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Ecological responses to substrates in electroactive biofilm: A review

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Substrate as the electron donor of bioelectrochemical system (BES) has fateful impacts on the microbial community composition of electroactive biofilm (EAB), via the selection upon functional microorganisms such as exoelectrogens, fermenters and methanogens, as well as their interactions. Electrochemical performance as the terminal reflects of electroactivity and the correspondence between community members have been summarized. Exoelectrogens responsible to the conversion towards electricity from their respective preferred substrates such as acetate, propionate, glucose and cellulose has been found to be finite in a small range, e.g., *Geobacter*, *Shewanella* and *Pseudomonas*. Their demands of micromolecular electron donors and the selective pressure of primary substrates facilitate the existence of competitive or cooperative biological processes to exoelectrogenesis. The inherent mechanisms of the dynamics of such interactions have been explored with electrochemical methods, defined co-culture experiments and community analysis. Complete view of the metabolic network in electroactive microbial communities has been shed light on, and appeals further investigation.

substrates, electroactive biofilm, bioelectrochemical systems, fermentation, methanogenesis

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1 Introduction

Bioelectrochemical system (BES) is a promising technology turning up in recent years. With exoelectrogens as the catalyst on electrodes, BES has the ability to achieve a plethora of practical applications such as hydrogen production, value-added product recovery, toxicity or organic pollution detection, desalination and electricity generation [1]. As proposed by scientists, BES can be named as MXC, where the "X" means the function, including microbial fuel cell (MFC), microbial electrolysis cell (MEC), microbial desalination cell (MDC), etc., [2–4]. Exoelectrogens are microorganisms capable of releasing or capturing electrons from an electrode, sometimes bidirectionally [5–7]. Through their metabolic reactions, electrons are exchanged between external donors (i.e., substrate) and acceptors (i.e., electrode) thus complete the circuit and enable the conversion of diverse energy forms. This ability known as extracellular electron transfer (EET) has been illuminated through at least three mechanisms: via outer-membrane proteins such as c-type cytochromes [8–10], conducted by nanowires [11–13] or mediated by self-produced soluble electron shuttles or added mediators [10,14].

From the practical point of view, mixed communities still possess the advantage of electricity generation compared to pure or defined co-cultures [15]. In a mixed community, what kinds of character exoelectrogens play and how to enrich an effective electroactive microbial community still remain questions worth dealing with. Substrates provide carbon and energy source in any biological process, thus have notable impact on the structure and functions of mixed communities, which is of vital importance in the enrichment and operation of electroactive microorganisms as well. Typical substrates preferred by type strains of exoelectrogens

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have been thoroughly illuminated such as acetate to *Geobacter sulfurreducens* [15,16] and lactate to *Shewanella oneidensis* [15,17]. However, speaking of the practical applications, BESs cannot be always operated with synthetic wastewater containing merely defined nutrients. The impacts of multiple substrates on the following aspects therefore need to be clear: (i) the structure of bacterial communities; (ii) the performance of BES along with its inherent mechanism and especially; (iii) the ecological interaction between exoelectrogens and their fermentative or syntrophic partners.

There have been efforts throwing light on the influence of substrate on the composition of bacterial community, especially in the initial stage or on fluctuant conditions. Yet these researches have not been reviewed. This article has been aimed at summarizing the literature pertaining the substrate-dependent dynamics of bacterial communities and the in-depth inter-species relationships related. The electrochemical performance as the consequence and reflect of the inner variation of anodic communities, has also been reviewed as the opening of story. Studies related to complex substrates such as undefined domestic, industrial or agricultural wastewater have been roundly reviewed elsewhere [18,19], most of which focus more on the applicative modification rather than the inherent composition and dynamics, therefore was not discussed here. For the further exploration in this field, the methodologies utilized up to now are also summarized.

