processes

MDPI

Review

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

Panagiota Stamatopoulou, Juliet Malkowski, Leandro Conrado, Kennedy Brown and Matthew Scarborough *

Department of Civil and Environmental Engineering, University of Vermont, Burlington, VT 05405, USA; Panagiota.Stamatopoulou@uvm.edu (P.S.); Juliet.Malkowski@uvm.edu (J.M.); Leandro.Fernandes@uvm.edu (L.C.); Kennedy.Brown@uvm.edu (K.B.)

* Correspondence: Matthew.Scarborough@uvm.edu and mscarbor@uvm.edu

Received: 14 October 2020; Accepted: 23 November 2020; Published: 28 November 2020

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 O_2 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. kluveryi*, 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. kluveryi* and *E. limosum*, Prevot and Taffanel isolated *Ramibacteriurn 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. galactitolvorans* 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*, *Caproicproducens*, and *Caproicbacter.* 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
Clostridium kluyveri	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]
Megasphaera elsdenii	Sheep rumen	Glucose, Fructose, Maltose, Mannitol, Sorbitol, Lactate	C2, C3, C4, C5, C6, CO_2 and H_2	[14,33–35]
Ruminococcaceae bacterium CPB6	Fermentation pit	Lactate, Ethanol, Glucose	C4, C6	[14,49,50]
Caproiciproducens galactitolivorans BS-1	Wastewater treatment plant	Galactitol, C2	Ethanol, Butanol, C2, C4, C6, H_2	[52,53]
Eubacterium limosum	Pea-blanching wastes	H_2 and CO_2	C2, C4, C5, C6	[14,27,28]
Eubacterium pyruvativorans	Sheep rumen	Formate, Acetate, Propionate, iso-butyrate, C4, Lactate	C4, C5, C6	[48]
Rhodospirillum rubrum	Dead mouse	Glucose, Fructose, Sucrose, CO	C6	[10,16–19]
Pseudoramibacter alactolyticus	Lung abscess	Glucose	Formate, C2, C4, C5, C6, C8	[29–31,63]
Megasphaera hexanoica	Cow rumen	Fructose, Mannose	C2, iso-butyrate, iso-valerate, C6, H_2, CO_2, H_2S	[56,57]
Clostridium sp. BL-3	Anaerobic bioreactor	Lactate, Ethanol, Xylose, Fructose	C2, C4, iso-butyrate, C6	[59,60]
Clostridium sp. BL-4	Anaerobic bioreactor	Lactate, Ethanol, Xylose, Fructose	C2, C4, iso-butyrate, C6	[59,60]
Clostridium sp. BL-6	Anaerobic bioreactor	Lactate, Ethanol, Xylose, Fructose	C2, C4, iso-butyrate, C6	[59,60]
Caproiciproducens sp. 7D4C2	Open-culture, chain-elongating bioreactor	Glucose, Fructose	Lactate, C2, C4, C6, H_2, CO_2	[61]

3. MCFA Production form 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.

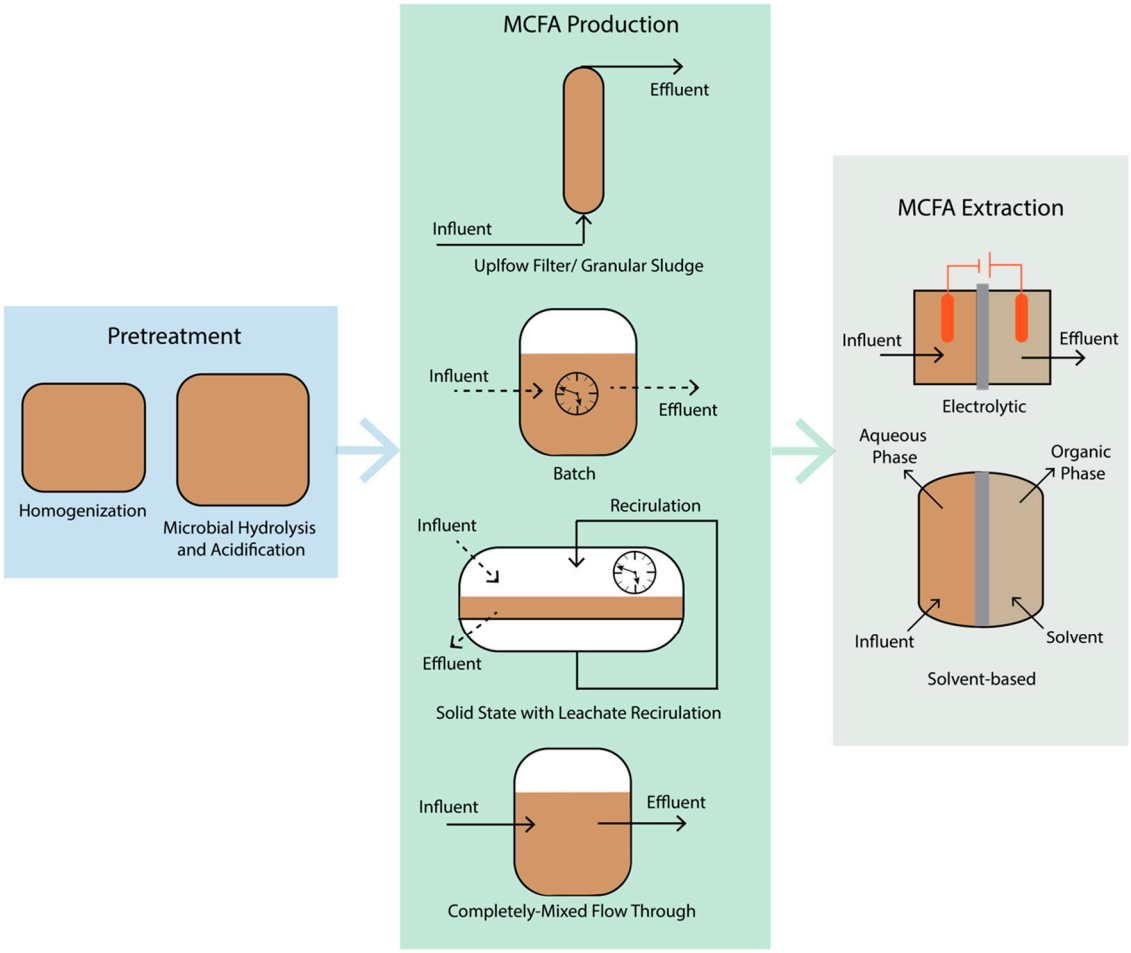

Figure 1. Processes for converting organic wastes to MCFAs. Several processes have been implemented for converting organic waste streams to MCFAs.

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, *C. kluyveri*	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, *C. kluyveri*	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	*Megasphaera elsdenii*	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	*Ruminococcaceae* 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. Base 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, H_2, or both ethanol and H_2 as supplemental electron donors in batch experiments. They found that lactate in the leachate reactor effluent was converted to C6 when H_2 or a combination of H_2 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 *Caproicproducens* [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 H_2 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-hydroxyacl-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}.

