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Review

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Electrochemical communication in anaerobic digestion

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ABSTRACT

Anaerobic microbial consortia in AD or fermentation oxidise or reduce a target organics (including nutrients) in order to share (i.e. extract and utilise) thermodynamic enthalpy energy (i.e. ATP with thermal energy). Herein, the oxidation and reduction (i.e. electrochemical reaction) is a type of biochemical reaction that involves a transfer of electrons or hydrogens between two species and or taxa. The review discusses an electrochemical communication in the bacterial communal society leading to a 'bacterial cartel' which can be a type of struggling for life (to obtain the biochemical energy constantly). Interestingly, syntrophic bacteria (mostly acetogenic bacteria) bind or flocculate the AD bacterial consortia and build two-layer biofilms or bioflocs to obtain the energy while producing a peculiar profile of fatty acids. The hydrolytic fermentative bacteria also dissociate with acidogenic bacteria for an association with the syntrophic bacteria when $\Delta\mu$ approaches at between -200 and -250 mV. Three examples (single-methanogenesis, long-chain fatty acid (LCFA) degradation and acid-fermentation process) explain in the electrochemical origin. This concept remains quite controversial, but if true, may have major implications in broad areas of environmental and biological processes.

KEYWORDS

Bioflocculation; Bioflocs Biofilms; ATP Energy Association; Taxonomic Phylogenetic Analysis; Anaerobic Digestion; Fermentation; Syntrophic Bacteria; Natural Selection

1. INTRODUCTION

Most microorganisms (i.e. biocatalyst in biological process) require a constant supply of 'energy' for cell growth, motility, reproduction, and differentiation. The energy (i.e. enthalpy energy) is provided by highly reactive and unstable compounds (ATPs), which are synthesised and consumed through glycolysis in cooperation with citric acid cycle (CAC). The ATPs are neither rapidly synthesised in cellular cytoplasm [1], nor preserved in any intracellular organs [2], so that the microorganisms intend to store possible energy-rich compounds replaced ATPs. Higher-

level organisms store glucose or ‘glyceraldehyde–3–phosphate (G3P)’ in cytoplasm, where glycolysis occurs for ATPs generation. Under anaerobic culture [3, 4], the energy–rich compounds may be transformed and accumulated as lactate. In prokaryotic cells including bacterial cells (i.e. lower–level organisms without mitochondria or chloroplasts), the glycolysis occurs in an extracellular matrix (between cell-membrane and wall) and the citric acid cycle (CAC) maintains a state of biochemical equilibrium in the cytoplasm. Thus, the prokaryotic cells (including bacterial phyla) can instantly respond to substrates, and the abundances (i.e. biomass X , mg L^{-1}) can be readily estimated by crystallization kinetics [5] or enzymatic kinetics [6, 7] in terms of the rate of substrate consumption (SS , BOD_5 , or COD) per gram cell weight per unit hour.

Anaerobic bacteria particularly store the energy–rich compounds in extracellular culture medium for the constant regulation of glycolysis with CAC [8–10]. An example is a hydrolytic fermentative taxon (e.g. *Saccharomyces*, *Aspergillus*, *Leuconostoc*; *Enterobacter*) attaching to a complex organic particle, where they produce extracellular enzymes to hydrolyse the organic matter and ingest the hydrolysed products (i.e. mainly mono– and/or di–saccharides) [11]. Hydrolytic fermentation occurs in an extracellular matrix of the taxon and cooperates with CAC. The cooperation is based on a trade-off of molecular hydrogen between hydrolytic fermentation and CAC. Once the rate (mol s^{-1}) of the hydrolytic fermentation (i.e. production rate of the energy–rich compound) is greater than the rate of the CAC, the energy–rich compound is slowly converted into fatty alcohols (through acetaldehyde), and then the anaerobic bacteria excrete the alcohols into the extracellular culture medium. Herein, the driving force of fermentation relies on the rate of the CAC which regulate a state of biochemical equilibrium in cytosol. The residual energy–rich compounds are significantly reduced into the alcoholic excreta, which may be further converted into long–chain fatty alcohols (particularly butanol) as reduces water content.

Once the alcoholic excreta are severely accumulated in the culture medium (e.g. >11% w/w ethanol), cell lysis is induced, impacting the cell-membranes and inhibiting the regulation of hydrolytic extracellular enzymes [12].

The anaerobic bacteria [13, 14] also build a communal society with 'syntrophic bacteria' to utilise the energy-rich excreta. Stams group [15] reported that when the syntrophic bacteria produce and spread an ubiquinone (NADH₂ dehydrogenase) out [16], the hydrolytic fermentative bacteria consume the ubiquinone to produce the energy-rich excreta (+ ATPs). A typical, industrial application of the community is an acid-fermentation [17, 18]. In the presence of hydrolytic fermentative bacteria, two communities (or taxa) are participated in acid-fermentation: (i) acidogenic bacteria and (ii) acetogenic bacteria including syntrophic (acetogenic) bacteria. As they are dissociated and associated with the other members of the community, the anaerobic biota are entirely integrated. Once the end-product (carbonates) of fermentation is oversaturated in the broth, the acidogenic taxon oxidising the energy-rich excreta into organic acids, is spontaneously bonded to the hydrolytic fermentative bacteria. They are relatively 'passive donors' of molecular hydrogen, providing as much as the hydrolytic fermentative taxon requires, and they obtain ATP energy building a two-layer structured bioflocs [19]. This is readily observed in a state of electrochemical equilibrium [20-23]. Acetogenic taxon (ii) supporting acidogenic bacterial taxon further converts the organic acids into 'acetate' including molecular hydrogen. The metabolic reaction is thermodynamically nonspontaneous ($\Delta S < 0$ and $\Delta G > 0$), but only feasible under the very low partial pressure of hydrogen (less than approximately 10^{-5} atm) [11, 24, 25]. Observed hydrogen partial pressures have been interpreted as the consequences of 'product inhibition' in acetogens, and the acetogenic efficiency depends strongly on the removal rate of molecular hydrogen in hydrogenophilic methanogenic species [24, 25]. Oh and Martin [11, 20, 26] also reported that the