2 Electricity conversion priorities of different substrates

Based on differences in the diets of exoelectrogens, there exist differences in the priorities of electricity conversion among substrates, reflecting as different electrochemical performances. Here, electrochemical performances depending on substrates in terms of mainly power density (PD) and Coulombic efficiency (CE) are reviewed to summarize the tendency. Fermentable and non-fermentable substances have been investigated, some of which showed decent performance such as acetate and glucose, while some of which found barely no electroactivity like methanol [20] and i-butyrate [21]. Such priorities due to the differences in microbial communities hint the importance of microbiological understanding in BESs. Therefore, functional microorganisms corresponding to specific substrates are also reviewed to make attempts to explain the mechanisms primarily. The comparison among different substrates has been summarized in Table 1 [10,14,17,20,22–32].

2.1 Electrochemical performance dependent on substrates

Standing for the electroacivity of BESs, the electrochemical performance has been reviewed here as the general reflection of the impacts from substrates. Considering the different facilities of biodegradation pertaining fermentable or non-fermentable substrates, the discussion is divided into two groups: nonfermentable substrates represented by volatile fatty acids (VFAs) for their recorded superior capabilities, and fermentable substrates such as ethanol and glucose.

2.1.1 VFAs

Fatty acids were evidenced to be favorable for the EET via facilitating the development of thinner biofilm thus higher per-biomass electron-donating rates [33] than fermentable substrates such as glucose and sucrose [34]. Acetate and propionate were found to be especially preferred as electron donors in MFCs fed with VFAs mixture [21,35]. Comparing with carbohydrates, BESs fed by VFAs attained filamentous biofilm structure in scanning electron microscope (SEM) rather than high cell densities, thus producing higher CE (45%–55% than 10%–30% respectively) and higher average biocatalytic rates (336±101 than 179±52 μmol-electron g-protein^{-1} min^{-1}) [33].

Among fatty acids, acetate has been reported more easily utilized by exoelectrogens since it has relatively rapid oxidation kinetics in BESs. Acetate usually achieves a higher CE and PD than other organic acids (however the PD can be lower than glucose sometimes). Electrochemical performance with acetate or butyrate was firstly compared by Liu et al. [36], showing that acetate-fed MFC reached a maximum power density of 661 mW/m^2 with a CE of 10%–31%, while 349 mW/m^2 and 8%–15% for butyrate. A more comprehensive study showed that MFC fed with acetate achieved the highest CE of 72%, followed by butyrate (43%), propionate (36%) and glucose (15%), while glucose had the greatest power density over twice of acetate [24]. Acetate also performed well in long-term operations [26], reflecting a stable property as a BES substrate. Hydrogen-recovering MECs fed with three volatile fatty acids (namely acetate, butyrate and propionate) showed the similar trend as above mentioned [27]. Acetate fed cycles attained a maximum current density of 6.0±0.28 A/m^2 (CE=87%±5.7%), which was 250% and 340% higher than those of butyrate and propionate. The cathodic hydrogen recovery decreased in the same order, with values of 98%, 79% and 71% respectively. By means of COD measurement and calculation, it was noted that the propionate and butyrate fed MECs achieved relatively higher biomass yields but lower current densities in contrast [27], probably because of the better bio-availability of acetate. Such feature has been deduced as the driving force behind the superior performance of acetate fed BES, quantified as lower Ohmic losses by a model [28]. Moreover, functional microorganisms directly stimulated by acetate have been verified superior in electricity generation, which is the inherent reason and will be discussed in Sect.

Table 1 Electrochemical performance and functional microorganisms of different substrates

