Leng L, Yang P, Singh S, Zhuang H, Xu L, Chen W-H, Dolfing J, Li D, Zhang Y,
Zeng H, Chu W, Lee P-H.

[A review on the bioenergetics of anaerobic microbial metabolism close to the thermodynamic limits and its implications for digestion applications.](#)

Bioresource Technology 2017, 247, 1095-1106

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DOI link to article:

<https://doi.org/10.1016/j.biortech.2017.09.103>

Date deposited:

13/12/2017

Embargo release date:

19 September 2018

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1 **A review on the bioenergetics of anaerobic microbial metabolism close to the**
2 **thermodynamic limits and its implications for digestion applications**

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Abstract: The exploration of the energetics of anaerobic digestion systems can reveal how microorganisms cooperate efficiently for cell growth and methane production, especially under low-substrate conditions. The establishment of a thermodynamically interdependent partnership, called anaerobic syntrophy, allows unfavorable reactions to proceed. Interspecies electron transfer and the concentrations of electron carriers are crucial for maintaining this mutualistic activity. This critical review summarizes the functional microorganisms and syntroph partners, particularly in the metabolic pathways and energy conservation of syntrophs. The kinetics and thermodynamics of propionate degradation to methane, reversibility of the acetate oxidation process, and estimation of microbial growth are summarized. The various routes of interspecies electron transfer, reverse electron transfer, and Poly- β -hydroxyalkanoate formation in the syntrophic community are also reviewed. Finally, promising and critical directions of future research are proposed. Fundamental insight in the activities and interactions involved in AD systems could serve as a guidance for engineered systems optimization and upgrade.

Keywords: Anaerobic digestion (AD); syntrophy; thermodynamics; kinetics; electron transfer

1. Introduction

Anaerobic digestion (AD) is a biological process in which microorganisms mineralize organic materials in the absence of molecular oxygen. This process is widely used in industrial and municipal wastewater treatment for biogas recovery. Recently, the global challenges in energy and environmental arena necessitate a facelift of AD, with respect to maximizing energy production and enhancing treatment efficiency. Insight in the microbial mutualism that underpins AD and functions close to the thermodynamic limits, is the key to elucidate the black box and upgrade this process to a new renaissance (Tan et al., 2016).

AD for methane production is less exergonic than aerobic degradation or alternative forms of anaerobic respiration; for example, the conversion of hexose to methane and carbon dioxide releases only 15% of the energy generated by aerobic degradation (Schink, 1997). This small amount of energy generation in AD forces the microorganisms into a very close and efficient cooperation. Syntrophy is a particularly mutualistic partnership in AD, defined as a thermodynamically interdependent life style where neither partner can operate without the other (Morris et al., 2013). Interspecies electron transfer and the concentrations of electron carriers

52 in the system are crucial for maintaining this cooperative metabolic activity. It has
53 been postulated that a bacterium needs a minimum of about -20 kJ mol^{-1} (one third of
54 the energy for the synthesis of an ATP molecule) to exploit the free energy change of
55 a reaction, which is the smallest quantum of metabolically convertible energy for an
56 ion transported across the cytoplasmic membrane and the amount for a living cell
57 cooperating in syntrophic fermentation (Schink, 1997). However, it was reported that
58 this minimum energy is considerably lower with the evidences from anaerobic mixed
59 culture chemostat studies (i.e. $-8.0 \pm 3.1 \text{ kJ mol}^{-1}$ of Gibbs free energy available at
60 growth equilibrium (ΔG_E) for propionate conversion to acetate plus hydrogen)
61 (McCarty & Bae, 2011).

62 The AD process begins with the microbial hydrolysis of proteins, fats,
63 carbohydrates, and some other biodegradable polymers, releasing amino acids, fatty
64 acids, and sugars. Hydrolytic bacteria are phylogenetically diverse but mostly fall into
65 two phyla, *Bacteroidetes* and *Firmicutes* (Venkiteshwaran et al., 2015). Acidogenic
66 bacteria then convert amino acids, fatty acids, and sugars into ammonia, short-chain
67 fatty acids (SCFAs), carbon dioxide (CO_2), hydrogen (H_2), and alcohols. Most species
68 of acidogenic bacteria belong to the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*,

69 *Chloroflexi*, and *Actinobacteria*. The genera under these phyla have been commonly
70 identified as *Clostridium* and *Bacillus*, *Bacteroides* and *Proteiniphilum*, *Desulfovibrio*
71 and *Geobacter*, *Chloroflexus*, and *Mycobacterium* (Cai et al., 2016). While acetate,
72 formate, H_2/CO_2 , and methyl compounds can be directly utilized by methanogens,
73 other compounds resulting from acidogenesis such as butyrate, propionate, lactate,
74 and ethanol are further biodegraded by a group of syntrophic acetogens into acetate,
75 formate, and H_2/CO_2 . Syntrophic acetogens generally include *Syntrophobacter*,
76 *Pelotomaculum*, *Smithella*, *Syntrophus*, *Syntrophomonas*, and *Syntrophothermus*. The
77 first three genera are typically involved in propionate degradation, whereas the others
78 are commonly responsible for the oxidation of butyrate and other fatty acids (Cai et
79 al., 2016; Venkiteshwaran et al., 2015).

80 Syntrophic acetogenesis is thermodynamically unfavorable under standard
81 conditions. The syntrophic partnership with methanogens, which maintain a low H_2
82 partial pressure (P_{H_2}) and low formate and acetate concentrations, allows this process
83 to occur (Stams & Plugge, 2009). For example, the P_{H_2} is crucial in the control of a
84 syntrophic partnership between *Syntrophobacter* bacteria and hydrogenotrophic
85 methanogens. P_{H_2} measured at steady-state conditions were in the range of $1\text{--}20 \times 10^{-5}$

86 atm, under which propionate consumption is thermodynamically favorable (McCarty
87 & Smith, 1986). In addition, *Smithella* bacteria in syntrophic partnership with
88 H₂-consuming methanogens have a larger H₂ window than the classical syntrophic
89 acetogens due to a different propionate degradation pathway (Dolfing, 2013).
90 Syntrophic electron flow during methanogenesis can also be achieved by interspecies
91 formate transfer. In microbial flocs, more than 90% of syntrophic ethanol conversion
92 to methane by *Desulfovibrio vulgaris* and *Methanobacterium formicicum* was
93 mediated via interspecies formate transfer (Thiele & Zeikus, 1988). There are four
94 main pathways for methane (CH₄) production: (i) acetoclastic methanogens utilize
95 acetate to directly produce CH₄ and CO₂; (ii) hydrogenotrophic methanogens use H₂
96 or formate to reduce CO₂ to CH₄; (iii) methylotrophic methanogens metabolize
97 methyl compounds to produce a small amount of CH₄; (iv) syntrophic partnerships of
98 acetate-oxidizing bacteria and hydrogenotrophic methanogens convert acetate to CH₄
99 via the intermediates H₂ and CO₂. In anaerobic wastewater treatment, around 70% of
100 the CH₄ is produced from acetate and the remainder mostly comes from H₂ and CO₂
101 (Venkiteshwaran et al., 2015). In terms of thermodynamics, the overall energy
102 generated via acetoclastic methanogenesis (Pathway i) is the same as the energy