metabolic reaction in acetogenic taxon (ii) is only thermodynamically spontaneous ($\Delta G \ll 0$) in the presence of methanogenic archaeobacteria, and identified the consortia depending on electrochemical potential (ψ). The electrochemical reaction relies strongly on the syntrophic bacteria. The syntrophic bacteria metabolite 'acetate' to support hydrogenophilic methanogenic bacteria, while they synthesise 'acetate' from carbonates to support acetoclastic methanogenic bacteria.

Anaerobic digestions (ADs) are derived from the 'communal society' where the relative bacterial abundance is intercorrelated with the initial ratio of the inocula. As the community is fully acclimatised in terms of 'biomass' (X , mg L^{-1}) [27], the works [28] seeking for the correlation may have a linear relation to a dynamic distribution of organic loading rates (OLR, $\text{gCOD L}^{-1} \text{d}^{-1}$). In case of hydrolytic fermentative bacteria always observed with acidogenic taxon, the relative abundance relies on the OLR, but strongly depends on a product–inhibition that will be explained in next section. Hydrogenophilic methanogenic bacteria (i.e. *Methanobacteriales*) also coexist with acetoclastic methanogenic bacteria (i.e. *Methanosarcinales*) and the relative abundance is corresponding to the same manners of the hydrolytic fermentative bacteria over acidogenic bacteria. From an ecological perspective, the empirical observations clearly represent a theory of spontaneous generation [29], in which the relative abundance has frequently changed in a product–inhibition (of molecular hydrogen) of the AD process. Hydrolytic fermentative bacteria diminish in a long–term operation [30], and the community disappears completely when methanogenesis (*Methanobacteriales* and *Methanosarcinales*) is dominant. The methanogenic archaeobacteria, thereafter, cooperate with syntrophic acetogenic bacteria struggling for life (to survive). The syntrophic bacteria support the methanogenic bacteria and control the relative abundance of hydrogenophilic methanogenic bacteria over acetoclastic methanogenic bacteria,

which were explained in previous section. It tells us that AD process may be subjected to the theory of Darwin's natural selection [31].

In addition, the AD communal society is based on kinetic studies (in the quasi-steady state), while largely ignoring the underlying electrochemical communication between the interspecies. The conventional approach cannot explain why methanogens and syntrophic acetogens have a great difficulty in metabolising the molecular hydrogen. The solubility of the molecular hydrogen in culture medium (Henry's law coefficient $< 8.58 \times 10^{-4}$ mole atm⁻¹ L⁻¹ in pure water) is very low as is the hydrogen diffusion coefficient ($< 4.50 \times 10^{-9}$ m² s⁻¹ in pure water) between members of the bacterial community. Together these directly prevent a high rate of interspecies transport required to support the observed rates of methanogenesis. More recently, the electrochemical interactions have been examined in terms of biochemical mediators (such as NAD⁺/NADH, NADP⁺/NADPH; HNQ⁺/HNQH). An example is 'quorum sensing' in biological systems [32]. Greater concentrations of NAD⁺ can be achieved (0.8–1.6 mM/g-biomass [33]) in culture medium, but NAD⁺/NADH diffusion is relatively low because of the relatively high molecular weight of these molecules. The proximity of cells tends to mitigate this effect by offering particularly short diffusion pathways in bioflocs (or on biofilms). Reguera group [34] observed peculiar 'nano-wires' between individual cells in microbial consortium (i.e. *Geobacteraceae* in a genus of Proteobacteria). Such wires may also offer routes for electrochemical communication between cells through complex ionic and/or hydrogen-bonded structures (i.e. mechanism of electric conduction). Hydrogen bonding of electrolytic solution is used to explain the anomalously high proton-diffusion coefficient observed in electrolytes [35]. These suggest that interspecies hydrogen transport can occur independently of conventional biochemical carriers, and the success of a bacterial community (particularly in AD process) is attributable to the efficiency of electron and proton transfer (i.e. electrochemical communication).

In this review, anaerobic microorganisms form a 'bacterial cartel' to share a maximum of biochemical energy (i.e. ATP with thermal energy) for cell growth, motility, reproduction, and differentiation. The members of the communal society electrochemically communicate in the bacterial cartel, which is corresponded to the 'struggle for life' that explained in Darwin's natural selection [31]. Herein, the authors discuss an electrochemical communication in the communal society driving bioflocculation in the AD process. The electrochemical communication is simply based on the electron transport between the interspecies, in where syntrophic bacteria particularly buffer the electric loading and manage the relative abundance and diversity. Conventional biokinetic (barrier and inhibition) AD phenomena are particularly discussed in electrochemical origins. Three peculiar examples (single methanogenesis, long-chain fatty acid (LCFA) degradation and acid-fermentation process) are shown in typical AD processes. The authors also support that the observed inhibitions of AD process are attributable to the electrochemical communication driving a bioflocculation of the society. This concept remains quite controversial, but if true, will have major implications in broad areas of environmental biological processes.