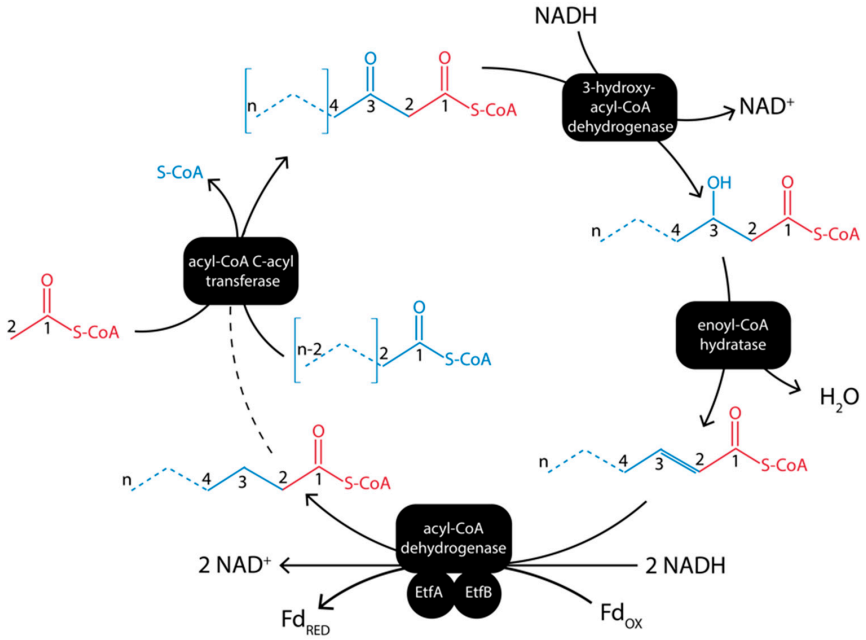

Figure 2. Reverse β-oxidation with an electron-bifurcating acyl-CoA dehydrogenase.

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].

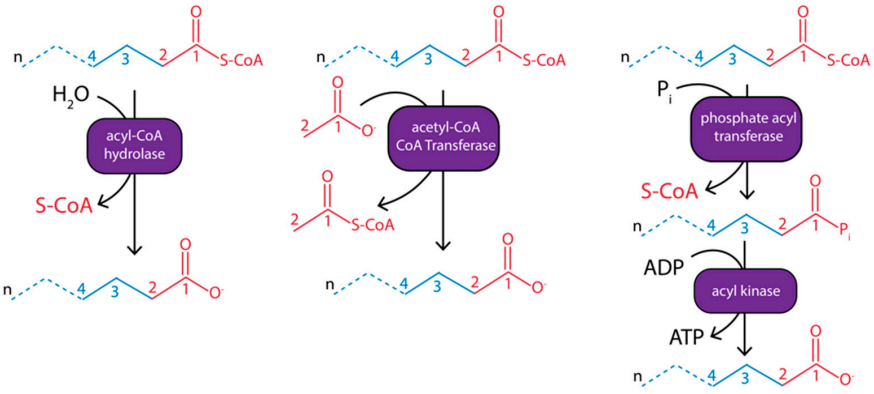

Figure 3. Terminal enzymes for hydrolyzing the CoA-ligated products of reverse β-oxidation to release a carboxylate and CoA.

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.

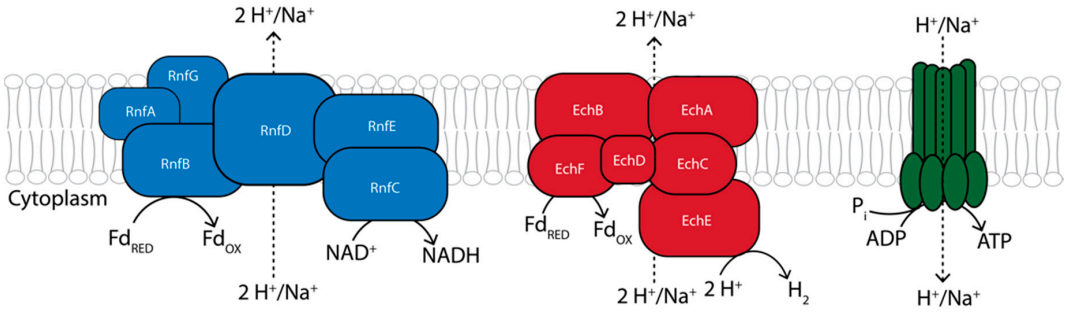

Figure 4. Membrane-bound enzymes for energy conservation in MCFA-producing bacteria. The RNF complex couples the reduction of NAD+ with Fd_{RED} with ion translocation to generate an ion motive force. Similarly, energy-conserving hydrogenase couples the reduction of protons with Fd_{RED} to ion translocation. 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 NADPH and can be used in reverse to produce Fd_{RED} and NADH from NADPH [26]. Another example is HydABC (Figure 5), which produces H_2 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 H_2.

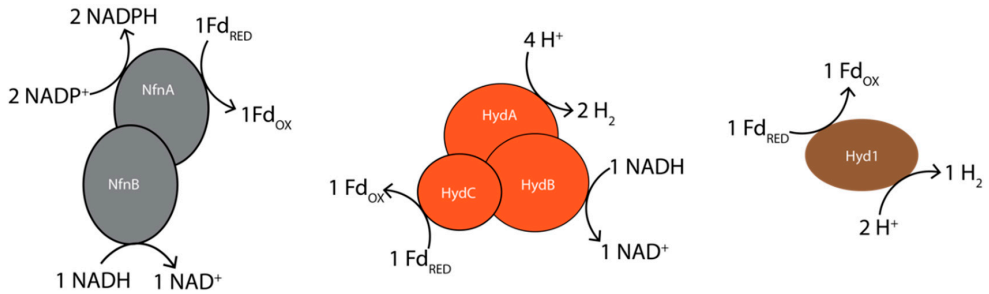

Figure 5. Additional enzymes for redox balancing in MCFA-producing bacteria. NfnAB couples NADH and Fd_{RED} oxidation with $NADP^+$ reduction. HydABC relies on electrons from both NADH and Fd_{RED} to reduce protons. A ferredoxin hydrogenase uses only Fd_{RED} for hydrogen production.

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).