generated via acetate oxidation and hydrogenotrophic methanogenesis based on anaerobic syntrophy (Pathway iv). The difference is that in acetoclastic methanogenesis all energy goes to one type of microorganisms. In syntrophic acetate oxidation, the energy is shared by two different species. Based on energetics alone, acetoclastic methanogens should outcompete syntrophic acetate-oxidizing bacteria. Hydrogenotrophic methanogens are crucial for the electron flow in the AD process because of their ability to scavenge H₂/formate at low levels and promote syntrophic acetogenesis. The most abundant genus of methanogens found in two anaerobic digesters was *Methanosarcina* (Cai et al., 2016), which are facultative acetoclastic methanogens that can also utilize H₂/CO₂ and C-1 compounds for methane production (Liu & Whitman, 2008). *Methanosaeta* are obligate acetoclastic methanogens that are known to use only acetate or acetate plus electrons obtained via direct interspecies electron transport (DIET) (Venkiteshwaran et al., 2015). DIET raises the intriguing possibility that the organism gets additional energy with electrons transferred via DIET (Shrestha et al., 2013). *Methanoculleus*, *Methanospirillum*, *Methanoregula*, *Methanosphaerula*, *Methanobacterium*, *Methanobrevibacter*, and *Methanothermobacter* are the most commonly observed hydrogenotrophic

methanogens in anaerobic digesters (Cai et al., 2016). The H_2 can also be thought of as protons (H^+) associated with electrons, and syntrophic bacteria share electrons with methanogens in the form of H_2 could also be possible in a particular form of interspecies electron transfer separately with H^+ . DIET proceeds via electrically conductive pili or c-type cytochromes from anaerobic syntrophs to methanogens resulting in methane production from ethanol, as observed for associations between *Geobacter* as electron producer and *Methanosaeta* or *Methanosarcina* as electron consumer (Rotaru et al., 2014a). The DIET will be discussed in more detail later in the review.

The focus of this review is on the literature that touches on anaerobic syntrophy as thermodynamically-limiting step in the AD process, with special attention to the functional microorganisms, syntrophic partners, microbial growth kinetics, and metabolic pathways involved in syntrophic processes, and to the molecular bioenergetics of syntrophic metabolism.

2. Thermodynamic and kinetic perspectives of propionate degradation

2.1 Thermodynamic perspective

Oxidation of propionate is energetically unfavorable because of the standard Gibbs free energy change, ΔG^0 , of this reaction is positive. Propionate can be oxidized only if a syntrophic association occurs between propionate-oxidizing bacteria and H_2 -consuming methanogens, such that the overall reaction is thermodynamically feasible (McInerney et al., 2009). The accumulation of propionate, an important intermediate, causes acidification of anaerobic digestion systems and deterioration of digestion performance (Smith & McCarty, 1989). Its degradation into acetate and H_2/CO_2 (and then to CH_4) accounts for approximately 6–35% of the total methanogenesis (Smith & McCarty, 1989). Therefore, the degradation of propionate is crucial, and as propionate degraders, syntrophic propionate-oxidizing bacteria (SPOB) play an imperative role in the metabolic network.

Thermodynamic laws can act as a vital tool to provide the theoretical basis for analyzing experimental results and providing important information regarding bacterial growth and metabolism. Thermodynamics also play an important role in understanding the pathway reversibility. The possible pathway reversibility of specific anaerobic catabolic reactions opens a new paradigm in the development of biofuels and chemicals with high energy density (Leng et al., 2017). As anaerobic

153 bioprocesses occur in an energy-scarce environment in which concentrations of
154 substrates remain at a relatively low level, the metabolic pathways take place very
155 close to thermodynamic equilibrium with minimum energy dissipation. Therefore, a
156 slight change in substrate/product concentrations or environmental conditions can
157 alter the direction of the pathway.

158 Using thermodynamic principles, the formation mechanisms of the
159 intermediate compounds can also be analyzed (Smith & McCarty, 1989). Later,
160 efforts relating the thermodynamics with the process kinetics were made to
161 characterize the operation of anaerobic digestion systems (McCarty and Bae, 2011).
162 In addition, the correlation between microbial yield and Gibbs free energy changes of
163 microbial conversions is a well-known application of thermodynamic principles.
164 Thermodynamics also performs an essential function in kinetic models. Reactions can
165 occur if the end products contain less free energy than the reactants, which means that
166 the net Gibbs free energy ($\Delta G'$) is negative. This understanding can help in the
167 investigation of the product concentrations that cause inhibition to a bio-reaction
168 operating close to its thermodynamic equilibrium. Upon reaching the dynamic
169 equilibrium, the reaction will cease before all of the substrate is converted. In 2007,

Oh and Martin (2017) reported the application of various thermodynamic equilibrium models to the anaerobic digestion process. McCarty and Bae (2011) proposed a model that couples anaerobic process kinetics with biological growth equilibrium thermodynamics. Subsequently, González-Cabaleiro et al. (2013) linked thermodynamics and kinetics to assess pathway reversibility in anaerobic bioprocesses. In this section, the thermodynamics related to propionate degradation in anaerobic digestion processes will be discussed.

It is claimed that the syntrophic propionate metabolism should have a free energy change of about -20 kJ mol^{-1} to allow the net synthesis of one third of an ATP molecule (Scholten & Conrad, 2000), but the measured change in free energy during propionate metabolism ranges from -30 kJ mol^{-1} to much lower than -10 kJ mol^{-1} depending on the growth conditions (Scholten & Conrad, 2000). Propionate degradation is generally a bottleneck in methanogenic bioreactors (Gallert & Winter, 2008). Thus, various thermodynamics calculations have been performed to elucidate the thermodynamics of propionate degradation (Smith & McCarty, 1989; McCarty & Bae, 2011).

2.2 Propionate oxidation to acetate and bicarbonate

There are essentially two known pathways that are responsible for propionate metabolism. Most of the syntrophic propionate oxidation is accomplished via the randomizing methylmalonyl-CoA (MMC) pathway, which is also referred to as the classical pathway (Kosaka et al., 2006). So far, at least ten species have been identified as SPOB, belonging to the genera *Syntrophobacter*, *Smithella*, *Pelotomaculum* and *Desulfotomaculum*. The MMC pathway is more common and can be found in many propionate oxidizers such as *Syntrophobacter* (Chen et al., 2005), *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Plugge et al., 2002), *Pelotomaculum thermopropionicum* (Imachi et al., 2002), and *Pelotomaculum schinkii* (de Bok et al., 2005).

Liu et al. (1999) identified a propionate-degrading syntroph of the genus *Smithella*, which produces less methane and more acetate than the previously identified syntrophic propionate degraders such as *Syntrophobacter*. It differed greatly in the substrate range and catabolic products, forming small amounts of butyrate during propionate degradation. *Smithella* spp. utilize propionate in a non-randomizing pathway in which propionate is dismutated to acetate and butyrate via a six-carbon intermediate before being degraded via β -oxidation (de Bok et al.,

204 2001). This novel dismutation pathway is also known as the *Smithella* pathway (de
205 Bok et al., 2001).

206 Recently, Dolfing (2013) calculated the Gibbs free energy for these two
207 pathways and concluded that the *Smithella* pathway (also referred to as the
208 dismutation pathway in this article) is less sensitive to H_2 and is more
209 thermodynamically favorable in a certain range of H_2 than the MMC pathway.
210 *Smithella* species are known to utilize the six-carbon pathway via butyrate and have
211 been detected in various anoxic environments (Liu et al., 2016a), suggesting that this
212 pathway is widespread. A significant range of conditions exist under which
213 propionate oxidation via the *Smithella* pathway is exergonic whereas the MMC
214 pathway is endergonic (Dolfing, 2013). Table 1 shows the windows of opportunity
215 under specific conditions for the MMC and *Smithella* pathways with
216 hydrogenotrophic methanogenesis at 25 °C and 55 °C, respectively, and Figure 1
217 shows the Gibbs free energy change over a wide range of P_{H_2} for both the processes
218 under specific conditions. Based on Table 1 and Figure 1, it can be concluded that for
219 both temperatures (i.e. 25 °C and 55°C), the *Smithella* pathway coupled to

hydrogenotrophic methanogenesis has a wider range of P_{H_2} to occur spontaneously ($\Delta G < 0$) compared to MMC pathway coupled to hydrogenotrophic methanogenesis.