2. SYNTROPHIC BACTERIA IN METHANOGENESIS

Methanogenic archaeobacteria (forming methane) metabolise a variety of organics (acetate, formate, methanol, methylamines, and even carbonates), but they are only divided into two metabolic groups (based on a specific substrate: molecular hydrogen): i) hydrogenophilic methanogens utilising molecular hydrogen together with formate, methanol, and/or carbonates. The hydrogenophilic methanogens (in the Phylum *Euryarchaeota*) have a unique wall structured by N-acetylglucosamine cross-linked to N-acetylalosaminuronic acid [36] which is capable of chelating with bacterial walls structured by peptide bonds. So, they are capable of flocculating with bacterial taxa, e.g. syntrophic acetogenic bacteria [37, 38]. In addition, they enable

syntrophic acetogenic bacteria (that have a hydrogenophilic metabolism with carbonates) utilising energy which is produced from hydrogenophilic methanogens. The metabolism of hydrogenophilic methanogens (cooperated with the syntrophic acetogenic bacteria) is analogous to the catabolic reaction of ii) acetoclastic methanogenic archaeobacteria, which have been simply described to produce methane in acetate solution [39, 40].

The methanogenic archaeobacteria also cooperate with syntrophic acetogenic bacteria (particularly, *Clostridium aceticum*, *Acetobacterium woodii*, and *Clostridium thermoaceticum*) in AD processes [41-43]. A few genera (*Methanosarcinaceae* and *Methanosaetaceae*) can attach or detach to the bioflocs of syntrophic acetogenic bacteria (as described above), but the attachment and/or detachment not only rely on the hydraulic retention times (HRTs) and the sludge retention times (SRTs) but also they are related to bacterial abundances [44, 45]. In particular, the syntrophic acetogenic bacteria supply acetate into the metabolic route of acetoclastic methanogens to produce energy or they accumulate and store acetate in the cytosol [46] or cysts [47] of acetoclastic methanogens under a starving condition of acetate. Thus, methanogenic archaeobacteria coexist with syntrophic acetogenic taxa [48, 49], which is corresponding to an association of the communal society for 'struggling life'. **Figure 1A** shows a schematic diagram of the methanogenic bioflocs.

<Figure 1> [19, 50]

In the theory of spontaneous generation (in ecological perspective), the relative abundance and diversity of the methanogenic archaeobacteria are correlated to the initial ratio of the inocula, but they can fluctuate in accordance with the distributional profile in organic loading rate (OLR, $\text{gCOD L}^{-1} \text{d}^{-1}$). Hydrolytic fermentative *Bacteroidetes* and syntrophic *Proteobacteria* are dominant in mesophilic AD systems [30], while they vanish in a long-term operation and

acetoclastic methanogenic archaeobacteria (i.e. *Methanosarcinaceae* and *Methanosaetaceae* in the Phylum *Euryarchaeota*) account for 50–75% of enrichment (shown in **Table 1**), where the methanogens consist of conventional members in the orders *Methanobacteriales*, *Methanococcales* and *Methanosarcinales*. In thermophilic conditions, hydrogenophilic methanogens of *Methanobacteriales* are particularly dominant, although acetoclastic methanogenic archaeobacteria (i.e. *Methanosarcina* and *Methanosaeta*) are present [51–53]. These observations are corresponding to the syntrophic bacteria (degrading acetate into carbonates) and strongly collaborating with the hydrogenophilic methanogenic bacteria.

<**Table 1**> [54–58]

Interestingly, syntrophic bacteria rarely studied in the microbial diversity of AD processes, but Oh and Martin [26] evaluated the catabolic reactions of methanogenic bioflocs associated with syntrophic bacteria in a electrochemical field ($\Delta\psi$). The electrochemical field ($\Delta\psi$) represents in a state of electrochemical equilibrium, but also exhibits a nearly instant kinetic behaviour in electron and proton transport. Thus, the electrochemical field ($\Delta\psi$) enable to represent typical kinetics of metabolic reactions transferring electrons and hydrogens between the interspecies of AD bioflocs. Herein, methanogenic archaeobacteria reside in electrochemically polarised areas between –250 and –200 mV SHE [59]. The overall metabolic reactions convert a high energy–rich compound (i.e. acetate produced by syntrophic acetogenic bacteria) into lower energy–rich compounds ($\text{CO}_2 + \text{CH}_4$), and then generate a maximum ATPs (including thermal energy) for the cell maintenance. Although the catabolism is exothermic, the lower energy–rich compounds ($\text{CO}_2 + \text{CH}_4$) consume the thermal energy to expand the vapour phase because of their low solubility under isobaric conditions. The proton concentration (pH) adjusts the solubility of carbon dioxide and affects the partial pressure of methane (pCH_4) in the vapour phase. The ratio of partial pressures (CO_2/CH_4) instantly re–evaluates the electrochemical field ($\Delta\psi$), directly

affecting the degradation of acetate in the methanogenic bioflocs. A previous study [26] find out that the solubility difference between carbon dioxide and methane positively influences the bioflocs to persistently demand thermal energy and to maintain to reside in electrochemically polarised regions between -250 and -200 mV SHE (**Figure 2**). The electrochemical polarisation of methanogenic bioflocs is further discussed in next section 3. This suggests that thermophilic process (in isochoric conditions) results in better digestion of molecular acetate, but it needs to prevent a vaporisation of carbon dioxide from the digested liquor. The thermophilic digestion may be much more efficient in a neutral range of pH (approximately pH 7–8 in a strong buffer solution), which makes overall catabolism exothermic.