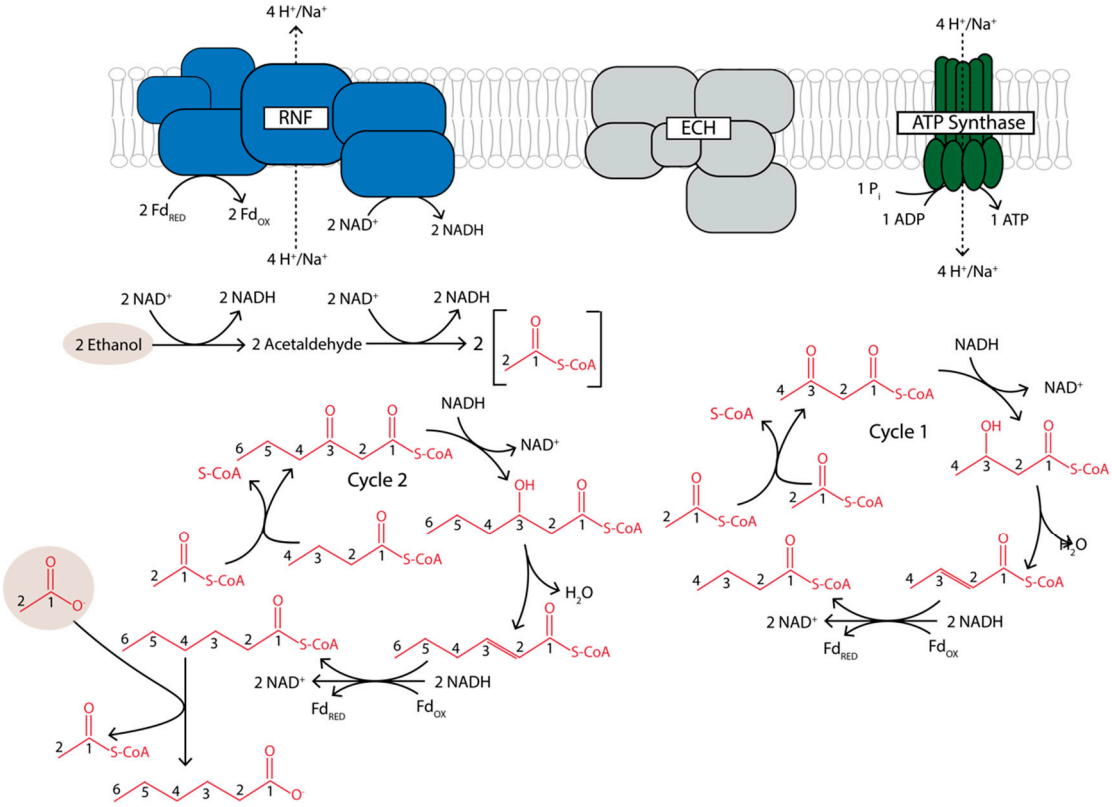

Figure 6. An example model of C6 production from ethanol (2 mols) and acetate (1 mol) without production of H_2.

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).

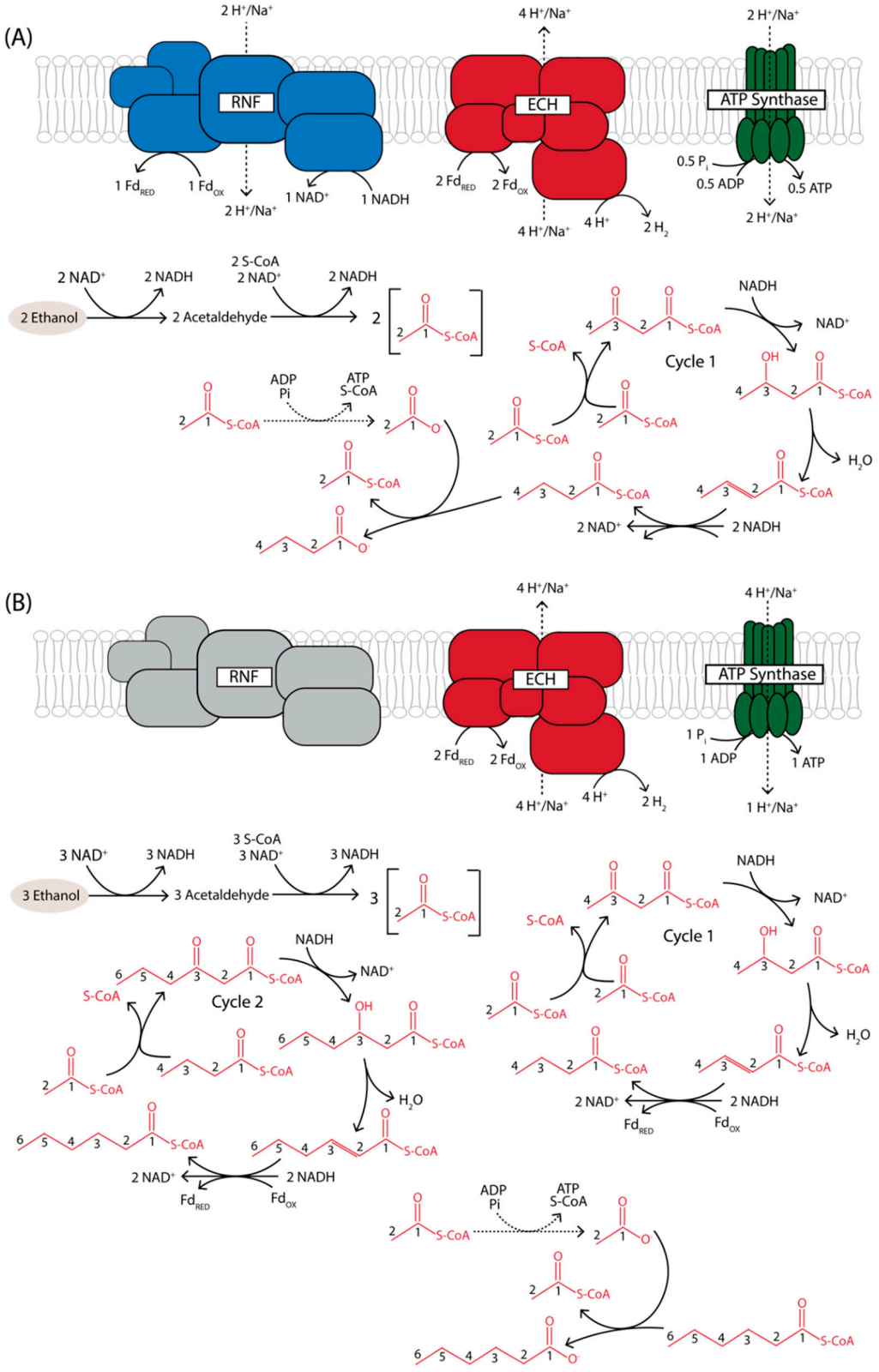

Figure 7. Example models of C4 production (**A**) and C6 production (**B**) when ethanol is used as a sole substrate without acetate as an electron acceptor. The conversion of C6 is predicted to increase the ATP yield per mol of ethanol by 33%.

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, H_2 is predicted to be produced for all scenarios, and it is shown that the amount of H_2 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].

$$1\,C_2H_6O\ +\ 1\,H_2O\ \rightarrow 1\,C_2H_3O_2^{-} + 2\,H_2 + 1\,H^{+} \quad \Delta G0' = +9.65\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{1}$$

$$1\,C_2H_6O \rightarrow \frac{1}{2}\,C_4H_7O_2^{-} + 1\,H_2 + \frac{1}{2}\,H^{+} \quad \Delta G0' = -14.5\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{2}$$

$$1\,C_2H_6O_2 \rightarrow \frac{1}{3}\,C_6H_{11}O_2^{-} + \frac{1}{3}\,H_2O + \frac{2}{3}\,H_2 + \frac{1}{3}H^{+} \quad \Delta G0' = -22.6\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{3}$$

$$1\,C_2H_6O_2 \rightarrow \frac{1}{4}\,C_8H_{15}O_2^{-} + \frac{1}{2}\,H_2O + \frac{1}{2}\,H_2 + \frac{1}{4}\,H^{+} \quad \Delta G0' = -27.4\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{4}$$

The co-consumption of ethanol and acetate reduces the need for H_2 production (Equations (5)–(8)) as acetate can accept electrons that would otherwise be used to produce H_2. When consuming 1 mol C2 per mol of product (Equations (5)–(8)), no H_2 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].

$$1\,C_2H_6O\ +\ 1\,C_2H_3O_2^{-} \rightarrow 1\,C_4H_7O_2^{-} + 1\,H_2O \quad \Delta G0' = -38.6\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{5}$$

$$1\,C_2H_6O\ +\frac{1}{2}C_2H_3O_2^{-} \rightarrow \frac{1}{2}\,C_6H_{11}O_2^{-} + 1\,H_2O \quad \Delta G0' = -38.7\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{6}$$

$$1\,C_2H_6O + \frac{1}{3}C_2H_3O_2^{-} \rightarrow \frac{1}{3}C_8H_{15}O_2^{-} + 1\,H_2O \quad \Delta G0' = -39.7\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{7}$$

$$1\,C_2H_6O\ +\frac{2}{3}\,C_2H_3O_2^{-} \rightarrow \frac{5}{6}\,C_4H_7O_2^{-} + \frac{2}{3}\,H_2O\ +\frac{1}{3}\,H_2\ +\frac{1}{6}H^{+} \quad \Delta G0' = -30.6\ \text{kJ mol}^{-1}\ \text{ethanol} \tag{8}$$

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 H_2 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 H_2 partial pressures for the predicted ATP production to be feasible (Figure 7). The co-utilization of C2 can obviate the need for H_2 production, and the predicted ATP yield (0.5 mol ATP per mol ethanol) when no H_2 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 H_2 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			$\Delta G^{0'}$/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 H_2 and hydrogenase enzymes, we performed a thermodynamic analysis to assess the feasibility of a subset of predictions (Table 4) with varying conditions of H_2 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 H_2 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 H_2 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 H_2 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 H_2 partial pressure of 3 atm, indicating that H_2 partial pressure may not be an appropriate control to drive MCFA production if Hyd1 is the hydrogenase.