Sulfate addition has been found to enhance the degradation of propionate (Qiao et al., 2016), presumably by acting as auxiliary electron acceptor. Based on thermodynamic calculations, Qiao et al. (2016) suggested that syntrophic propionic acid degradation, coupled with methanogenesis, was unfavorable with a ΔG of +3 kJ mol⁻¹, whereas the consumption of propionic acid by sulfate-reducing bacteria (SRB) would be much more favorable with a ΔG of -180 kJ mol⁻¹. Later it was identified that these calculations were based on a temperature of 25 °C rather than 55 °C. This glitch affects the $\Delta G^{0'}$ of the important reactions by 8.6 kJ/reaction, which subsequently indicated that propionate degradation via MMC pathway was exergonic rather than endergonic and notably alters the narrative (Dolfing, 2017). However, the phenomenon reported by Qiao et al. (2016) suggests that the mechanism of propionate degradation in the presence of small amounts of sulfate should be seriously considered and further investigated. Elucidating the mechanism and routes of propionate degradation warrants further experimentation and thermodynamic studies.

Reactions involved in syntrophic conversion of propionate to methane along with responsible microbes and standard Gibbs free energy values are provided in Table 2. Many factors, including operational conditions such as pH, temperature, P_{H_2} , and the presence of volatile fatty acids (VFAs), organic compounds, and toxins, as well as the reactor configuration, affect the biodegradation of propionate and lead to different biokinetic characteristics. Numerous investigations into the anaerobic oxidation of short-chain fatty acids have been conducted in various fermentation modes. Table 3 summarizes pertinent kinetic parameters of anaerobic degradation of propionate, which were reported from previous studies in single- and two-phase fermentation systems.

2.3 Methanogenesis

Acetate based methanogenesis is responsible for almost 70% of the total methane production in anaerobic wastewater treatment (Kim et al., 2013). Acetate oxidation is thus potentially a very important process in anaerobic digestion. The syntrophic partnership of acetate-oxidizing bacteria and hydrogenotrophic methanogens produces CH_4 from acetate via the intermediates H_2 and CO_2 , and will be focused upon in this review. The reversibility of acetate oxidation reaction is of

253 interest. The thermodynamic equilibrium between acetate oxidation and
254 homoacetogenesis is highly dependent on the P_{H_2} . It has been observed once that only
255 by increasing the P_{H_2} that an acetate-oxidizing system can be changed to a
256 CO_2 -reducing system (Lee & Zinder, 1988). In accordance with Le Chatelier's
257 principle, the system will adjust itself in such a way that the effect of the change will
258 be nullified whenever a system in equilibrium is disturbed. However, this might not
259 be the only case. It would be interesting to know the conditions under which such
260 process reversibility is performed by a specific micro-organism. González-Cabaleiro
261 et al. (2013) have used the thermodynamics of the various steps in biochemical
262 pathways along with kinetic and physiological constraints to constrict the reversibility
263 of the pathways by evaluating effects of changes in process conditions. To ensure cell
264 growth and maintenance, $\Delta G_{catabolism}$ must release more energy than $\Delta\mu_{H^+}$ (at pH=7),
265 and the metabolite concentration should also be within a physiological range. For
266 example, if the metabolite concentration is under 1 μM , it will kinetically disable the
267 reactions in the cell, whereas metabolite concentrations of over 10 mM are not
268 physiologically feasible (Bar-Even et al., 2011). The authors studied the reversibility
269 of homoacetogenesis under two sets of conditions: (1) without energy dissipation and

only $\Delta\mu_{H^+}$ energy recovery at the CO oxidation site, and (2) with energy dissipation and $\Delta\mu_{H^+}$ energy investment at the methyl-THF oxidation site. The results suggest that under the former condition, both pathways are disabled due to either low $\Delta G_{\text{catabolism}}$ or too high and low metabolite concentration, and under the latter condition, acetate oxidative direction will be favorable at low P_{H_2} , whereas CO_2 reductive direction will be favorable at high P_{H_2} .

In anaerobic digestion systems treating recalcitrant substances, hydrolysis is considered as the rate-limiting step that affects the overall process kinetics. In contrast, if the feedstock mostly consists of easily biodegradable materials, methanogenesis is considered to be the rate-limiting step (Narihiro et al., 2016). Methanogens are relatively slow-growing microorganisms and have a limited range of available substrates (Liu & Whitman, 2008). The typical CH_4 production reactions from major methanogenic substrates are listed in Table 4 (Liu & Whitman, 2008; Thauer et al., 1977). The growth rates of hydrogenotrophic methanogens (0.05 to 4.07 d^{-1}) are normally faster than those of acetoclastic methanogen (0.08 to 0.7 d^{-1}) (Demirel & Scherer, 2008). Table 5 summarizes the kinetic parameters of acetoclastic methanogenesis.

Various factors influence the CH₄ production rate from methanogenic substrates, including the microbial community structure and sludge/substrate composition. In sludge digestion systems, the methanation rate from typical methanogenic precursors follows the order H₂/CO₂ > acetate > formate (Pan et al., 2016).

As a significant methanogenic precursor and interspecies electron carrier, formate is equivalent to H₂ both thermodynamically and stoichiometrically, but with higher solubility. Some hydrogenotrophic methanogens utilize formate as both an electron donor and an electron acceptor to produce CH₄ and CO₂. Moreover, the formate concentration can be balanced via the interconversion reaction between formate and H₂/CO₂, which can be described as $\text{HCOO}^- + \text{H}^+ \rightarrow \text{H}_2 + \text{CO}_2$; $\Delta G^{0'} = 1.3 \text{ kJ mol}^{-1}$. The direction of the reaction depends on the system conditions, specifically pH and temperature (Thauer et al., 1977). In general, both the forward and reverse reactions can occur in anaerobic digestion systems in the presence of formate, although this can depend on the real situation, such as operational conditions and microbial composition. Interestingly, analogous to syntrophic acetate oxidation, syntrophic formate oxidation has also been observed (Dolfing et al., 2008).

2.4 Biological kinetic models in anaerobic systems

To prevent performance deterioration, it is important to estimate the biological growth kinetics, which are mainly based on the fundamental relationships between growth rate and substrate utilization rate. In continuous-flow or steady-state digestion systems, the simple empirical model of the Monod kinetic equation can be applied to describe microbial growth:

$$\mu = \frac{\mu_{max} S}{K_s + S} - b \quad (1)$$

where μ_{max} is the maximum specific growth rate (T^{-1}), S is the concentration of the growth-limiting substrate ($M L^{-3}$), K_s is the half-velocity coefficient (i.e., substrate concentration at one-half maximum specific growth rate, $M L^{-3}$), and b is the specific microbial decay rate (T^{-1}).