<Figure 2> [26]

3. SYNTROPHIC BACTERIA IN ACETOGENESIS

Broughton [60] suggested that acetogenic bacteria (including syntrophic acetogenic bacteria) degrade long-chain fatty acids (LCFAs) to acetate via β -oxidation and require the methanogenic bioflocs to reduce the inhibitory levels of acetate (and molecular hydrogen) produced from acetogenic bacteria. The acetogenesis is the major 'limiting step' [39, 61-65] of AD processes, which is closely related to the concentration of LCFAs in the feedstock, and the high level of concentration in LCFAs leads to a failure in conventional AD processes. However, some researchers [66-69] reported that the failure is mainly attributable to the methanogenic bioflocs floating on the surface of the digested liquor, and simultaneously the LCFAs inhibition (of the methanogenic bioflocs) in the result of LCFAs-adsorption to the cell wall (and/or membrane), affecting the metabolic processes of transportation [70-72].

In electrochemical thermodynamic perspective, the acetogenesis is entirely nonspontaneous and endothermic ($\Delta H > 0$, $\Delta S < 0$ and $\Delta G > 0$) [11]. The acetogenic taxon requires the spontaneous methanogenic bioflocs so that the overall bioflocs process (i.e. acetogenesis together with methanogenesis) becomes spontaneous although the microbial consortia require a long-term operation to be associated in the complex liquors. Nonspontaneous acetogenesis also requires enthalpy-driving energy (released from the spontaneous methanogenesis) and releases electrons and protons. Strong methanogenesis uses the electrons and protons to release the enthalpy-driving energy [11, 24, 25]. There is no empirical evidence that the enthalpy energy (i.e. ATPs including thermal energy) is exchanged between the AD interspecies, but a few community (acetogenic bacteria conjugated with methanogens) is identified and isolated in the AD bioflocs [73-75]: *Smithella* and *Syntrophobacter* of *Delta-proteobacteria* in butyrate solutions [38, 43, 76, 77] and *Syntrophomonas* of *Firmicutes* in propionate solutions [78, 79]. The empirical evidence indicates that the acetogenic bacteria coexist in methanogenic bioflocs including syntrophic bacteria. **Figure 1B** shows a schematic diagram of the two-layer biofilm (covered with acetogenic bacteria), indicating electrochemical pressurising on the hydrogenophilic methanogens to form methane. This may explain why most methanogens reside (between -250 and -200 mV SHE [80]) in electrochemically polarised regions.

The acetogenesis was studied [11] on methanogenic bioflocs (including syntrophic bacteria) for determining the limits of the electrochemical potential (ψ) which is an intensity of electrochemical communication between interspecies. The intensity is instantly self-adjusted through the gradient of ψ and always reaches a state of electrochemical equilibrium. A change in the $\Delta\psi$ leads to the production of enthalpy energy (ATPs + thermal energy by methanogenic bioflocs) and the energy is distributed to the counterpart of members (i.e. acetogens including syntrophic bacteria) requiring the energy. **Figure 3A** shows the overall enthalpy changes in the

ADs of individual LCFAs solutions respectively. Two contrasting cases are shown in the ADs of LCFAs: exothermic but nonspontaneous process ($\Delta H < 0$ and $\Delta S < 0$) in a low LCFA concentration; endothermic but spontaneous process ($\Delta H > 0$ and $\Delta S > 0$) in a high LCFA concentration. A high concentration of LCFAs may be better digested when additional thermal energy is provided in thermophilic digesters, and the overall driving force results from the thermal energy (which is linearly correlated to the LCFA concentrations). However, in the former case, the AD is completely nonspontaneous at low LCFA concentrations. In this circumstance, low LCFA concentrations (at $\Delta H \approx 0$) may be in an insoluble state such as ‘hydrophobic aggregation including emulsion state’ in the aqueous phase. The process may limit the overall AD and, if so, the ADs of the LCFAs are only governed by the kinetics of dissociation (or disintegration) from hydrophobic aggregation. This is not supporting any empirical evidence, but **Figure 3C** shows that as the hydrophobic tail is elongated, the solubility of LCFA is decreased. **Figure 3B** shows that as the hydrophobic tail is elongated, the acetogenic bioflocs (with methanogens) promote a methane formation. This suggests that acetogens readily absorb the LCFAs into the cell wall or membranes (c.f. **Figure 1B**) and oxidise the LCFAs along with the $\Delta\psi$ generated by methanogenic bioflocs. Although it is stoichiometrically verified, this appears in sharp contrast to numerous empirical results [63, 81-86]. The most of empirical observations explain that the microbial inhibition is directly related to the inhibition of ψ network (following a short proximity distance of the $\Delta\psi$). In the observed biochemical reaction (propionate to acetate) [87, 88], the electron transport occurs instantly in between acetogens and methanogens in two-layer bioflocs and Oh’s $\Delta\psi$ model supports the empirical lines of evidence in electron interspecies [24, 25, 89, 90]. The ADs of LCFAs are illustrated in metabolic coordination shown in **Figure 3D**.

