstructions of ethanol consumption predict that hydrogen-producing enzymes play a role in determining the most energetically favorable products (Table 4). If C₂ is produced from ethanol, no net ATP production is predicted with Hyd1. With ECH, up to 1.0 mol ATP per mol ethanol is predicted, but the amount of free energy needed to generate this much ATP is only feasible at H₂ partial pressures below 10⁻⁵ atm, assuming a minimum quantum of -50 kJ per mol ATP [102] (Figure 7). With Hyd1, longer products are predicted to increase the ATP yield. With ECH, shorter products increase the ATP yield, but these scenarios all rely on low H₂ partial pressures for the predicted ATP production to be feasible (Figure 7). The co-utilization of C₂ can obviate the need for H₂ production, and the predicted ATP yield (0.5 mol ATP per mol ethanol) when no H₂ is produced is higher than all scenarios using ethanol as a sole substrate and Hyd1 as the hydrogenase. If Hyd1 is the only hydrogenase available, co-utilization of C₂ with ethanol is always predicted to produce more ATP per mol ethanol than consumption of ethanol as a sole substrate, and all products result in the same ATP yield (0.5 mol ATP per mol ethanol). However, if ECH is available, the ATP yield is higher if ethanol is the sole substrate, but the overall free energy release is lower per mol ATP produced. This suggests that ethanol consumers with ECH may only co-utilize acetate under high H₂ partial pressures.

Table 4. Predicted ATP production and free energy release for the conversion of ethanol to acetate, butyrate, hexanoate, and octanoate with either a ferredoxin hydrogenase (Hyd1) or energy-conserving hydrogenase (ECH).

| Ethanol: Acetate | Product | Hydrogenase | ATP/mol Ethanol | | | ΔG^0 /mol ATP | Model |
|---------------------|---------|-------------|------------------|-------------------|-------|--------------------------|------------|
| | | | SLP ¹ | ATPS ² | Total | | |
| 1:0 | C2 | Hyd1 | 1.00 | −1.00 | 0 | − | Figure S1 |
| 1:0 | C4 | Hyd1 | 0.500 | −0.250 | 0.250 | −58.0 | Figure S2 |
| 1:0 | C6 | Hyd1 | 0.333 | 0 | 0.330 | −68.5 | Figure S3 |
| 1:0 | C8 | Hyd1 | 0.250 | 0.125 | 0.375 | −73.1 | Figure S4 |
| 1:0 | C2 | ECH | 1.00 | 0 | 1.00 | +9.65 | Figure S5 |
| 1:0 | C4 | ECH | 0.500 | 0.250 | 0.750 | −19.3 | Figure S6 |
| 1:0 | C6 | ECH | 0.333 | 0.333 | 0.678 | −33.7 | Figure S7 |
| 1:0 | C8 | ECH | 0.250 | 0.375 | 0.625 | −43.8 | Figure S8 |
| 1:1 | C4 | None | 0 | 0.500 | 0.500 | −65.4 | Figure S9 |
| 2:1 | C6 | None | 0 | 0.500 | 0.500 | −77.4 | Figure S10 |
| 3:1 | C8 | None | 0 | 0.500 | 0.500 | −79.4 | Figure S11 |
| 3:2 | C4 | Hyd1 | 0.167 | 0.250 | 0.417 | −73.4 | Figure S12 |
| 3:2 | C4 | ECH | 0.167 | 0.417 | 0.584 | −52.4 | Figure S13 |

¹ The ATP predicted to be produced from substrate-level phosphorylation (SLP). ² The ATP predicted to be produced via ATP synthase (ATPS).

To further illustrate the potential importance of H₂ and hydrogenase enzymes, we performed a thermodynamic analysis to assess the feasibility of a subset of predictions (Table 4) with varying conditions of H₂ partial pressure. We considered either Hyd1 or ECH as the sole enzyme for hydrogen production. All products are predicted to result in thermodynamically favorable reactions up to a partial pressure of 10^{−1} atm (Figure 8A). At H₂ partial pressures greater than 10^{−1} atm, the production of acetate as the sole product is predicted to be infeasible ($\Delta G^0 > 0$). Because the overall reaction is the same regardless of the type of hydrogenase assumed, there is only one line per product shown in Figure 8A. We then incorporated the predicted ATP yields from our metabolic reconstructions (Figure 8B). With ECH, the predicted free energy release per mol ATP is greater than −50 kJ at increasing H₂ partial pressures for each potential product (C2 = 10^{−5} atm; C4 = 10^{−4} atm; C6 = 10^{−3} atm; C8 = 10^{−1} atm). This suggests that H₂ partial pressure may be a control to drive the production of MCFAs if ECH is used as a hydrogenase. With Hyd1, the production of acetate from ethanol is predicted to produce a net of 0 ATP in order to maintain redox balance (Figure S1). ATP predictions from all other products are feasible up to a H₂ partial pressure of 3 atm, indicating that H₂ partial pressure may not be an appropriate control to drive MCFAs production if Hyd1 is the hydrogenase.