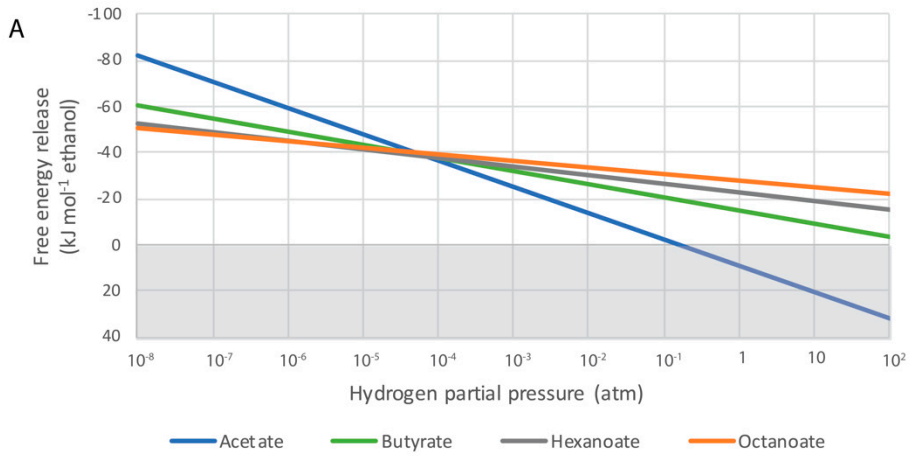

Figure 8. *Cont.*

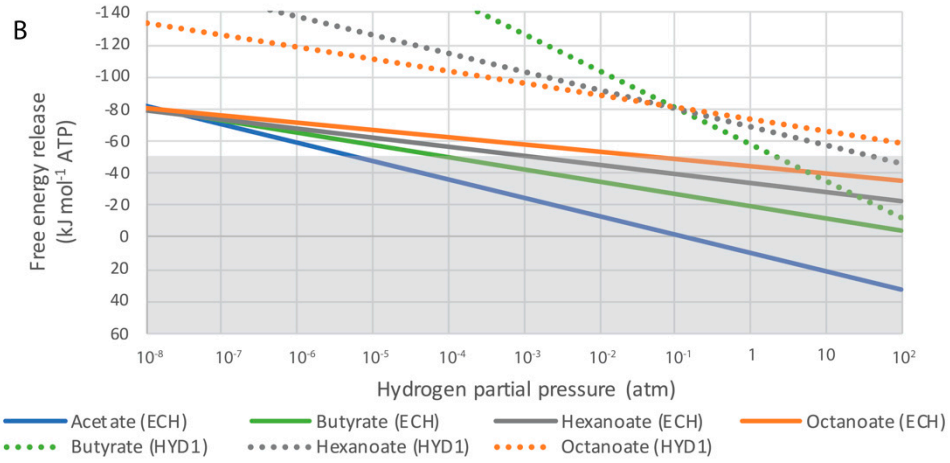

Figure 8. Free energy release for different products from ethanol under a range of H_2 partial pressures. Plots show the overall free energy release (**A**) and the free energy release per mol of ATP (**B**). Areas shaded in gray are considered infeasible and the dashed lines represent scenarios with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

Author Contributions: Conceptualization, M.S. and P.S.; review of literature—P.S., J.M., and L.C.; writing—original draft preparation, M.S. and P.S.; writing—review and editing, P.S., J.M., L.C., K.B. and M.S.; supervision, M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: We thank the College of Engineering and Mathematical Sciences at the University of Vermont for supporting M.S. with start-up funding and J.M. and K.B. with a summer research experience for undergraduates.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. McGovern, P.E.; Zhang, J.; Tang, J.; Zhang, Z.; Hall, G.R.; Moreau, R.A.; Nunez, A.; Butrym, E.D.; Richards, M.P.; Wang, C.S.; et al. Fermented beverages of pre- and proto-historic China. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17593–17598. [CrossRef]
2. He, P.J. Anaerobic digestion: An intriguing long history in China. *Waste Manag.* **2010**, *30*, 549–550. [CrossRef] [PubMed]
3. World Biogas Association. *Global Potential of Biogas*; World Biogas Association: London, UK, 2019.
4. Mancini, A.; Imperlini, E.; Nigro, E.; Montagnese, C.; Daniele, A.; Orru, S.; Buono, P. Biological and Nutritional Properties of Palm Oil and Palmitic Acid: Effects on Health. *Molecules* **2015**, *20*, 17339–17361. [CrossRef] [PubMed]
5. Marten, B.; Pfeuffer, M.; Schrezenmeir, J. Medium-chain triglycerides. *Int. Dairy J.* **2006**, *16*, 1374–1382. [CrossRef]
6. Bach, A.C.; Babayan, V.K. Medium-chain triglycerides: An update. *Am. J. Clin. Nutr.* **1982**, *36*, 950–962. [CrossRef]
7. Sun, Y.; Bu, D.P.; Wang, J.Q.; Cui, H.; Zhao, X.W.; Xu, X.Y.; Sun, P.; Zhou, L.Y. Supplementing different ratios of short- and medium-chain fatty acids to long-chain fatty acids in dairy cows: Changes of milk fat production and milk fatty acids composition. *J. Dairy Sci.* **2013**, *96*, 2366–2373. [CrossRef]
8. Vyas, D.; Teter, B.B.; Erdman, R.A. Milk fat responses to dietary supplementation of short- and medium-chain fatty acids in lactating dairy cows. *J. Dairy Sci.* **2012**, *95*, 5194–5202. [CrossRef]
9. Zentek, J.; Buchheit-Renko, S.; Ferrara, F.; Vahjen, W.; Van Kessel, A.G.; Pieper, R. Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets. *Anim. Health Res. Rev.* **2011**, *12*, 83–93. [CrossRef]
10. Angenent, L.T.; Richter, H.; Buckel, W.; Spirito, C.M.; Steinbusch, K.J.J.; Plugge, C.M.; Strik, D.; Grootscholten, T.I.M.; Buisman, C.J.N.; Hamelers, H.V.M. Chain Elongation with Reactor Microbiomes: Open-Culture Biotechnology To Produce Biochemicals. *Environ. Sci. Technol.* **2016**, *50*, 2796–2810. [CrossRef]
11. Sarria, S.; Kruyer, N.S.; Peralta-Yahya, P. Microbial synthesis of medium-chain chemicals from renewables. *Nat. Biotechnol.* **2017**, *35*, 1158–1166. [CrossRef]
12. Harvey, B.G.; Meylemans, H.A. 1-Hexene: A renewable C6 platform for full-performance jet and diesel fuels. *Green Chem.* **2014**, *16*, 770–776. [CrossRef]
13. Kim, S.; Chen, J.; Cheng, T.; Gindulyte, A.; He, J.; He, S.; Li, Q.; Shoemaker, B.A.; Thiessen, P.A.; Yu, B.; et al. PubChem 2019 update: Improved access to chemical data. *Nucleic Acids Res.* **2019**, *47*, D1102–D1109. [CrossRef] [PubMed]
14. Han, W.H.; He, P.J.; Shao, L.M.; Lu, F. Road to full bioconversion of biowaste to biochemicals centering on chain elongation: A mini review. *J. Environ. Sci.* **2019**, *86*, 50–64. [CrossRef] [PubMed]
15. Venkateswar Reddy, M.; Kumar, G.; Mohanakrishna, G.; Shobana, S.; Al-Raoush, R.I. Review on the production of medium and small chain fatty acids through waste valorization and CO2 fixation. *Bioresour. Technol.* **2020**, *309*, 123400. [CrossRef] [PubMed]
16. Molisch, H. *Die Purpurbakterien Nach Neuen Untersuchungen: Eine Mikrobiologische Studie*; Verlag von Gustav Fischer: Jena, German, 1907.
17. Esmarch, E. Über die Reinkultur eines Spirillum. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. *Abteilung I* **1887**, *1*, 225–230.
18. Munk, A.C.; Copeland, A.; Lucas, S.; Lapidus, A.; Del Rio, T.G.; Barry, K.; Detter, J.C.; Hammon, N.; Israni, S.; Pitluck, S.; et al. Complete genome sequence of Rhodospirillum rubrum type strain (S1). *Stand. Genomic Sci.* **2011**, *4*, 293–302. [CrossRef]
19. Gest, H. A serendipic legacy: Erwin Esmarch's isolation of the first photosynthetic bacterium in pure culture. *Photosynt. Res.* **1995**, *46*, 473–478. [CrossRef]
20. Barker, H.A. The production of caproic and butyric acids by the methane fermentation of ethyl alcohol. *Arch. Mikrobiol.* **1937**, *8*, 415–421. [CrossRef]
21. Barker, H.A.; Taha, S.M. Clostridium kluyverii, an Organism Concerned in the Formation of Caproic Acid from Ethyl Alcohol. *J. Bacteriol.* **1942**, *43*, 347–363. [CrossRef]
22. Gildemyn, S.; Molitor, B.; Usack, J.G.; Nguyen, M.; Rabaey, K.; Angenent, L.T. Upgrading syngas fermentation effluent using Clostridium kluyveri in a continuous fermentation. *Biotechnol. Biofuels* **2017**, *10*, 83. [CrossRef]