Contois (1959) found that the half-velocity coefficient is proportional to the influent substrate concentration (S_0), i.e., $K_s = a \cdot S_0$, where a is a proportionality constant. By neglecting the decay coefficient b , Contois derived the following equation by incorporating the microorganism concentration (X) into the Monod equation:

$$\mu = \frac{u_m S}{BX + S} \quad (2)$$

where $u_m = \mu_{max}/(1+a)$ and $B = a/Y(1+a)$.

Then, based on the Contois model, Chen and Hashimoto developed the following equation (Chen and Hashimoto, 1978):

$$\mu = \frac{\mu_{max}S}{KS_0 + (1-K)S} \quad (3)$$

where $K = a/(1 + a)$ is a constant.

2.5 Effects of H₂ on kinetics

Generally, although less than one third of methane production comes from hydrogenotrophic activities, the utilization of H₂ and its interspecies transfer is critical as it regulates the rate and extent of H₂-producing reactions by control the P_{H_2} . Previous study using thermodynamics estimation indicated that at a high P_{H_2} (>200 Pa), homoacetogenesis is preferred over hydrogenotrophic methanogenesis (Fukuzaki et al., 1990). The efficient elimination of H₂ produced during the fermentative degradation of polymers and acetogenesis is necessary to allow these reactions, even more for syntrophic reactions, to proceed continuously under natural and physiological conditions.

2.6 Inhibition of kinetics

Propionate degradation is inhibited by propionate, acetate, and H₂. For the inhibition by H₂ and acetate, a noncompetitive product inhibition model is applied to

describe the inhibition. Inhibition of propionate utilization can be analyzed by a second-order substrate inhibition model. Specifically, the inhibition by propionate and acetate is due to the free acids rather than the anions because it depends on the pH. The inhibition constant (K_P) for acetate of 48.6 μM is of the same order of magnitude as the constant for H_2 , which was 71.5 μM (dissolved H_2), which suggests that the removal of both H_2 and acetate by methanogens is critical to maintain efficient rates of propionate degradation (Fukuzaki et al., 1990). Substrate self-inhibition behavior was observed in some of the anaerobic digestion systems, and the Andrews equation was applied to estimate the inhibition coefficient (K_I , mg L^{-1}) (Fukuzaki et al., 1990):

$$\mu = \mu_{max} \frac{S}{K_S + S + S^2/K_I} \quad (4)$$

3. Interspecies electron flow in anaerobic digestion

In the AD process, methane production is achieved by a collaborative mechanism between acidogenic and acetogenic bacteria and methanogens. This cooperation is not just a simple dependency on the food chain, but also has a thermodynamic rational. The anaerobic degradation of short-chain fatty acids (SCFAs) in the absence of inorganic electron acceptors is endergonic and cannot be spontaneous under standard conditions (298K, pH 7, and 1 atm). However, the methanogens can consume the

degradation products (e.g., acetate and H₂) and make the SCFA oxidation thermodynamically favorable. Meanwhile, as precursors to methane, the degradation products promote methane production. In the syntrophic association, interspecies electron transfer (IET) plays a predominant role. IET from the electron donors (syntrophic bacteria) to electron acceptors (methanogens) determines whether organic matter degradation and methane production can be carried out in an efficient and orderly manner. This combination of different species is also necessary to break through the thermodynamic barrier to capture energy for growth (Liu et al., 2016b). Over the years, it has been recognized that the process of syntrophic methane production mainly involves the two mechanisms of mediated interspecies electron transfer (MIET) and DIET.

3.1 Mediated interspecies electron transfer

One of the electron carriers in syntrophic methanogenic associations that has received the most attention is H₂. Other than H₂, formate is another important extracellular electron carrier in syntrophic metabolism and can be detected in most methanogenesis systems. Proof of principle was provided in a co-culture of *Desulfovibrio vulgaris* and *Methanobacterium formicicum*, in which more than 90%

372 of methane production was mediated via interspecies formate transfer (Thiele &
373 Zeikus, 1988). Moreover, during syntrophic propionate metabolism, the level of
374 formate dehydrogenase (FDH) activity for *Syntrophobacter fumaroxidans* is high (de
375 Bok et al., 2004). The redox potential of formate/CO₂ ($E_0' = -0.432$ V) is close to that
376 of H₂/H⁺ ($E_0' = -0.421$ V), both formate and H₂ have the same niche as an
377 intermediate. Currently, H₂ and formate are known to exist in syntrophic communities,
378 but their concentrations are difficult to measure because of their low concentrations
379 and fast metabolic rate. The solubility of formate is higher than that of H₂. A flux
380 analysis by de Bok et al. (2004) indicated that the transfer speed and diffusion
381 distance of formate are much higher than those of H₂ in syntrophic propionate and
382 butyrate degradation. The model proposed by Batstone et al. (2006) suggested that the
383 electron fluxes mediated by H₂ and formate are at the same level, but the model by
384 Schink et al. (2017) indicated that the levels of H₂ and formate will be affected by the
385 environmental conditions. pH can affect Gibbs free energy of formate to H₂
386 conversion which would change the ratio of H₂ and formate in AD systems. This
387 suggests that methanogens able to consume both H₂ and formate have a better niche
388 for survival.

A genomic analysis of microorganisms in syntrophic methanogenesis gave insight in the mechanism of H₂ and formate generation: multiple hydrogenase and FDH genes were found in the genomes of syntrophic bacteria (Sieber et al., 2012). *Aminobacterium colombiense*, *Clostridium sporogenes*, and *Thermanaerovibrio acidaminovorans* do not contain FDH genes, which means they can only operate via H₂ electron transfer in syntrophic metabolism (Sieber et al., 2012).

Some other substances besides H₂ and formate have been reported as electron carriers in syntrophic metabolism. During the co-culture of *Geobacter sulfurreducens* and *Geobacter metallireducens*, OmcS, a multiheme c-type cytochrome of *G. sulfurreducens*, can accept electrons from c-type cytochromes of *G. metallireducens* (Summers et al., 2010). Kaden et al. (2002) reported that cysteine was used as the interspecies electron shuttle in the co-culture of *Geobacter sulfurreducens* and *Wolinella succinogenes*. Quinone-mediated interspecies electron transfer was observed in the co-culture of *Geobacter metallireducens* and *Geobacter sulfurreducens* on ethanol (Smith et al., 2015).

3.2 Direct interspecies electron transfer

In an upflow anaerobic sludge blanket reactor treating brewery wastewater, it was found that the methanogenic aggregates were conductive. These aggregates were dominated by *Geobacter daltonii* (25% of bacteria) and *Methanosaeta concilii* (90% of archaea), and the methane production rate from added formate and acetate in the aggregates was much lower than that from ethanol degradation (Morita et al., 2011). Observations of Summers et al. (2010) and Rotaru et al. (2014b) indicate that methanogens can directly accept electrons through direct electron transfer for methane production and that DIET can outcompete interspecies H₂/formate transfer in methanogenic systems because of the higher electron transfer efficiency by electric current and the lower loss of intermediates. These results demonstrated that DIET is likely to be an important electron transfer mechanism for methanogenic systems. Detailed mechanism of this direct electron transfer based syntroph is discussed later in the review.