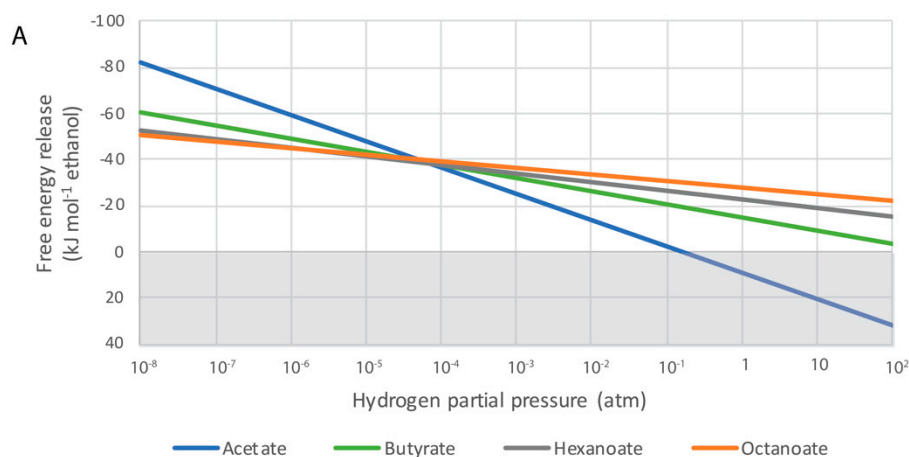


Figure 8. Cont.

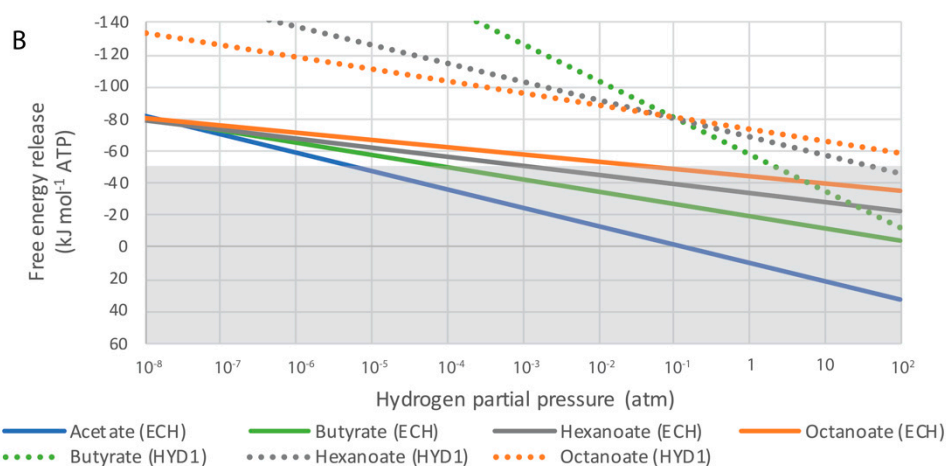


Figure 8. Free energy release for different products from ethanol under a range of H_2 partial pressures. Plots show the overall free energy release (A) and the free energy release per mol of ATP (B). Areas shaded in gray are considered infeasible and the dashed lines represent scenarios with Hyd1 as a hydrogenase instead of ECH.

5. Path Forward

Taken together, the metabolic reconstructions and thermodynamic analysis suggests that (1) the most energetically advantageous products depend on enzymes other than those directly performing reverse β -oxidation, and (2) the infinite number of combinations of substrate ratios and product formation make simulating MCFA production a challenge. Still, as we learn more about the behavior of microbes producing MCFAs and how these microbes interact with complex microbial communities, we should improve our ability to design, diagnose, and control MCFA-producing bioreactors. We have identified specific knowledge gaps below.