23. Seedorf, H.; Fricke, W.F.; Veith, B.; Bruggemann, H.; Liesegang, H.; Strittmatter, A.; Miethke, M.; Buckel, W.; Hinderberger, J.; Li, F.; et al. The genome of Clostridium kluyveri, a strict anaerobe with unique metabolic features. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2128–2133. [CrossRef] [PubMed]
24. Richter, H.; Molitor, B.; Diender, M.; Sousa, D.Z.; Angenent, L.T. A Narrow pH Range Supports Butanol, Hexanol, and Octanol Production from Syngas in a Continuous Co-culture of Clostridium ljungdahlii and Clostridium kluyveri with In-Line Product Extraction. *Front. Microbiol.* **2016**, *7*, 1773. [CrossRef] [PubMed]
25. Li, F.; Hinderberger, J.; Seedorf, H.; Zhang, J.; Buckel, W.; Thauer, R.K. Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from Clostridium kluyveri. *J. Bacteriol.* **2008**, *190*, 843–850. [CrossRef] [PubMed]
26. Wang, S.; Huang, H.; Moll, J.; Thauer, R.K. NADP+ reduction with reduced ferredoxin and NADP+ reduction with NADH are coupled via an electron-bifurcating enzyme complex in Clostridium kluyveri. *J. Bacteriol.* **2010**, *192*, 5115–5123. [CrossRef]
27. Genthner, B.R.; Davis, C.L.; Bryant, M.P. Features of rumen and sewage sludge strains of Eubacterium limosum, a methanol- and H2-CO2-utilizing species. *Appl. Environ. Microbiol.* **1981**, *42*, 12–19. [CrossRef]
28. Chen, W.S.; Ye, Y.; Steinbusch, K.J.J.; Strik, D.P.B.T.B.; Buisman, C.J.N. Methanol as an alternative electron donor in chain elongation for butyrate and caproate formation. *Biomass Bioenergy* **2016**, *93*, 201–208. [CrossRef]
29. Prevot, A.; Taffanel, J. Action of water soluble vitamins on the fermentation of glucose by Plectridium tetani. *Compte Rendu des Seances de la Societe de Biologie* **1942**, *136*, 384–385.
30. Holdeman, V.L.; Cato, E.P.; Moore, W.E.C. Amended Description of Ramibacterium Alactolyticum Prevot and Taffanel With Proposal of a Neotype Strain. *Int. J. Syst. Bacteriol.* **1967**, *17*, 323–341. [CrossRef]
31. Moore, W.E.C.; Holdeman, L.V. New Names and Combinations in the Genera Bacteroides Castellani and Chalmers, Fusobacterium Knorr, Eubacterium Prevot, Propionbacterium Delwich, and Lactobacillus Orla-Jensen. *Int. J. Syst. Bacteriol.* **1973**, *23*, 69–74. [CrossRef]
32. Integrative Human Microbiome Project Research Network Consortium. The Integrative Human Microbiome Project. *Nature* **2019**, *569*, 641–648. [CrossRef]
33. Elsden, S.R.; Lewis, D. The production of fatty acids by a gram-negative coccus. *Biochem. J.* **1953**, *55*, 183–189. [CrossRef] [PubMed]
34. Gutierrez, J.; Davis, R.E.; Lindahl, I.L.; Warwick, E.J. Bacterial Changes in the Rumen During the Onset of Feed-lot Bloat of Cattle and Characteristics of Peptostreptococcus elsdenii n. sp. *Appl. Microbiol.* **1959**, *7*, 16–22. [CrossRef] [PubMed]
35. Rogosa, M. Transfer of Peptostreptococcus elsdenii Gutierrez et al. to a New Genus, Megasphaera [M. elsdenii (Gutierrez et al.) comb. nov.]. *Int. J. Syst. Bacteriol.* **1971**, *21*, 187–189. [CrossRef]
36. Riley, L.A.; Hatmaker, E.A.; Guss, A.M.; Westpheling, J. Development of emerging model microorganisms: Megasphaera elsdenii for biomass and organic acid upgrading to fuels and chemicals. In Proceedings of the 2019 Genomic Sciences Program Annual Principal Investigator (PI) Meeting, Tysons, VA, USA, 25 February 2019.
37. Nelson, R.; Peterson, D.; Karp, E.; Beckham, G.; Salvachúa, D. Mixed Carboxylic Acid Production by Megasphaera elsdenii from Glucose and Lignocellulosic Hydrolysate. *Fermentation* **2017**, *3*, 10. [CrossRef]
38. Weimer, P.J.; Moen, G.N. Quantitative analysis of growth and volatile fatty acid production by the anaerobic ruminal bacterium Megasphaera elsdenii T81. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 4075–4081. [CrossRef]
39. Prabhu, R.; Altman, E.; Eiteman, M.A. Lactate and Acrylate Metabolism by Megasphaera elsdenii under Batch and Steady-State Conditions. *Appl. Environ. Microb.* **2012**, *78*, 8564–8570. [CrossRef]
40. Henning, P.H.; Horn, C.H.; Leeuw, K.J.; Meissner, H.H.; Hagg, F.M. Effect of ruminal administration of the lactate-utilizing strain Megasphaera elsdenii (Me) NCIMB 41125 on abrupt or gradual transition from forage to concentrate diets. *Anim. Feed Sci. Tech.* **2010**, *157*, 20–29. [CrossRef]
41. Soto-Cruz, O.; Favela-Torres, E.; Saucedo-Castaneda, G. Modeling of growth, lactate consumption, and volatile fatty acid production by Megasphaera elsdenii cultivated in minimal and complex media. *Biotechnol. Progr.* **2002**, *18*, 193–200. [CrossRef]
42. Soto-Cruz, O.; Chavez-Rivera, R.; Saucedo-Castaneda, G. Stimulation of the Megasphaera elsdenii's butyrate production in continuous culture by a yeast additive. *Braz. Arch. Biol. Technol.* **2001**, *44*, 179–184. [CrossRef]
43. Hino, T.; Kuroda, S. Presence of lactate dehydrogenase and lactate racemase in Megasphaera elsdenii grown on glucose or lactate. *Appl. Environ. Microbiol.* **1993**, *59*, 255–259. [CrossRef]