Two mechanisms were reported for DIET in methanogenic metabolism: (i) The electrons are transferred directly to the receptor via c-type cytochrome on the cell membrane (Leang et al., 2010); (ii) The electrons are transferred through pili and other cell appendages (Lovley, 2011). Summers et al. (2010) reported that *G.*

metallireducens and *G. sulfurreducens* are capable of inter-aggregate electron transport in their conductive syntrophic ethanol-oxidizing systems, and that this interspecies electron transfer is mediated by *G. sulfurreducens* c-type cytochrome. Low-iron media inhibited extracellular electron transport of *G. sulfurreducens* because of a decrease in cytochrome concentration, which indirectly demonstrated the necessity of cytochromes in extracellular electron transport (Estevez-Canales et al., 2015). Using conducting-probe atomic force microscopy, Reguera et al. (2005) demonstrated that *G. sulfurreducens* pili are highly conductive, and could serve as biological nanowires to transfer electrons from the surface of the cell to extracellular insoluble Fe(III) oxides. It has been predicted that electrons can also be transferred via the pili to other species. Malvankar et al. (2014) reported that proteinaceous filaments on the pili of *G. sulfurreducens* strain KN400 act as a molecular conductor for long-range extracellular electron transfer by pili and c-type cytochromes.

Co-culture systems of *G. metallireducens* or *Pelobacter carbinolicus* with *Methanosarcina barkeri* demonstrated that *M. barkeri* can interact with *G. metallireducens* and share electrons via DIET during ethanol-based methanogenesis, and can perform H₂ based interspecies electron transfer with *P. carbinolicus* for the

production of methane (Rotaru et al., 2014a). A *pilA*-defective strain of *G. metallireducens* was unable to initiate syntrophic growth with *M. barkeri*; however, syntrophic metabolism could be restored by adding granular activated carbon (GAC). The restoration of DIET indicated that the conductive material could replace the pili and allow the DIET mechanism. There have been many reports on the promotion of methane production via enhancing DIET during syntrophic methanogenesis by the addition of conductive materials, such as GAC (e.g. Rotaru et al., 2014a), and (semi)conducting iron oxide minerals (e.g. Liu et al., 2012).

4. Energy conservation in anaerobic syntrophy

Even when the products of syntrophic acetogenesis are at low concentrations, the Gibbs free energy change for syntrophic metabolism is about -15 to -20 kJ mol⁻¹ (McInerney et al., 2009). It is the smallest quantum of metabolically convertible energy for an ion transported across the cytoplasmic membrane, which is lower than the energy required for the synthesis of ATP from ADP (40–70 kJ per mole of ATP) (McInerney et al., 2009; Schink & Stams, 2013). Syntrophic bacteria couple substrate metabolism directly to ATP synthesis by classical phosphoryl transfer reactions (Jackson & McInerney, 2002), and are well adapted to an energetically stressed

lifestyle with low growth rate and low yield. The marginal energy economy of syntrophs based on oxidation of energetically unfavorable substrates requires an efficient interspecies transfer to enable the survival of the microbes involved under thermodynamically demanding conditions; for instance, extremely low H_2 concentrations are required for syntrophic bacteria to acquire energy through the oxidation of energetically challenging substrates (Kouzuma et al., 2015). For thermodynamically unfavorable reduction of protons (H^+) to H_2 , energy conservation systems, such as reverse electron transfer and electron bifurcation are necessary (Narihiro et al., 2016). Whole-genome and metagenome sequencing approaches have been used to investigate how the syntrophic microorganisms conserve energy when their thermodynamic driving force is very low (McInerney et al., 2009).

4.1 Reverse electron transfer

Reverse electron transfer, an energy conservation system, is a key requirement in syntrophic interactions. Via biochemical mechanisms, microorganism can perform endergonic chemical transformations under prevailing conditions using energy from other exergonic transformation steps (Stams & Plugge, 2009). In anaerobic syntrophs when metabolizing saturated fatty acids such as butyrate, H_2 (E' of -261 mV at 1 Pa

H₂) and formate (E' of -258 mV at 1 μ M formate) production with electrons generated in the oxidation of acyl-CoA intermediates to their respective enoyl-CoA intermediates (E' of -10 mV) is energetically unfavorable and can occur only with energy input from a reverse electron transfer (i.e. a reverse quinone loop model with the energy supplied by a proton motive force) (Sato et al., 1999; Sieber et al., 2015).

4.2 Energy conservation systems

Three modes of energy conservation are generally known: substrate level phosphorylation (SLP), electron transport phosphorylation (ETP), and flavin-based electron bifurcation (FBEB) (Buckel & Thauer, 2013; Thauer et al., 1977). SLP is a metabolic reaction that results in the phosphorylation of ADP with a phosphoryl (PO_3) group, which is directly transferred from another phosphorylated compound, to form ATP. ETP, via chemiosmotic ion-gradient-driven phosphorylation, uses the electrochemical potential between redox partners to drive ATP synthesis by a membrane-bound ATP synthase, and the ATP synthesis is formally coupled to H₂-consuming reactions, such as Wood–Ljungdahl pathway with H₂ as electron donor and CO₂ as electron acceptor in strictly anaerobic bacteria (Thauer et al., 1977). Specifically, the generation of the transmembrane electrochemical ion gradient (an

electrical or ion gradient) across the membrane is induced by an electron-transfer reaction of different redox partners (Schuchmann & Muller, 2014). FBEB was first described for *Clostridium kluyveri* containing a butyryl-CoA dehydrogenase (Bcd), with two closely located subunits of an electron-transferring flavoprotein (EtfAB) forming a complex and binding a flavin cofactor (FMN or FAD) (Li et al., 2008). Interestingly, the mechanism of FBEB involves endergonic redox reactions coupled to exergonic redox reactions, in which the exergonic reduction reaction of one acceptor drives the endergonic reduction of the second acceptor (Schuchmann & Muller, 2014). For example, in *Clostridia*, an endergonic ferredoxin reduction with NADH is coupled to an exergonic crotonyl-CoA reduction with NADH catalyzed by the Bcd/Etf complex (Buckel & Thauer, 2013).

Table 6 summarizes the catabolism information and energy conservation systems of different syntrophic metabolizers. Substrates for syntrophic bacteria generally include alcohols, fatty acids, and aromatic acids (Stams & Plugge, 2009). Therein, propionate and acetate represent the most energetically unfavorable compounds for syntrophs. Syntrophic benzoate degradation remains an enigma in terms of the mechanism through which substrate-level phosphorylation and

ion-translocating reactions can provide sufficient energy for the activation of benzoate, the reduction of benzoyl-CoA, and the production of H₂ or formate by reverse electron transfer (McInerney et al., 2009).

4.3 H₂ and formate-dependent syntrophs

S. fumaroxidans, *P. thermopropionicum*, and *Smithella propionica* are commonly known propionate-oxidizing syntrophic metabolizers. *S. fumaroxidans* and *P. thermopropionicum* both possess a menaquinone (reverse quinone loop) system involved in electron transport in membranes (Kosaka et al., 2008), which could function as the electron carrier for membrane-associated dehydrogenase/hydrogenase or dehydrogenase/hydrogenase complexes. Succinate oxidation via a menaquinone to fumarate is the most energy-dependent reaction in the MMC pathway; the reaction is endergonic and the P_{H_2} is not sufficient to allow this reduction, and, thus, requires a transmembrane proton gradient for this mechanism to function (Plugge et al., 2012). It was hypothesized that the electrons released during the oxidation of succinate are shifted to a lower redox potential via reverse electron transport (Scholten & Conrad, 2000). Schink estimated that approximately 0.67 molecules of ATP must be invested to make this reaction energetically possible at a P_{H_2} of 1 Pa and a formate