<Figure 3> [11]

In conclusion, syntrophic acetogenic bacteria construct a methanogenic block (sharing biochemical metabolites) associating with hydrogenophilic methanogenic bacteria and acetoclastic methanogenic bacteria. The acetogenic bacteria show a peculiar (obverse or reverse) metabolism in accordance to the strength of $\Delta\mu$ (built by hydrogenophilic methanogenic bacteria). The $\Delta\mu$ is correlated to the proximity distance of the interspecies and indirectly related to the microbial acclimatisation of the bioflocs. As the bioflocs excretes methane and carbonates, the bioflocs produces and shares ATPs with the other members of the bacterial community. A vaporisation of carbonates shifts from exotherm to endotherm in the overall AD process. This suggests that a fixed pH value (under an isochoric process) may prevent significant vaporisation of carbonates exhausting a large amount of thermal energy. Interestingly, acetogenic bacteria (oxidising LCFAs on the extracellular membrane) demand a large amount of enthalpy energy (ATP + thermal energy), and the process is entirely nonspontaneous. The energy includes cracking a state of hydrophobic aggregation on the bacterial wall or membrane. The methanogenic bioflocs adjusts a thermodynamic spontaneity of acetogenesis as supplying the energy to the acetogenic bacteria. The overall AD process in the methanogenic bioflocs relies on the electric driving force of the hydrogenophilic methanogens, which drive syntrophic acetogens to reverse the metabolism. A vaporisation of carbonates also induces a disintegration of the interspecies (syntrophic bacteria with hydrogenophilic methanogenic bacteria) and then promotes acetoclastic methanogens. In this case, acetogenesis is steadily inhibited although a large amount of thermal energy is supplied.

4. SYNTROPHIC BACTERIA IN ACID-FERMENTATION

Most hydrolytic fermentative bacteria included in the phyla *Actinomycetes* [91], *Bacteroidetes* [92], *Chloroflexi* [93], *Firmicutes* [94] and *Proteobacteria* [95] participate in acidogenesis of sugars to produce volatile fatty acids (VFAs), while they significantly accumulate VFAs in AD

processes [52, 53, 98, 99] associating with acidogenic bacteria (genus *Anaerolineaceae* in the phylum *Chloroflexi* [96], and genera *Bifidobacterium* and *Paludibacter* in the phylum *Bacteroidetes* [97]). Ahring group [66], Lettinga group [67] and Nielsen group [100] investigated an range of high organic loading rates (approximately $1.4\text{--}3\text{ kg VS m}^{-3}\text{ d}^{-1}$) causing a high growth of hydrolytic fermentative bacteria over acidogenic bacteria, but also leading to a sudden accumulation of VFAs in overall AD processes. The accumulated VFAs are further slowly degraded and accumulated into ‘acetate’ by the two-layer biofilms (the acetogenic bacteria together with methanogenic bioflocs as described above). However, the empirical observations are sharply contrasted in Le Chatelier's principle under the electrochemical communication in the bioflocs. The acidogenesis is corresponding to the acetogenesis in a strongly nonspontaneous process ($\Delta G > 0$ and $\Delta S < 0$). The acetogenic taxon having a positive field ($\Delta\psi$) also repels from the analogous field ($\Delta\psi$) established in the acidogenic taxon, and then attracts to methanogenic bioflocs producing ATPs. When the proximity between the interspecies (acetogenic taxon–acidogenic taxon) is steadily decreased, the $\Delta\psi$ increases and acetogenic metabolism is readily inhibited (**Figure 1B**), but when the $\Delta\psi$ nearly vanishes, the acetogenic taxon (together with syntrophic acetogens) not only supports the existing hydrolytic fermentative bacteria producing ATPs, but also synthesises ATPs by themselves (**Figure 4A**). This directly indicates that the overall AD process relies on electron (and proton) transport in acetogenesis [11, 24, 25]. The exchange of electrons and protons ($\Delta\psi$) between the interspecies (acetogenic taxon–acidogenic taxon), substantially affects a ‘bioflocculation’ in (between hydrolytic fermentative bacteria and methanogenic bacterial community) which may impair the kinetic performance of AD processes.

<Figure 4>

In particular, the hydrolytic fermentative bacteria associate with syntrophic bacteria (acetogenic bacteria) to produce shorter volatile fatty acids and dissociate with acidogenic bacteria. The microbial association (**Figure 5A**) was electrochemically studied in disaccharides–fermentation [101]. The electron and proton (produced in the fermentation) are assumed to conduct through the ψ network between the interspecies. The hydrolytic fermentative bacteria ferment disaccharides (maltose) into fatty alcohols through the intracellular, metabolic pathway, and then produce enthalpy energy (i.e. ATP including thermal energy). The process entirely relies on a peculiar metabolism designed in the industrial microbial species (or the hydrolytic fermentative bacteria cooperated with an enzymatic catalyst) [102, 103], which may produce butanols in the extracellular matrix. **Figure 5B** shows a process coordination in glucose–fermentation. The red solid line represents the fermentation of hydrolytic fermentative bacteria and the red dotted line represents the biosynthesis of ethanol (up to butanols) that may occur in the extracellular matrix. There is no evidence that the enthalpy energy produced by hydrolytic fermentative bacteria is distributed to acidogenesis (along with acetogenesis), but as both (acidogenic and acetogenic) bacteria provide electrons and protons into the overall metabolism of hydrolytic fermentative bacteria, they convert fatty alcohols into long–chain VFAs (caproate $\ll$ valerate $\approx$ butyrate). They further oxidise the long–chain VFAs to shorter–chain VFAs (propionate or acetate). The acidogenic bacteria produce a small amount of enthalpy energy, although acetogenic bacteria (explained in the previous section) require more energy (than that produced from acidogenesis). The transferred energy is not sufficient to completely decompose whole VFAs through the β oxidation, so that the bacterial community (acidogens with acetogens) utilises the enthalpy energy produced by hydrolytic fermentative bacteria.