5.1. Controlling Product Length

Controlling the terminal product length remains a challenge. While we have illustrated that some conditions should favor the production of MCFAs over C2 and C4, almost all studies to date create mixtures of end products, varying in length from C2 to C6 or C8 (Table 3). The highest specificities of C6 and C8 production have resulted from the incorporation of pertraction systems [103]. This real-time removal of C6 and C8 is thought to increase their production in part because the protonated forms of MCFAs are toxic [104–106]. Thus, their production may be limited by their toxic effects. While there has been recent work on the toxic effects of these compounds in MCFA-producing bioreactors [107], an improved understanding of the toxicity of these products is needed. Quantifying these effects, for instance via Monod-based inhibition terms, would be particularly valuable for modeling the impacts of MCFA toxicity and the favorable production of specific carbon chain lengths across various systems.

Enzyme specificity is also expected to control the length of the terminal product. The enzymes directly involved in reverse β -oxidation (Figure 2) and the termination of reverse β -oxidation (Figure 3) are all likely to play a role in dictating the predominant product length. Individual enzymes of the fatty acid biosynthesis (Fab) pathway are well characterized, and metabolic engineering approaches have been used to produce specific chain lengths [108,109]. Additional kinetic studies of reverse β -oxidation enzymes can inform modeling approaches, and structural studies could enable metabolic engineering to improve enzyme specificity towards target products. Genetically tractable MCFA-producers, however, are only beginning to emerge [36]. As metabolic engineering tools continue to improve and additional MCFA-producing isolates become available, isolate-based studies should elucidate strategies to improve the control of target chain lengths.

5.2. Microbial Community Assembly, Function, and Resiliency

The many microbial community-based studies for MCFA production have elucidated common functional guilds that become enriched in these communities, while phylogenetically distinct, functional roles tend to be conserved. As an example, several reactors fed carbohydrates have been enriched in *Lactobacillus* species and lactate-consuming MCFA producers. A framework for these functional guilds was recently proposed [86].

Further, these microbial communities have demonstrated resilience (sustained production of MCFAs) when operated as open cultures, often fed with non-sterile feedstocks. This resilience may be due to the presence of MCFAs, which select for only organisms with some tolerance to these products. In addition, although these communities contain organisms that rely on oxygen-sensitive ferredoxin [110], they survive in conditions with feedstocks that likely contain small amounts of oxygen. Thus, we predict that facultative anaerobes (such as *Lactobacillus*-related organisms) may play a role in quenching oxygen. When separating stages of fermentation (e.g., two-stage processes, Figure 1) or implementing pertraction, it is important to consider the traits that may make these microbial communities resilient.

5.3. Product Uses

MCFA production from renewable feedstocks may allow for reduced dependence on palm refining and fossil fuels. While MCFAs currently have high market prices, it is expected that the widespread production of MCFAs would reduce their value. Thus, more work on refining MCFAs to other products is needed. Kolbe electrolysis has already been demonstrated as a potential option to convert MCFAs to alkanes for use as a liquid transportation fuel. Another option may be to purify MCFAs and use them as a feed supplement. MCFAs are expected to have many of the same potential benefits as MCTs and may have potential as a dietary supplement for both humans and animals. More work on the role of MCFAs as a platform chemical is needed, and this work should consider the potential societal and environmental benefits along with the economic benefits. Thus, emphasis on life cycle assessment of MCFA-production is needed, and the study performed by Chen et al. provides a good example [87]. If implemented with sustainable approaches, MCFA production from organic wastes will be a step towards local circular bioeconomies which are critical for addressing persistent environmental and social challenges [111].

Supplementary Materials: The following are available online at <http://www.mdpi.com/2227-9717/8/12/1571/s1>, Figure S1: Proposed model for conversion of ethanol to acetate with Hyd1, Figure S2: Proposed model for conversion of ethanol to butyrate with Hyd1, Figure S3: Proposed model for conversion of ethanol to hexanoate with Hyd1, Figure S4: Proposed model for conversion of ethanol to octanoate with Hyd1, Figure S5: Proposed model for conversion of ethanol to acetate with ECH, Figure S6: Proposed model for conversion of ethanol to butyrate with ECH, Figure S7: Proposed model for conversion of ethanol to hexanoate with ECH, Figure S8: Proposed model for conversion of ethanol to octanoate with ECH, Figure S9: Proposed model for conversion of ethanol and acetate to butyrate, Figure S10: Proposed model for conversion of ethanol and acetate to hexanoate, Figure S11: Proposed model for conversion of ethanol and acetate to octanoate, Figure S12: Proposed model for conversion of ethanol and acetate to butyrate with an ethanol-to-acetate ratio of 3:2 and Hyd1, Figure S13: Proposed model for conversion of ethanol and acetate to butyrate with an ethanol-to-acetate ratio of 3:2 and ECH.

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