44. Marx, H.; Graf, A.B.; Tatto, N.E.; Thallinger, G.G.; Mattanovich, D.; Sauer, M. Genome sequence of the ruminal bacterium Megasphaera elsdenii. *J. Bacteriol.* **2011**, *193*, 5578–5579. [CrossRef] [PubMed]
45. Hatmaker, E.A.; Klingeman, D.M.; O'Dell, K.B.; Riley, L.A.; Papanek, B.; Guss, A.M. Complete Genome Sequences of Two Megasphaera elsdenii Strains, NCIMB 702410 and ATCC 25940. *Microbiol. Resour. Announc.* **2019**, *8*. [CrossRef] [PubMed]
46. Bag, S.; Ghosh, T.S.; Das, B. Whole-Genome Sequence of a Megasphaera elsdenii Strain Isolated from the Gut of a Healthy Indian Adult Subject. *Genome Announc.* **2017**, *5*. [CrossRef] [PubMed]
47. Lee, N.R.; Lee, C.H.; Lee, D.Y.; Park, J.B. Genome-Scale Metabolic Network Reconstruction and In Silico Analysis of Hexanoic acid Producing Megasphaera elsdenii. *Microorganisms* **2020**, *8*, 539. [CrossRef] [PubMed]
48. Wallace, R.J.; McKain, N.; McEwan, N.R.; Miyagawa, E.; Chaudhary, L.C.; King, T.P.; Walker, N.D.; Apajalahti, J.H.A.; Newbold, C.J. Eubacterium pyruvativorans sp. nov., a novel non-saccharolytic anaerobe from the rumen that ferments pyruvate and amino acids, forms caproate and utilizes acetate and propionate. *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 965–970. [CrossRef]
49. Zhu, X.; Tao, Y.; Liang, C.; Li, X.; Wei, N.; Zhang, W.; Zhou, Y.; Yang, Y.; Bo, T. The synthesis of n-caproate from lactate: A new efficient process for medium-chain carboxylates production. *Sci. Rep.* **2015**, *5*, 14360. [CrossRef] [PubMed]
50. Zhu, X.; Zhou, Y.; Wang, Y.; Wu, T.; Li, X.; Li, D.; Tao, Y. Production of high-concentration n-caproic acid from lactate through fermentation using a newly isolated Ruminococcaceae bacterium CPB6. *Biotechnol. Biofuels* **2017**, *10*, 102. [CrossRef]
51. Tao, Y.; Zhu, X.; Wang, H.; Wang, Y.; Li, X.; Jin, H.; Rui, J. Complete genome sequence of Ruminococcaceae bacterium CPB6: A newly isolated culture for efficient n-caproic acid production from lactate. *J. Biotechnol.* **2017**, *259*, 91–94. [CrossRef]
52. Jeon, B.S.; Kim, B.C.; Um, Y.; Sang, B.I. Production of hexanoic acid from D-galactitol by a newly isolated Clostridium sp. BS-1. *Appl. Microbiol. Biotechnol.* **2010**, *88*, 1161–1167. [CrossRef]
53. Andersen, S.J.; De Groof, V.; Khor, W.C.; Roume, H.; Props, R.; Coma, M.; Rabaey, K. A Clostridium Group IV Species Dominates and Suppresses a Mixed Culture Fermentation by Tolerance to Medium Chain Fatty Acids Products. *Front. Bioeng. Biotechnol.* **2017**, *5*, 8. [CrossRef]
54. Bengelsdorf, F.R.; Poehlein, A.; Daniel, R.; Durre, P. Genome Sequence of the Caproic Acid-Producing Bacterium Caproiciproducens galactitolivorans BS-1(T) (JCM 30532). *Microbiol. Resour. Announc.* **2019**, *8*. [CrossRef] [PubMed]
55. Kim, B.C.; Seung Jeon, B.; Kim, S.; Kim, H.; Um, Y.; Sang, B.I. Caproiciproducens galactitolivorans gen. nov., sp. nov., a bacterium capable of producing caproic acid from galactitol, isolated from a wastewater treatment plant. *Int. J. Syst. Evol. Microbiol.* **2015**, *65*, 4902–4908. [CrossRef] [PubMed]
56. Jeon, B.S.; Kim, S.; Sang, B.I. Megasphaera hexanoica sp. nov., a medium-chain carboxylic acid-producing bacterium isolated from a cow rumen. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 2114–2120. [CrossRef] [PubMed]
57. Kim, H.; Jeon, B.S.; Sang, B.I. An Efficient New Process for the Selective Production of Odd-Chain Carboxylic Acids by Simple Carbon Elongation Using Megasphaera hexanoica. *Sci. Rep.* **2019**, *9*, 11999. [CrossRef] [PubMed]
58. Flaiz, M.; Baur, T.; Brahner, S.; Poehlein, A.; Daniel, R.; Bengelsdorf, F.R. Caproicibacter fermentans gen. nov., sp. nov., a new caproate-producing bacterium and emended description of the genus Caproiciproducens. *Int. J. Syst. Evol. Microbiol.* **2020**, *70*, 4269–4279. [CrossRef] [PubMed]
59. Liu, B.; Popp, D.; Strauber, H.; Harms, H.; Kleinsteuber, S. Draft Genome Sequences of Three Clostridia Isolates Involved in Lactate-Based Chain Elongation. *Microbiol. Resour. Announc.* **2020**, *9*. [CrossRef]
60. Liu, B.; Popp, D.; Sträuber, H.; Harms, H.; Kleinsteuber, S. Lactate-based microbial chain elongation for n-caproate and iso-butyrate production: Genomic and metabolic features of three novel Clostridia isolates. *Res. Sq.* in press. **2020**. [CrossRef]
61. Esquivel-Elizondo, S.; Bağcı, C.; Temovska, M.; Jeon, B.S.; Bessarab, I.; Williams, R.B.H.; Huson, D.H.; Angenent, L.T. The isolate Caproiciproducens sp. 7D4C2 produces n -caproate at mildly acidic conditions from hexoses: Genome and rBOX comparison with related strains and chain-elongating bacteria. *BioRxiv* **2020**. [CrossRef]