Substrate	Power density (mW/m^2)	Coulumbic efficiency (%)	Functional microorganisms	Reference
Acetate	48.4±0.3	72	*Geobacter sulfurrenducens*	[22]
	360	42	–	[23]
	64.3	72.3	*Geobacter*-like species	[24]
	1256±23	71±3	*Rhodobacter gluconicum*	[25]
	556±48	19.9±0.6	*Pelobacter propionicus*	[26]
	6.0±0.3	87±5.7	–	[27]
	2 2.28±0.62	12.6	–	[28]
Glucose	40.3±3.9	64	*Geobacter sulfurrenducens* and *Firmicute*	[22]
	9.8	3	–	[23]
	156	15	*Geobacter*-like species	[24]
	72±1	23.5±0.1	*Clostridium* and *Bacilli*	[29]
	1519±21	62±8	*Firmicutes*	[25]
Propionate	58	36	–	[10]
	3.2	93	–	[14]
	46.2	31.5±0.6	*Geobacter* and *Bacillius*	[17]
	1.6±0.1	51±6.4	–	[27]
	745±19	47±5	*Propionibacteriaceae bacterium*	[25]
Butyrate	51.4	43	*Geobcater*-like species	[24]
	2.5±0.06	72±2	–	[27]
	836±18	56±1	*Acinetobacter johnsonii*	[25]
Ethanol	488±12	10	–	[20]
	289±180	7.7±0.8	*Pelobacter propionicus*	[26]
Lactate	52.0±4.7	61	*Geobacter sulfurrenducens*	[22]
	474±25	13.4±0.3	*Pelobacter propionicus*	[26]
Cellulose	143	47	*Clostridium cellulolyticum* and *Geobacter sulferrenducens,* defined co-culture	[30]
	1070	25–50	*Clostridium thermocellum* and *Geobacter sulferreducen*	[31]
	4.9±0.01	–	*Enterobacter cloacae*	[32]
Formic acid	30±1	3.9±2.0	–	[26]
Succinic acid	340±34	16.2±5.2	*Pelobacter propionicus*	[26]
Mixed VFAs	240	–	*Xanthomonas*	[22]

2.2.

As homolog to acetate, propionate is also relatively prior for electricity generation as electron donors. Propionate-fed MFCs could generate a power density of 3.2 mW/m^2 and achieve a Coulombic efficiency of 93% [37], with another MFC fed with 5 mM propionate achieving a power density of 46.24 W/m^3 [38]. However, unlike acetate, the mechanism of propionate conversion to electricity and its functional microorganisms are still under controversy, which will be discussed in Sect. 2.2. Besides VFAs, lactic acid and succinic acid are also known to be capable of electricity generation with mediocre performances [22,26], for the responsible electroactive communities have relatively inferior abilities.

2.1.2 *Fermentable substrates*

Fermentable substrates such as ethanol, sucrose, glucose, and cellulose are also frequently used in BESs. In spite of indirect degradation path, their degradation *per se* has actually more practical meanings than VFAs. Glucose is the most investigated one especially when compared with acetate as typical substrate for BESs, but the results varied among reports. MFC fed with acetate achieved a Columbic efficiency of 72%, with 15% for glucose as aforementioned, while glucose-fed MFC showed the greatest power density 143% higher than acetate [24]. In a two-chambered MFC, acetate and glucose reached a similar level of PD (48±0.3 mW/m^2 and 40±4 mW/m^2) [22]. A PD_{max} of 1519±21 mW/m^2 was

found in glucose-fed single-chamber MFC in a later report, higher than the three VFAs including acetate. Yet acetate-fed ones showed the highest Coulombic efficiency of 71% and the lowest internal resistance [25]. In another report, the PD_{max} of acetate-fed MFC (360 mW/m^2) was extremely higher than glucose (9.8 mW/m^2) [23]. Current accounted for 71% and 49% of electron sink in acetate and glucose-fed MFCs, with biomass as the second (15% and 26%). It was therefore speculated that the low content of exoelectrogens and a concentration gradient in the thick biofilm were responsible to the low power density when feeding glucose [23]. In a decolorization biocathode, cyclic voltammetry (CV) showed acetate-fed biofilm had higher absolute currents, while the electrochemical impedance spectroscopy (EIS) results displayed that the total internal resistance of glucose was 73% lower than acetate [39], indicating that acetate induced higher reductive activity while glucose facilitated the catabolism in electron transfer process [40]. It is likely that glucose can achieve a higher current with the aid of fermenters, while acetate can be directly utilized by exoelectrogens to reach a higher CE. The demand of fermenters to convert glucose into acetate or hydrogen to enable the electricity generation stimulates the biological diversity in glucose-fed BESs. Such hypothesis was supported by the switch experiments between acetate and glucose. For acetate, the recovery of current needed a lag time of ~3 d after the switching towards glucose. But glucose enriched MFCs did not, and even produced higher PD and CE than acetate-enriched stage [29]. This superior tolerance of substrate switching was deduced to be on account of its bacterial structure diversity [24]. Evidence supporting such presumptive hierarchical structure of microbial community is reviewed in Sect. 3.2.