524 concentration of 10 μ M (Schink, 1997). *S. fumaroxidans* was also reported to perform
525 reverse electron transfer from a *Rhodobacter* nitrogen fixation (Rnf) complex, which
526 probably re-oxidizes NADH with ferredoxin reduction, followed by ferredoxin
527 oxidation by the induced formate dehydrogenases and hydrogenases (Worm et al.,
528 2011). *S. fumaroxidans* and *P. thermopropionicum* achieve energy harvesting (ATP
529 formation) from SLP via acetyl-CoA:propionate HS-CoA transferase and
530 succinyl-CoA synthase, respectively to drive reverse electron transport. *Syntrophus*
531 *aciditrophicus*, *Syntrophomonas wolfei*, and *Syntrophothermus lipocalidus* utilize
532 other fatty acids such as butyrate for metabolism through a β -oxidation pathway
533 (McInerney et al., 2009; Sekiguchi et al., 2000). Together with menaquinones for
534 electron transport, a novel reverse electron transport system involving
535 electron-transferring flavoprotein (ETF) and a membrane-bound iron-sulfur
536 oxidoreductase have been found in *S. aciditrophicus* and *S. wolfei* (McInerney et al.,
537 2007; McInerney et al., 2009). In addition, an Rnf-like complex in *S. aciditrophicus*
538 might be involved in syntrophic electron transfer (McInerney et al., 2007). *S. wolfei*
539 and *S. lipocalidus* also encode an electron-bifurcating ETF-associated butyryl-CoA
540 dehydrogenase (Bcd/EtfAB), which, however, may support these organisms to

541 perform crotonate reduction (Narihiro et al., 2016). *S. wolfei* and *S. lipocalidus*
542 acquire ATP by acetate kinase, whereas *S. aciditrophicus* achieves energy harvesting
543 via acetyl-CoA synthetase in a β -oxidation pathway (McInerney et al., 2007;
544 McInerney et al., 2009; Sekiguchi et al., 2000). The β -oxidation of butyrate results in
545 a net synthesis of one ATP per butyrate by SLP. Two thirds of the ATP made by SLP
546 are predicted to be used to drive reverse electron transport and the remaining one third
547 of the ATP is available for growth (Sieber et al., 2010).

548 *S. wolfei* was reported capable of producing and utilizing
549 Poly- β -hydroxyalkanoate (PHA) for intra-cellular energy regulation in both pure
550 culture and co-culture with *Methanospirillum hungatei* (Amos & McInerney, 1989). It
551 is hypothesized that such PHA formation is regulated by an endogenous energy
552 balance when the concentrations of H_2 or acetate are too high for the degradation of
553 the growth substrate to be thermodynamically favorable (McInerney et al., 1992).
554 PHA could temporarily serve as an energy sink and be subsequently utilized for
555 SLP-ATP synthesis at thermodynamically-strict-limited condition, which induces the
556 reverse electron transfer to facilitate syntrophic reactions. However, PHA formation

in other syntrophic bacteria utilizing butyrate as substrate or other intermediates is still unclear.

Bryant et al. revealed a syntrophic relationship between *D. vulgaris* Hildenborough and H₂-utilizing methanogens, in which the electrons transferred from ethanol or lactate could be used for methane production via H₂ (Bryant et al., 1977). *D. vulgaris* acquires ATP via SLP with acetate kinase, and a flavin-based electron bifurcation might be involved in the reverse electron transfer of *D. vulgaris* (Meyer et al., 2013).

4.4 Syntrophs involved in direct electron transfer

Microbial interspecies transfer of reducing equivalents can occur as electrical current through biotic (e.g., pili) and abiotic (e.g., conductive mineral and carbon particles) electrical conduits (Kouzuma et al., 2015). The syntrophic metabolizers with DIET capacity may have evolved by interacting with niche-associated microbes and contain flagella and pili with an important role in the establishment and synchronization of contact-dependent syntrophic consortia (McInerney et al., 2009). Molecular mechanisms showed that inner- and outer-membrane cytochromes are presumed to be needed to transfer electrons to nanowires (Shi et al., 2016).

574 *Pelobacter carbinolicus* and *Geobacter metallireducens* are two syntrophic
575 alcohol-consuming metabolizers with the capacity for direct interspecies electron
576 transfer (Aklujkar et al., 2012; McInerney et al., 2009; Rotaru et al., 2014a; Summers
577 et al., 2010). *P. carbinolicus* synthesizes ATP by SLP with acetate kinase/propanoate
578 kinase/butanoate kinase (Aklujkar et al., 2012), whereas *G. metallireducens* achieves
579 energy harvesting by SLP with acetate kinase and acetyl-CoA synthetase (Rotaru et
580 al., 2014a; Summers et al., 2010). In the syntroph of *G. metallireducens* and
581 *Methanosaeta harudinacea*, the complete conversion of ethanol to methane occurs not
582 only via the conversion of the acetate produced from ethanol by *G. metallireducens*
583 but also via the reduction of carbon dioxide by the electrons produced from ethanol
584 oxidation ($8\text{H}^+ + 8\text{e}^- + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$) (Rotaru et al., 2014a). *G.*
585 *metallireducens* has been reported to conduct extracellular electron transfer by
586 employing a multi-heme c-type cytochrome (OmcS) that promotes electron transfer to
587 insoluble Fe(III) oxides and electrodes. *P. carbinolicus* contains an Rnf-type
588 ion-translocating electron-transfer complex, a membrane-bound ion-translocating
589 hydrogenase, and NADH dehydrogenase complex I, which are involved in reverse

electron transfer with ATP compensation from SLP (Aklujkar et al., 2012; McInerney et al., 2009).

4.5 Acetate-oxidizing syntrophs

Another example of syntrophy in AD involves acetate-oxidizing bacteria and hydrogenotrophic methanogens. Acetate-oxidizing bacteria is few and the process is not typical in AD (Venkiteshwaran et al., 2015). However, this process is especially prevalent at high ammonia concentrations and/or high temperature with inhibitory to acetoclastic methanogens (Westerholm et al., 2011). The species of microorganisms have been identified to perform syntrophic acetate oxidization in conjunction with H₂-consuming methanogens, which include *Clostridium ultunense*, *Syntrophaceticus schinkii*, *Tepidanaerobacter acetatoxydans*, *Thermacetogenium phaeum*, *Thermotoga lettingae* (Balk et al., 2002). Acetate-oxidizing bacteria conserve energy in a different way. For example, *Syntrophaceticus schinkii* utilizes acetate through the oxidative Wood–Ljungdahl pathway with no SLP for ATP synthesis; an ATP synthase operon is expressed for converting an electrochemical gradient that is generated by cytoplasmatic proton consumption with hydrogenases and ferredoxins into ATP (Manzoor et al., 2016). Membrane-integral [Ni–Fe] hydrogenases, an

energy-conserving hydrogenase, and a Fd:NADH oxidoreductase/heterodisulfide reductase might be involved in the reverse electron transfer (Manzoor et al., 2016).