62. Scarborough, M.J.; Myers, K.S.; Donohue, T.J.; Noguera, D.R. Medium-Chain Fatty Acid Synthesis by "Candidatus Weimeria bifida" gen. nov., sp. nov., and "Candidatus Pseudoramibacter fermentans" sp. nov. *Appl. Environ. Microbiol.* **2020**, *86*. [CrossRef]
63. Willems, A.; Collins, M.D. Phylogenetic relationships of the genera Acetobacterium and Eubacterium sensu stricto and reclassification of Eubacterium alactolyticum as Pseudoramibacter alactolyticus gen. nov., comb. nov. *Int. J. Syst. Bacteriol.* **1996**, *46*, 1083–1087. [CrossRef]
64. Andersen, S.J.; Candry, P.; Basadre, T.; Khor, W.C.; Roume, H.; Hernandez-Sanabria, E.; Coma, M.; Rabaey, K. Electrolytic extraction drives volatile fatty acid chain elongation through lactic acid and replaces chemical pH control in thin stillage fermentation. *Biotechnol. Biofuels* **2015**, *8*, 1–14. [CrossRef] [PubMed]
65. Grootscholten, T.I.M.; Strik, D.P.B.T.B.; Steinbusch, K.J.J.; Buisman, C.J.N.; Hamelers, H.V.M. Two-stage medium chain fatty acid (MCFA) production from municipal solid waste and ethanol. *Appl. Energy* **2014**, *116*, 223–229. [CrossRef]
66. Grootscholten, T.I.M.; dal Borgo, F.K.; Hamelers, H.V.M.; Buisman, C.J.N. Promoting chain elongation in mixed culture acidification reactors by addition of ethanol. *Biomass Bioenergy* **2013**, *48*, 10–16. [CrossRef]
67. Nzeteu, C.O.; Trego, A.C.; Abram, F.; O'Flaherty, V. Reproducible, high-yielding, biological caproate production from food waste using a single-phase anaerobic reactor system. *Biotechnol Biofuels* **2018**, *11*, 108. [CrossRef]
68. Roghair, M.; Liu, Y.C.; Strik, D.P.B.T.B.; Weusthuis, R.A.; Bruins, M.E.; Buisman, C.J.N. Development of an Effective Chain Elongation Process From Acidified Food Waste and Ethanol Into n-Caproate. *Front. Bioeng. Biotech.* **2018**, *6*. [CrossRef]
69. Reddy, M.V.; Hayashi, S.; Choi, D.; Cho, H.; Chang, Y.C. Short chain and medium chain fatty acids production using food waste under non-augmented and bio-augmented conditions. *J. Clean. Prod.* **2018**, *176*, 645–653. [CrossRef]
70. Contreras-Davila, C.A.; Carrion, V.J.; Vonk, V.R.; Buisman, C.N.J.; Strik, D.P.B.T.B. Consecutive lactate formation and chain elongation to reduce exogenous chemicals input in repeated-batch food waste fermentation. *Water Res.* **2020**, *169*, 115215. [CrossRef]
71. Lonkar, S.; Fu, Z.; Holtzapple, M. Optimum alcohol concentration for chain elongation in mixed-culture fermentation of cellulosic substrate. *Biotechnol. Bioeng.* **2016**, *113*, 2597–2604. [CrossRef]
72. Scarborough, M.J.; Lynch, G.; Dickson, M.; McGee, M.; Donohue, T.J.; Noguera, D.R. Increasing the economic value of lignocellulosic stillage through medium-chain fatty acid production. *Biotechnol. Biofuels* **2018**, *11*, 200. [CrossRef]
73. Weimer, P.J.; Nerdahl, M.; Brandl, D.J. Production of medium-chain volatile fatty acids by mixed ruminal microorganisms is enhanced by ethanol in co-culture with Clostridium kluyveri. *Bioresour. Technol.* **2015**, *175*, 97–101. [CrossRef]
74. Khor, W.C.; Andersen, S.; Vervaeren, H.; Rabaey, K. Electricity-assisted production of caproic acid from grass. *Biotechnol. Biofuels* **2017**, *10*, 180. [CrossRef] [PubMed]
75. Urban, C.; Xu, J.; Sträuber, H.; dos Santos Dantas, T.R.; Mühlenberg, J.; Härtig, C.; Angenent, L.T.; Harnisch, F. Production of drop-in fuels from biomass at high selectivity by combined microbial and electrochemical conversion. *Energy Environ. Sci.* **2017**, *10*, 2231–2244. [CrossRef]
76. Carvajal-Arroyo, J.M.; Candry, P.; Andersen, S.J.; Props, R.; Seviour, T.; Ganigué, R.; Rabaey, K. Granular fermentation enables high rate caproic acid production from solid-free thin stillage. *Green Chem.* **2019**, *21*, 1330–1339. [CrossRef]
77. Wu, Q.; Feng, X.; Guo, W.; Bao, X.; Ren, N. Long-term medium chain carboxylic acids production from liquor-making wastewater: Parameters optimization and toxicity mitigation. *Chem. Eng. J.* **2020**, *388*. [CrossRef]
78. Kucek, L.A.; Xu, J.J.; Nguyen, M.; Angenent, L.T. Waste Conversion into n-Caprylate and n-Caproate: Resource Recovery from Wine Lees Using Anaerobic Reactor Microbiomes and In-line Extraction. *Front. Microbiol.* **2016**, *7*. [CrossRef]
79. Xu, J.; Hao, J.; Guzman, J.J.L.; Spirito, C.M.; Harroff, L.A.; Angenent, L.T. Temperature-Phased Conversion of Acid Whey Waste Into Medium-Chain Carboxylic Acids via Lactic Acid: No External e-Donor. *Joule* **2018**, *2*, 280–295. [CrossRef]