Ethanol is another important substrate for BESs, especially in the treatment of industrial wastewater. The possibility of ethanol as a sole substrate for electricity conversion was firstly confirmed by Kim et al. [20], with a PD of 488±12 mW/m^2 and a CE of 10%. But PDs of long-term-operated MFCs fed with ethanol could only achieve half the value of acetic acid fed ones in contrast experiments [25].

Cellulose as a fermentable substance has been proved to act as the electron donor directly or indirectly for electricity conversion. MFC constructed with defined co-culture of a cellulolytic fermenter *Clostridium cellulolyticum* and *Geobacter sulferrenducens* achieved a maximum power density of 143 mW/m^2 and a Coulombic efficiency of 47% with cellulose as a sole substrate [30]. But a pure culture of *Enterobacter cloacae* was also verified to have the ability of accomplish both cellulose degradation and electricity generation without exogenous mediators [32]. In an electroactive mixed microbial community enriched beforehand in MFC, cellulose achieved a notably high current density of 1070 mW/m^2 [31]. Similarly, MFC operated with corn stover containing high content of cellulose attained a power density of 406 mW/m^2 with the dealing of steam explosion in another report [41]. In BES, the conversion of cellulose includes both direct and indirect routes, by which exoelectrogens utilize cellulose with or without the aids of fermenters. This difference can also influence the terminal performance and will be discussed next.

2.2 Substrate selected functional microorganisms in anodic biofilms

Knowledge of the corresponding relationship between different substrates and their functional microorganisms is of vital importance to understand the in-depth mechanism of the impact of substrates on the BES performance. Two roles of functional microbes are considered to thrive on the electrode, namely the exoelectrogens who respire with electrodes as the electron acceptor, and fermenters who convert complex substrate to small-molecule electron donors for the former.

2.2.1 Geobacter

The genus *Geobacter* is a ubiquitous microorganism thoroughly studied in BESs, known to be capable of utilizing acetate, hydrogen [16], ethanol, and propionate [42], etc., as substrates. Considering the great capability of EET of this genus, their enrichment in BESs fed with a broad range of substrate is not surprising. *Geobacter* typically dominates the microbial communities in BESs when acetate served solely as the electron donor [42,43]. *Geobacter* was also found to be common (15%–20%) in complex substrate such as glycerol, milk and starch-fed MFCs with pyrosequencing [44]. Interestingly, the preference to substrates varies from different species within the genus *Geobacter* such as *G. sulfurrenducens* and *G. metallireducens*, resulting in different enrichment and dominance with different substrates. *G. sulfurrenducens* was once found to be enriched in all anode communities in a two-chamber MFC fed with lactate, acetate and glucose [22], and the usage of acetate-based artificial wastewater yielded the dominance of *G. sulferreducens* in an anodic consortium [45]. While the total percentage of OTUs from *Geobacter* genus was found to be 47% (by RFLP screening of 16S rRNA gene clone libraries) on a propionate-fed anodic biofilm, a substrate cannot be directly utilized by *G. sulfurrenducens* [38]. According to the absence of acetate in the effluent of a propionate-fed MFC and the dominance of *Geobacter* on its anodic community [38], it was speculated that the degradation of propionate to current was either directly performed by a propionate-consuming exoelectrogen such as *G. metallireducens* [42] or via the syntrophic association of H_2-producing syntrophus and *Geobacter sulferreducens* [46]. In a recent report, the *Geobacter* genus was found to dominate all the anodic biofilms of acetate, lactate,

propionate and butyrate-fed MECs with a property of 62.4% [47]. The substrate-selected distribution within the *Geobacter* genus was observed, that *Geobacter sulfurreducens* was deduced to be responsible to the conversion of lactate and acetate, while *G. toluenoxydans* was predominant with propionate as electron donor and *G. pelophilus* formed a butyrate syntrophic relationship with butyrate-oxidizing *G. metallireducens*. The "barrier effect" caused by the low metabolic versatility of *Geobacter sulfurreducens* preventing propionate oxidation was suggested [47]. These results indicate that dynamic changes within the genus *Geobacter* are influenced by the added substrates, and the metabolic differences among different species of *Geobacter* warrant further investigation.