5. Perspectives

In energy-limited systems, the syntrophic reaction kinetics is crucial for the degradation efficiency of substrates and the growth rate of organisms, which remains to be determined. Whether growth rate close to the thermodynamic limit jumps in quantum steps or transitions smoothly along the energy spectrum is also unclear. Estimation of the microbial growth kinetics in AD systems and evaluation of the degradation kinetics for different degradation pathways would be meaningful and promising directions for further study; especially, the difference of kinetics and thermodynamics between organisms that use either of the classical MMC or the *Smithella* propionate degradation pathways and, similarly, for syntrophic acetate oxidizers versus acetoclastic methanogens. Detailed studies into the affinities for H₂ and formate of the various methanogens would be helpful. Additionally, the energy conservation systems of some syntrophic metabolizers, such as *S. propionica* remain unclear and require further investigation. Next-generation sequencing (NGS), an ultra-high throughput DNA sequencing method, has been developed for microbial

624 whole-genome sequencing and 16S rRNA sequencing, which provides a platform to
625 open the black box of AD. Significant advances in NGS techniques and biocomputing
626 speeds can generate information on putative functional gene candidates, and map
627 those genes to the reference genomes in the optimal performance of microbial
628 communities. Then, functional biomarkers for the putative digestion microbes of
629 interest can be tailor-made and measured, along with physical and chemical
630 parameters (e.g. H₂, VFAs, pH, etc.), to yield input signals for *in-situ* system
631 biocomputation. Of special interest for monitoring the performance of digesters,
632 especially the association between syntrophs and methanogens, key microbial
633 biomarkers and chemical parameters can be assigned to estimate microbial energetics
634 and kinetics as efficient input signals. An artificial intelligence algorithm can be built
635 for automated *in-situ* system feedback adjustments for digester optimization.
636 Simultaneously, smart stability operation can be achieved by cyber-physical control
637 and thus to drive digesters close to the thermodynamic limits for system optimization.
638 A proteomic approach is also warranted to reveal the regulatory mechanisms and
639 energy metabolism in these PHA-forming syntrophs. Further, of special interest for
640 optimizing the performance of digesters is to integrate the knowledge gained into

smart *in-situ* bio-computation systems to achieve the goal of sustainability in organic waste management.

6. Conclusions

This critical review focuses on the bioenergetics of anaerobic syntrophy including a summary of the functional microorganisms, their metabolic pathways and energy conservation systems, their reaction kinetics as controlled by thermodynamics versus typical Monod reaction kinetics, reversibility of the acetate oxidation process, and estimation of microbial growth. The various routes of interspecies electron transfer, reverse electron transfer, and poly- β -hydroxyalkanoate (PHA) formation in syntrophic communities are also reviewed. Our increased understanding in these areas is gradually opening up the route to strategies to engineer and manage these communities at the limits of thermodynamics.

Acknowledgments

The authors wish to acknowledge the Research Grants Council (RGC) Early Career Scheme Fund (539213), General Research Fund (15273316), Germany/Hong Kong Joint Research Scheme (G-PolyU504/13), Collaborative Research Fund (C7044-14G)

658 and Theme-based Fund (T21-711/16-R), as well as the Hong Kong Polytechnic
659 University-Beijing University of Technology Cooperation Scheme and National Rail
660 Transit Electrification and Automation Engineering Technology Research Center
661 (1-BBYL) for providing financial support. JD acknowledges funding from the
662 Biotechnology and Biological Sciences Research Council (BB/K003240/1;
663 Engineering synthetic microbial communities for biomethane production).

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Table 1. The window of opportunity corresponding to estimated range of H₂ concentration comparing the *smithella* pathway and the methylmalonyl CoA pathway coupled to hydrogenotrophic methanogenesis.

Pathway	Hydrogen concentration at 25 °C (Pa)	Hydrogen concentration at 55 °C (Pa)	Reference
<i>Smithilla</i> pathway with hydrogenotrophic methanogenesis	0.002 - 500.34 ^a	1.8 - 580 ^b	^a This article ^b Dolfing (2017)
Methylmalonyl-CoA pathway with hydrogenotrophic methanogenesis	0.002 - 1.82 ^a	1.8 - 7.3 ^b	^a This article ^b Dolfing (2017)

Conditions: [acetate] = [propionate] = 1 mM; [bicarbonate] = 50 mM; P_{CH₄} = 1 atm; pH=7.

Table 2. Reactions involved in propionate conversion to methane and responsible microbes along with standard Gibbs free energy.

Metabolism	Reactions involved	$\Delta G^{\circ'}$ (kJ/reaction)	Responsible microbes	Reference
Propionate oxidation	$\text{CH}_3\text{CH}_2\text{COO}^- + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 3\text{H}_2 + \text{H}^+$	76.5 ^a	<i>Syntrophobacter fumaroxidans</i> ; <i>Desulfotomaculum thermobenzoicum</i> subsp. <i>thermosyntrophicum</i> ; <i>Pelotomaculum thermopropionicum</i> ; <i>Pelotomaculum schinkii</i>	Chen <i>et al.</i> , 2005; Plugge <i>et al.</i> , 2002; Imachi <i>et al.</i> , 2002; de Bok <i>et al.</i> , 2005
	$2\text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 3\text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{H}^+$	48.4	<i>Smithella propionica</i>	Liu <i>et al.</i> 1999
Hydrogenotrophic methanogenesis	$4\text{H}_2 + \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$	-135.6 ^a	Most methanogens	-
Acetoclastic methanogenesis	$\text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{HCO}_3^- + \text{CH}_4$	-30.96	<i>Methanosarcina</i> and <i>Methanosaeta</i>	Cai <i>et al.</i> 2016; Rotaru <i>et al.</i> (2014b)
Methanogenesis from formate	$4\text{HCOO}^- + 4\text{H}^+ \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O}$	-144.5	Many of hydrogenotrophic methanogens	-

^a Calculation based on G_f° values tabulated by Thauer *et al.* (1977).

Table 3. Kinetic parameters of anaerobic degradation of propionate reported in the literature.

System	Retention time d	Temp. °C	k mg COD /mg VSS-d	K _s mg COD/L	μ _{max} d ⁻¹	Y mg VSS/mg COD	b d ⁻¹	Reference
Steady state, Mixed Cultures	—	25	7.8	1145	0.358	0.051	0.04	Lawrence and McCarty (1969)
	—	33	6.2	246	0.156	0.025	—	Gujer and Zehnder (1983)
	—	35	7.7	60	0.313	0.042	0.01	Lawrence and McCarty (1969)
	14.5	35	—	17	0.13	—	—	Heyes and Hall (1983)
	8.2	35	—	500	1.2s	—	—	Heyes and Hall (1983)
Steady state, single-phase	17	35	6.2	69	—	—	—	Zamanzadeh et al. (2013)
	17	55	17.1	281	—	—	—	
	3.5	35	11	210	—	—	—	
	3.5	55	23.6	504	—	—	—	
Single-phase	7.2	35	11	20	—	—	—	Siegrist et al. (2002)

	6.2	55	33	150	—	—	—	Siegrist et al. (2002)
Single-phase	14	35	3.7	16.5	—	—	—	Heyes and Hall (1983)
	8.2	35	34	495	—	—	—	Heyes and Hall (1983)
Single-phase	20	35	3.5	60	—	—	—	Kalfas et al. (2006)
	20	55	37.8	225	—	—	—	Kalfas et al. (2006)
Single-phase	—	35	13	100	—	—	—	Batstone et al. (2002)
	—	55	20	300	—	—	—	Batstone et al. (2002)
Two-phase	20	35	9	200	—	—	—	Blumensaat and Keller (2005)
	2-4	55	16	400	—	—	—	Blumensaat and Keller (2005)

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Table 4. Comparison of syntrophic propionate degradation using *Smithella* pathway and methylmalonyl CoA pathway.