As the fermentation is almost completed, carbonates are saturated in the liquor and then syntrophic acetogenesis (accumulating acetate) spontaneously increases. The accumulated

acetate in the culture medium can be correlated to the ATPs production of the syntrophic acetogenesis. Residual shorter-chain VFAs (valerate $\approx$ butyrate $\ll$ acetate) show the largest proportion of the digested liquor (except for water). It was known as an '*acetoclastic inhibition*' in conventional fermentation processes, but Oh group [101] represented it as a microbial association excreting 'acetate' to store a maximum amount of residual energy and building a peculiar structure of biofloculation. **Figure 5A** shows a mole-production of acetate in the initial mole of palm oil waste [104], lactose [105], and cassava [106]. However, the overall process (in a short-term operation) carry out cell lysis (i.e. disinfection process) on a high concentration ($<$ approximately 13.7% v/v) of acetate [107], and this has been widely applied for organic food preservations (approximately 15% acetate v/v in industrial pickling applications [108]; 3–5% v/v in household sauerkraut applications [109]).

As the carbonates (produced from CAC of hydrolytic fermentative bacteria) are oversaturated and significantly vaporised, the single-phase fermentation (was explained) is separated into two-phases (vapour and liquid). The phase transition (utilising a large amount of thermal energy) shows an inhibition of the accumulation in acetate, but the proton concentration is dramatically increased in the culture medium (**Figure 5B**). As the pH value is decreased, the bioflocs are clearly dissociated between syntrophic acetogens and fermentative bacteria in the bacterial community. Once the pH value reaches the steady state, the bioflocs accumulates long-chain VFAs (mostly caproate, valerate, and butyrate). This was known as '*acetogenic inhibition* or pH inhibition', but it was found that the inhibition is inevitable in acid-fermentative process. **Figure 5C** shows that Δp slowly and steadily increases as the maltose concentration increases (in the single phase fermentation), whereas the Δp rapidly and dramatically increases in the two-phase fermentation. This, in part, explains why fermentation should be operated in a closed system to maintain syntrophic acetogens. Together with these empirical observations of acid-fermentation

indicate that the inhibition behaviour is attributable to the microbial dissociation/association (in terms of bioflocculation) underlining thermodynamic spontaneity, rather than exclusively to biokinetic sources. It also indicates that an acidification in fermentation (or anaerobic digestion) can be simply resolved by mixing (or recirculation) of the fermented broth and the physical vaporisation of carbonates regulates the reversible syntrophic acetogenesis [110].

<Figure 5> [104-106, 111]

Interestingly, Lee group [112] observed a peculiar glucose digestion of which the formation of methane could not be observable in fully acclimatised bioflocs. The digestion of glucose initially followed a conventional fermentation through the α route (**Figure 6B**). The fermentative products (i.e. fatty alcohols) might be steadily and slowly converted into VFAs (i.e. butyrate and valerate) by an (acidogenic–acetogenic bacterial) consortium (already explained), whereas the acetate production was completely nonspontaneous. As carbonates were fully accumulated in approximately 10 days operation of the AD, syntrophic acetogens (producing acetate) appeared and converted carbonates into acetate in the ADs. Acetoclastic methanogens simultaneously converted acetate into methane and carbonates. After 15 days of the operation, acetogens spontaneously accumulated shorter–chain volatile fatty acids, revealing a linear relationship with the accumulation of 'propionate including butyrate'. It still remains unclear whether the hydrolytic fermentative bacteria produce and diffuse acetate around the extracellular membranes, but it was found that the hydrolytic fermentative bacteria discharged 'acetate' through the CAC route using syntrophic acetogenesis, and then the existing acetogenic bacteria slowly synthesised 'acetate' into longer chain VFAs (propionate and butyrate). This process was thermodynamically spontaneous underpinning the assumption of *reversible acetogenesis* [113, 114]. **Figure 6A** shows the enthalpy changes in the coordinated process, shifting from the initial α route to the β

route. After 30 days of the operation, hydrogenophilic methanogens secondarily increased a methane formation.

<Figure 6> [111].

The empirical observation has a good agreement with the works of Oh and Martin [111], who observed a network of Δp that mutually transferred electrons and protons in AD interspecies of bioflocs. The transport of electrons and protons is assumed to be instantaneous in a quasi-steady state of AD microbial growth. The glucose injected is completely converted into methane and carbonates, although residual concentrations of VFAs are observed in very small numeric values over the range of glucose concentration. The Δp network shows that acetate is consistently presented at the highest concentration of VFAs. In the presence of acetoclastic methanogens, acetate accumulation is unfavourable over the range of glucose concentrations (though slightly less than propionate). This result is no overall thermodynamic basis for ‘feedstock inhibition’ observed by the Ahring group [66], Lettinga group [67], and Nielsen group [100]. Interestingly, **Figure 7A** shows a plot of the carbon fraction of initial substrate remaining in acetate at equilibrium versus the mole fraction (x) of glucose. At a low range, $x < 0.001$, the acetate fraction is strongly dependent on the concentrations of ethanol, indicating that ethanol production is logarithmically correlated with acetate production. The dependence becomes less strong as the concentration increases, demonstrating that glucose concentration adjusts to the fermentation α route. In the range $0.001 < x < 0.1$, as x is increased, the acetate fraction (largely independent of ethanol concentration) decreases. The rapid transition (between ethanol dependence and independence) is attributable to the rapid increase in proton transport. **Figure 7B** shows the rapid decrease in pH value, where the existing ammoniacal nitrogen is completely oxidised. The agreement is the best at higher initial ethanol concentrations compared to empirical