80. Duber, A.; Jaroszynski, L.; Zagrodnik, R.; Chwialkowska, J.; Juzwa, W.; Ciesielski, S.; Oleskowicz-Popiel, P. Exploiting the real wastewater potential for resource recovery—n-caproate production from acid whey. *Green Chem.* **2018**, *20*, 3790–3803. [CrossRef]
81. Domingos, J.M.B.; Martinez, G.A.; Scoma, A.; Fraraccio, S.; Kerckhof, F.-M.; Boon, N.; Reis, M.A.M.; Fava, F.; Bertin, L. Effect of Operational Parameters in the Continuous Anaerobic Fermentation of Cheese Whey on Titers, Yields, Productivities, and Microbial Community Structures. *ACS Sustain. Chem. Eng.* **2017**, *5*, 1400–1407. [CrossRef]
82. Zhang, W.; Yin, F.; Dong, H.; Cao, Q.; Wang, S.; Xu, J.; Zhu, Z. Bioconversion of swine manure into high-value products of medium chain fatty acids. *Waste Manag.* **2020**, *113*, 478–487. [CrossRef]
83. Zoghlami, A.; Paes, G. Lignocellulosic Biomass: Understanding Recalcitrance and Predicting Hydrolysis. *Front. Chem.* **2019**, *7*, 874. [CrossRef]
84. Badgett, A.; Newes, E.; Milbrandt, A. Economic analysis of wet waste-to-energy resources in the United States. *Energy* **2019**, *176*, 224–234. [CrossRef]
85. Scarborough, M.J.; Lawson, C.E.; Hamilton, J.J.; Donohue, T.J.; Noguera, D.R. Metatranscriptomic and Thermodynamic Insights into Medium-Chain Fatty Acid Production Using an Anaerobic Microbiome. *mSystems* **2018**, *3*. [CrossRef] [PubMed]
86. Scarborough, M.J.; Hamilton, J.J.; Erb, E.A.; Donohue, T.J.; Noguera, D.R. Diagnosing and Predicting Mixed-Culture Fermentations with Unicellular and Guild-Based Metabolic Models. *mSystems* **2020**, *5*. [CrossRef] [PubMed]
87. Chen, W.S.; Strik, D.; Buisman, C.J.N.; Kroeze, C. Production of Caproic Acid from Mixed Organic Waste: An Environmental Life Cycle Perspective. *Environ. Sci. Technol.* **2017**, *51*, 7159–7168. [CrossRef] [PubMed]
88. Dellomonaco, C.; Clomburg, J.M.; Miller, E.N.; Gonzalez, R. Engineered reversal of the beta-oxidation cycle for the synthesis of fuels and chemicals. *Nature* **2011**, *476*, 355–359. [CrossRef]
89. Thauer, R.K.; Jungermann, K.; Henninger, H.; Wenning, J.; Decker, K. The energy metabolism of Clostridium kluyveri. *Eur. J. Biochem.* **1968**, *4*, 173–180. [CrossRef]
90. Buckel, W.; Thauer, R.K. Flavin-Based Electron Bifurcation, Ferredoxin, Flavodoxin, and Anaerobic Respiration With Protons (Ech) or NAD(+) (Rnf) as Electron Acceptors: A Historical Review. *Front. Microbiol.* **2018**, *9*, 401. [CrossRef]
91. Buckel, W.; Thauer, R.K. Flavin-Based Electron Bifurcation, A New Mechanism of Biological Energy Coupling. *Chem. Rev.* **2018**, *118*, 3862–3886. [CrossRef]
92. Hartmanis, M.G.; Gatenbeck, S. Intermediary Metabolism in Clostridium acetobutylicum: Levels of Enzymes Involved in the Formation of Acetate and Butyrate. *Appl. Environ. Microbiol.* **1984**, *47*, 1277–1283. [CrossRef]
93. Kuhns, M.; Trifunovic, D.; Huber, H.; Muller, V. The Rnf complex is a Na(+) coupled respiratory enzyme in a fermenting bacterium, Thermotoga maritima. *Commun. Biol.* **2020**, *3*, 431. [CrossRef]
94. Tremblay, P.L.; Zhang, T.; Dar, S.A.; Leang, C.; Lovley, D.R. The Rnf complex of Clostridium ljungdahlii is a proton-translocating ferredoxin:NAD+ oxidoreductase essential for autotrophic growth. *mBio* **2012**, *4*, e00406–e00412. [CrossRef] [PubMed]
95. Biegel, E.; Schmidt, S.; Gonzalez, J.M.; Muller, V. Biochemistry, evolution and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes. *Cell. Mol. Life Sci.* **2011**, *68*, 613–634. [CrossRef] [PubMed]
96. Biegel, E.; Schmidt, S.; Muller, V. Genetic, immunological and biochemical evidence for a Rnf complex in the acetogen Acetobacterium woodii. *Environ. Microbiol.* **2009**, *11*, 1438–1443. [CrossRef] [PubMed]
97. Westphal, L.; Wiechmann, A.; Baker, J.; Minton, N.P.; Muller, V. The Rnf Complex Is an Energy-Coupled Transhydrogenase Essential To Reversibly Link Cellular NADH and Ferredoxin Pools in the Acetogen Acetobacterium woodii. *J. Bacteriol.* **2018**, *200*. [CrossRef]
98. Buckel, W.; Thauer, R.K. Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation. *Biochim. Biophys. Acta* **2013**, *1827*, 94–113. [CrossRef] [PubMed]
99. Schut, G.J.; Adams, M.W. The iron-hydrogenase of Thermotoga maritima utilizes ferredoxin and NADH synergistically: A new perspective on anaerobic hydrogen production. *J. Bacteriol.* **2009**, *191*, 4451–4457. [CrossRef]
100. Spirito, C.M.; Marzilli, A.M.; Angenent, L.T. Higher Substrate Ratios of Ethanol to Acetate Steered Chain Elongation toward n-Caprylate in a Bioreactor with Product Extraction. *Environ. Sci. Technol.* **2018**, *52*, 13438–13447. [CrossRef]

101. Spirito, C.M.; Richter, H.; Rabaey, K.; Stams, A.J.; Angenent, L.T. Chain elongation in anaerobic reactor microbiomes to recover resources from waste. *Curr. Opin. Biotechnol.* **2014**, *27*, 115–122. [CrossRef]
102. Thauer, R.K.; Jungermann, K.; Decker, K. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **1977**, *41*, 100–180. [CrossRef]
103. Kucek, L.A.; Spirito, C.M.; Angenent, L.T. High n-caprylate productivities and specificities from dilute ethanol and acetate: Chain elongation with microbiomes to upgrade products from syngas fermentation. *Energy Environ. Sci.* **2016**, *9*, 3482–3494. [CrossRef]
104. Royce, L.A.; Yoon, J.M.; Chen, Y.X.; Rickenbach, E.; Shanks, J.V.; Jarboe, L.R. Evolution for exogenous octanoic acid tolerance improves carboxylic acid production and membrane integrity. *Metab. Eng.* **2015**, *29*, 180–188. [CrossRef] [PubMed]
105. Fu, Y.; Yoon, J.M.; Jarboe, L.; Shanks, J.V. Metabolic flux analysis of Escherichia coli MG1655 under octanoic acid (C8) stress. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 4397–4408. [CrossRef] [PubMed]
106. Borrull, A.; Lopez-Martinez, G.; Poblet, M.; Cordero-Otero, R.; Rozes, N. New insights into the toxicity mechanism of octanoic and decanoic acids on Saccharomyces cerevisiae. *Yeast* **2015**, *32*, 451–460. [CrossRef] [PubMed]
107. Roghair, M.; Liu, Y.; Adiatma, J.C.; Weusthuis, R.A.; Bruins, M.E.; Buisman, C.J.N.; Strik, D.P.B.T.B. Effect of n-Caproate Concentration on Chain Elongation and Competing Processes. *ACS Sustain. Chem. Eng.* **2018**, *6*, 7499–7506. [CrossRef]
108. Deng, X.; Chen, L.; Hei, M.; Liu, T.; Feng, Y.; Yang, G.Y. Structure-guided reshaping of the acyl binding pocket of 'TesA thioesterase enhances octanoic acid production in E. coli. *Metab. Eng.* **2020**, *61*, 24–32. [CrossRef]
109. Tan, Z.; Yoon, J.M.; Chowdhury, A.; Burdick, K.; Jarboe, L.R.; Maranas, C.D.; Shanks, J.V. Engineering of E. coli inherent fatty acid biosynthesis capacity to increase octanoic acid production. *Biotechnol. Biofuels* **2018**, *11*, 87. [CrossRef]
110. Petering, D.; Fee, J.A.; Palmer, G. The oxygen sensitivity of spinach ferredoxin and other iron-sulfur proteins. The formation of protein-bound sulfur-zero. *J. Biol. Chem.* **1971**, *246*, 643–653.
111. D'Amato, D.; Droste, N.; Allen, B.; Kettunen, M.; Lähtinen, K.; Korhonen, J.; Leskinen, P.; Matthies, B.D.; Toppinen, A. Green, circular, bio economy: A comparative analysis of sustainability avenues. *J. Clean. Prod.* **2017**, *168*, 716–734. [CrossRef]