	<i>Smithella</i> pathway	ΔG^0 (kJ/reaction)	Methylmalonyl-CoA pathway	ΔG^0 (kJ/reaction)
Propionate oxidation	$\text{CH}_3\text{CH}_2\text{COO}^- + \text{H}_2\text{O} \rightarrow 3/2\text{CH}_3\text{COO}^- + 1/2\text{H}^+ + \text{H}_2$	24.18	$\text{CH}_3\text{CH}_2\text{COO}^- + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + \text{H}^+ + 3\text{H}_2$	76.5
Hydrogenotrophic methanogenesis	$\text{H}_2 + 1/4\text{HCO}_3^- + 1/4\text{H}^+ \rightarrow 1/4\text{CH}_4 + 3/4\text{H}_2\text{O}$	-33.9	$3\text{H}_2 + 3/4\text{HCO}_3^- + 3/4\text{H}^+ \rightarrow 3/4\text{CH}_4 + 9/4\text{H}_2\text{O}$	-101.7
Acetoclastic methanogenesis	$3/2\text{CH}_3\text{COO}^- + 3/2\text{H}_2\text{O} \rightarrow 3/2\text{HCO}_3^- + 3/2\text{CH}_4$	-46.44	$\text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{CH}_4$	-30.96
Overall equation	$\text{CH}_3\text{CH}_2\text{COO}^- + 7/4\text{H}_2\text{O} \rightarrow 7/4\text{CH}_4 + 5/4\text{HCO}_3^- + 1/4\text{H}^+$	-56.16	$\text{CH}_3\text{CH}_2\text{COO}^- + 7/4\text{H}_2\text{O} \rightarrow 7/4\text{CH}_4 + 5/4\text{HCO}_3^- + 1/4\text{H}^+$	-56.16

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Table 5. Kinetic parameters of acetoclastic methanogenesis reported in the literature.

System	Retention time d	Temp. °C	K mg COD /mg VSS-d	K_s mg COD/L	$\mu_{\max}$ d⁻¹	Y mg VSS/mg COD	b d⁻¹	Reference
Continuous, Mixed Cultures	—	25	5	930	0.25	0.05	0.011	Lawrence and McCarty (1969)
	—	30	5.1	356	0.275	0.054	0.037	
	—	35	8.7	165	0.357	0.041	0.015	
Batch, Mixed Cultures	—	20	2.6	—	—	0.05	—	van den Berg (1977)
	—	30	2.6-5.1	—	—	0.02	—	
	—	35	2.6-5.1	—	0.08-0.09	0.02	—	
Continuous, Mixed Cultures	4.5	35	11.6	421	—	—	—	Noike et al. (1985)
	6.5	35	6.6	43	—	—	—	
	9.6	35	4.4	15	—	—	—	

<i>Methanosarcina</i>	—	55	46.7	320	1.4	0.03	—	Zinder and Mah (1979)
<i>Methanosarcina bakeri</i>	—	36	15	320	0.44	0.05		Smith and Mah (1978)
<i>Methanothrix</i>	—	33	3.7	30	0.11	0.03		Zehnder et al. (1980)
Acetate enriched culture	—	35	8.5	185	0.34	0.04	0.036	Kugelman and Chin (1971)
Batch	—	30	26	11	0.26	0.01	—	Cappenberg (1975)
Continuous, <i>Methanosarcina bakeri</i>	—	37	8.6	257	0.206	0.024	0.004	Wandrey and Aivasidis (1983)
Methane phase CSTR	—	37	10	642	3.4	0.04		Pohland and Ghosh (1971)
Methane phase CSTR	—	36		4200	0.49			Klass et al. (1978)
Methane phase CSTR	—			395	0.43			Massey and Pohland (1978)
<i>Methanosarcina bakeri</i>	—	37				0.03		Weimer and Zeikus (1978)
<i>Methanothrix soehngenii</i>	—	37	8	45	0.16	0.02		Huser et al. (1982)

Table 6. Catabolism information and energy conservation systems of different syntrophic metabolizers.

Syntrophic metabolizer	Favorable substrate	Syntrophic partner	Metabolic pathway	Substrate level phosphorylation	Electron transport phosphorylation	Electron bifurcation	References
<i>Syntrophobacter fumaroxidans</i>	Propionate	H ₂ - and formate-utilizing <i>Methanospirillum hungatei</i>	Methylmalonyl-CoA pathway	Acetyl-CoA: propionate HS-CoA transferase	Menaquinones; Rnf complex	Soluble electron bifurcation	Plugge et al. (2012) Worm et al. (2011)
<i>Pelotomaculum thermopropionicum</i>	VFAs and alcohols	Hydrogenotrophic methanogens	Methylmalonyl-CoA pathway	Succinyl-CoA synthase	menaquinones	-	Kosaka et al. (2008)
<i>Smithella propionica</i>	Propionate	<i>Methanospirillum hungatei</i>	<i>Smithella</i> pathway	Acetate kinase	-	-	de Bok et al. (2001); Liu et al. (1999)

<i>Syntrophus aciditrophicus</i>	Fatty acids and aromatic acids	H ₂ - and formate-utilizing methanogens or sulfate reducers	Central pathways for benzoate and benzoyl-CoA metabolism; β -oxidation	Acetyl-CoA synthetase (ADP-forming)	Menaquinones; ETF system; Rnf-type ion-translocating electron transfer complex; Membrane-bound iron-sulfur oxidoreductase	-	McInerney et al. (2007)
<i>Syntrophomonas wolfei</i>	4 to 8 carbon saturated fatty acids; unsaturated fatty acids	H ₂ - and formate-utilizing microorganisms	β -oxidation	Acetate kinase	Menaquinones; ETF and a membrane-bound iron-sulfur oxidoreductase; Fix system comprised of a ETF; Soluble acyl-CoA dehydrogenase: ETF complex	Bcd-EtfAB	McInerney et al. (2009); Narihiro et al. (2016)
<i>Syntrophothermus lipocalidus</i>	4-10 carbon saturated fatty acids	Hydrogenotrophic methanogens	β -oxidation	Acetate kinase	ETF-linked ion-sulfur binding (Fe-S) reductase; FixABCX system; Flavin oxidoreductase-heterodisulfide reductase (Flox-Hdr) system along with FixABCX	Bcd-EtfAB	Narihiro et al. (2016); Sekiguchi et al. (2000)

<i>Pelobacter carbinolicus</i>	Alcohols (2,3-butanediol, acetoin and ethanol)	H ₂ - and formate-utilizing methanogens	Glycerol, 1,3-propanediol, 1,2-ethanediol, ethanolamine, choline, ethanol oxidation	Acetate kinase; propanoate kinase; butanoate kinase	Rnf-type ion-translocating electron transfer complex; Membrane-bound, ion-translocating hydrogenases; NADH dehydrogenase complex I	Soluble electron bifurcation	Aklujkar et al. (2012); McInerney et al. (2007)
<i>Geobacter metallireducens</i>	Ethanol	<i>Methanosaeta harundinacea</i> ; <i>Methanosarcina barkeri</i>	Ethanol oxidation	Acetate kinase; acetyl-CoA synthetase	OmcS, a multiheme c-type cytochrome that promotes electron transfer to insoluble Fe(III) oxides and electrodes	-	Rotaru et al. (2014b) Summers et al. (2010)
<i>Desulfovibrio vulgaris</i>	Lactate; Ethanol	Hydrogenotrophic methanogen	Lactate, ethanol oxidization to H ₂ and acetate	Acetate kinase	Cytochrome <i>c3</i>	Flavin-based electron bifurcation	Meyer et al. (2013); Walker et al. (2009)
<i>Syntrophaceticus schinkii</i>	Acetate	Hydrogenotrophic methanogens	Oxidative Wood-Ljungdahl pathway	-	Rnf complex (Unexpressed); Membrane integral [Ni-Fe] hydrogenases; Energy conserving hydrogenase; Fd:NADH oxidoreductase reductase	-	Manzoor et al. (2016)

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