observations [112, 115]. Together with these results suggest that in the digestion of ethanol, the observed "inhibition" of acetoclastic methanogenesis (relative to glucose digestion) is attributed to overall electrochemical origins (particularly the depletion of enthalpy energy to decompose acetate by syntrophic acetogens) rather than exclusively biokinetic sources. **Figure 7C** shows that the electrochemical potential ψ is dramatically increased as glucose concentration is increased to approximately $x = 0.001$, where the metabolism is shifted from the α route to the β route.

<**Figure 7**> [112, 115, 116]

In conclusion, hydrolytic fermentative bacteria catabolized disaccharides for consistent ATP energy and then utilised the by-products (i.e. electron and proton, or NADH_2) spontaneously for the production of fatty alcohols. Subsequent acidogenesis produces VFAs from fatty alcohols, but it is found that hydrolytic fermentative bacteria electrochemically cooperated with syntrophic acetogenic bacteria accumulate acetate (known as acetoclastic inhibition). The syntrophic acetogenic process is energetically related to the CAC in the metabolic pathway of hydrolytic fermentative bacteria, so that VFAs are not detected (except for the highest concentration of acetate) in single-phase fermentation. So, the overall process continues to accumulate 'acetate' in the culture medium and the pH value steadily decreases. In a long-term operation (> 15 days), the reverse metabolism of (slow but spontaneous) syntrophic acetogens produces shorter-chain VFAs (butyrate $\approx$ valerate $\ll$ acetate). The spectrum of VFAs is entirely dependent on the overall production of enthalpy, although acetate is the largest concentration followed by butyrate. Interestingly, as carbonates are significantly vaporised, the syntrophic acetogens detach from the hydrolytic fermentative bacteria. Residual energy may be rapidly utilised for the β oxidation of residual VFAs as encouraging acidogenesis from the fermentative products. The spectrum

(acetate < butyrate <<valerate) of VFAs is entirely dependent on the balance of residual enthalpy energy (i.e. ATPs with thermal energy). As the vapour phase hypothetically increases, the feeding sugars are converted and accumulated into 'acetone' around the saturated vapour pressure of water in the isochoric condition [101].

5. PERSPECTIVE

In this review, the authors discussed a bacterial community (i.e. syntrophic bacteria) driving bioflocculation (in terms of microbial association) in anaerobic digestions (ADs) and fermentation. The bacterial community builds a 'bacterial cartel' to share a maximum of biochemical energy (i.e. ATP with thermal energy) for cell growth, motility, reproduction, and differentiation. As the members of the bacterial cartel electrochemically communicate with each other, the electron and hydrogen are transported to share a maximum of energy between the interspecies. Particularly, syntrophic bacteria buffer the electric loading and manage the relative abundance and diversity in between the interspecies. The relative abundance and microbial diversity in the AD process can be correlated with the initial ratio of the inocula in the theory of spontaneous generation, but corresponded to the 'struggle for life' that explained in Darwin's natural selection. However, the dynamic bacterial community converges into a biofloc (or biofilm) to utilise the internal energy of peculiar organics (including nutrients) while the microbial association shares thermodynamic enthalpy energy (i.e. ATP with thermal energy). This review may be supported by the theorem of Darwin's Natural Selection (DNS) in part of microbial evolution (i.e. genetic heritage through evolution), but it strongly emphasizes a 'microbial association' against struggling for bacterial life (in the AD process and or acid-fermentation). This concept remains quite controversial, but if true, may have major implications in broad areas of environmental and biological processes. This approach not only provides useful

information on structural bioflocs (and/or biofilms) dynamically associated (or dissociated) with the other member of the bacterial consortia but also adds new enrichment strategies.

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FIGURE CAPTIONS

Figure 1. Schematic diagrams of methanogenic bioflocculation, based on electrochemical thermodynamics with taxonomical analysis. (A) The methanogenic bioflocs consist of hydrogenophilic methanogenic bacteria, acetoclastic methanogenic bacteria, syntrophic acetogenic bacteria, and anaerobic ammonium oxidising bacteria; (B) The bioflocs consist of acetogenic bacteria and methanogenic bioflocs; Reprinted from Plumb group [50] observed bacteria with archaeobacteria in an anaerobic baffled reactor (ABR) sludge flocs, which was detected with probes the ARC915 (red) and EUB338 (green); Sekiguchi group [19] observed bacteria with archaeobacteria in sludge granules in an anaerobic sludge blanket reactor (ASBR); Copyright © 1999, American Society for Microbiology

Figure 2. Enthalpy changes in methanogenesis at 25 °C and 1 atm [26]. One kilogram of acetate solution (injected into a closed batch AD reactor) was simulated at constant P (1 atm) and T (298.15 K). As the initial moles of acetate (HAc) in the system was increased, the initial moles of solvent water (H₂O) was decreased to conserve the system mass of 1 kg ($H_2O \cdot 18.02 + HAc \cdot 60.04 = 1000(g)$). The enthalpy changes in methanogenesis according to the change in end-products: (CO₂ (aq.) + CH₄ (aq.)), (CO₂ (aq.) + CH₄ (g)) and (CO₂ (g) + CH₄ (g)) respectively against the increase in the initial mole fraction (x) for HAc. The CO₂ vaporisation significantly increases the overall enthalpy change rather than other vaporisation of metabolites.

Figure 3. Energy balance between consumption of acetogenesis and production of methanogenesis in anaerobic LCFA-digestion. The electrochemical potential ψ equilibrium was simulated in an isothermal and isobaric (298.15 K and 1 atm) batch digester. The initial substrates were chosen from saturated fatty acids from acetic acid (CH₃COOH) to stearic acid (CH₃(CH₂)₁₆COOH). The AD of the saturated LCFAs is assumed to be initially operated under the best composition of microorganisms (acetogens and methanogens). Acetogens degrade the LCFAs to produce acetate and residual shorter-chain fatty acids. The residual acetate, carbon dioxide and hydrogen are removed by methanogenic bacteria (methanogenesis). The process was based on the reaction coordinate from the LCFAs degradation to the methane formation. (A) The enthalpy change versus the initial mole fraction of LCFAs in a variety of anaerobic LCFAs digestions [11]. (B) Ratio of partial pressure (CH₄:CO₂) in the LCFA-digestion [11], (C) The ratios of yield (CH₄:CO₂) and solubility of LCFAs, calculated by stoichiometric relationship at the state of equilibria (in phase transition, dissociation, and electrochemical potential), (D) enthalpy consumption and production in the reaction coordinate, where the LCFAs are acetic (HC2), propionic (HC3), butyric (HC4), hexanoic (HC6), octanoic (HC8), decanoic (HC10), lauric (HC12), myristic (HC14), palmitic (HC16) and stearic (HC18) acids.

Figure 4. Schematic diagram of the bioflocculation in fermentation without methanogenesis, where (A) two feasible routes of metabolic pathway and (B) enthalpy changes in fermentation via metabolic coordinate

Figure 5. Thermodynamic inhibition in disaccharide-fermentation [111], where the single-phase was separated into two-phases at the dotted line; (A) accumulated acetate concentration (B) pH value, and (C) electrochemical potentials (ψ) versus mole fraction of disaccharides (at 1 atm and 298.15 K), ● empirical observation of palm oil waste-fermentation [104], ○ lactose-fermentation [105], and ◇ cassava-fermentation [106]; The range $10^{-2} < x < 1$ shows that as the mole fraction of maltose was increased, the acetate fraction steadily decreased. The low range, $10^{-3} < x < 10^{-2}$ shows that the acetate fraction was strongly dependent on the concentrations of maltose. The 'rapid transition' in the range $10^{-4} < x < 10^{-3}$, was attributable to the rapid phase transition that occurred as carbonates were vaporised into the vapour phase.

Figure 6. Energy balance with energy consumption and production in anaerobic glucose digestion (A) [111], based on reaction enthalpies. Fermentation is thermodynamically coupled with methanogenesis through syntrophic acetogenesis as the fermentation is coupled with acidogenesis. The LCFAs are acetic (HAc), propionic (Hpro), and butyric (HButy) acids. (B) Schematic diagram of bioflocculation in the AD process.

Figure 7. Batched AD fed ethanol stillage in an isobaric 1atm and 298 K, where x is mole fraction of glucose in 1 litre of batch scale. (A) Fraction of initial substrate carbon remaining in acetate versus mole fraction (x) of glucose in anaerobic ethanol stillage digestion. Empirical biogas production: ○ Typical batched glucose digestion process [112] with 0.05 mole L⁻¹ ammonium concentration, shows reasonable agreement throughout the digestion period. ● Typical batched ethanol digestion process [115] shows qualitative agreement with the model. (B) pH value in the

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TABLE CAPTIONS

Table 1. Relative abundance of methanogens and bacteria at family/genus level in mesophilic AD

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Phylum	Class	Order	Genus/family	Single-phase AD [55]	Single-phase AD [56]	Two-staged AD (Acidogenesis) [57, 58] [54]	Two-staged AD (Methanogenesis) [57, 58] [54]
Euryarchaeota	Methanomicrobia	Methanomicrobiales		10±2%	8.10%	6.20%	17.60%
			Incertae sedis (Methanoregula)	36±34%	3.90%	2.70%	1.00%
			Methanospirillaceae		0.40%	1.10%	0.70%
			Methanomicrobiaceae(Methanoculleus)		3.20%	1.70%	1.90%
			Incertae sedis (Methanocalculus)				
			Methanocorpusculum				
		Methanosarcinales		51±30%			
			Methanosetaceae		0.10%	0.30%	8.20%
			Methanosarcinaceae		0.20%	0.20%	5.80%
		Methanocellales	Methermicoccaceae				
			Methanocellaceae				
	Methanobacteria	Methanobacteriales		0.2±0.3%			
	Methanococci	Methanococcales					
	Methanopyri	Methanopyrales					

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Highlights

- A microbial association is described in a dynamic bacterial community.
- The dynamic bacterial community is subjected in Darwin's natural selection.
- The strong microbial association shares thermodynamic enthalpy energy.
- Biokinetic observations are discussed in electrochemical origins.
- This discussion enables to revisit a fundamental bio-flocculation.