Inner mechanism of the substrate-dependent EET pathway of *Geobacter* was recently revealed by metatranscriptomics methods by Ishii et al. [48]. Application of the acetate and propionate stimulus induced more than 1000 genes to respond positively or negatively. Among them, numerous multi-heme c-type cytochromes (MH-cytCs) mainly correlated to the dominant *Geobacter* operational candidate species (OCSs) were positively stimulated, suggesting the correlation of the availability of non-fermentable fatty acids and the activity of EET. Deepgoing analysis showed that six outer membrane c-type cytochromes (OM-cytCs) played the key electron carriers for EET especially under respiratory conditions, with *omcB*/*omaB*, *omcS*, *omcX*, *omcZ*, *omcY*, and *omcQ* gene significantly upregulated by the stimulation of fatty acids. A key gene proposed as an activity marker for acetate consumption via the TCA cycle in family *Geobacteraceae* was notably upregulated, while key genes associated with formate oxidation were highly downregulated during the stimulus of acetate and propionate addition for all *Geobacter*/*Pelobacter* OCSs, indicating the metabolic responses of different substrates [48]. The comprehensive EET-related metabolism pathway of four abundantly shown *Geobacteraceae* OCS was summarized as in Figure 1.

2.2.2 *Shewanella*

Shewanella oneidensis is considered as a model exoelectrogen and is routinely cultured using lactic acid as the carbon source [43], known to be capable of extracellular electron transition via two mechanisms till now [49]: (i) direct electron transfer with outer membrane redox proteins [50] and (ii) mediated electron transfer with the assistance of self-produced mediators [51]. The typical substrate for the respiration with electrodes of *Shewanella* is lactate [43], which is, in the other hand, a common product of complex compounds fermentation. However, Rosenbaum et al. [49] reported that *S. oneidensis* showed an indifferent performance in pure culture with lactate and co-culture with a homolactic fermenter *Lactococcus lactis* utiliazing glucose in a continuous flow BES, in terms of both electricity production and physiological state (i.e., gene expression). The presence of *Lactococcus lactis* as the metabolic partner converting glucose to lactate was observed to have no impact on the physiological activity of *S. oneidensis*, thus the interaction between them was speculated as a merely substrate level one rather than deeper.

Besides the *S. oneidensis*, other species in this genus can be the responsible for formic acid uptaking in BESs. Current generation through formic acid was summarized to a three-way mechanism [43] including: (i) direct oxidation via mediator compounds such as humic acids or anthraquinone-2,6-disulfonate (AQDS) [52]; (ii) indirect conversion to current without any mediator by acetoclastic exoelectrogen *Shewanella putrefaciens* with the aid of homoacetogens [53]; (iii) cooperation of formate-consuming hydrogen producer *Paracoccus denitrificans* and hydrogenotrophic *Geobacteraceae* [16].

2.2.3 *Functional microorganisms for other substrates*

Propionate as a preferred electron donor [35] similar to but different from acetate, has been found to facilitate completely disparate microbial communities in BESs, therefore its conversion path to electricity stays uncertain. It was deduced by Kiely et al. [43] that the propionate oxidation in BES was via a direct route rather than syntrophic processes in according to the absence of *Geobacteraceae* [24,37]. But in an MFC fed with propionate, the total percentage of OTUs from *Geobacter* genus was found to be 47.5% in the anodic biofilm [38], indicating that *Geobacter* might be in charge of current production. *Geobacter spp.* and *Comamonas testosterone* were enriched by feeding propionate during a substrate switching [54]. Nevertheless, *Bacillus* was also reported to predominate in a propionate-fed MFC (55.6%) while no *Geobacter*-like sequence was detected, indicating a different route of electricity generation [24]. This Gram-positive genus along with *Pseudomonas* were later speculated to be responsible to the conversion of butyrate and propionate into current [35]. In another propionate-fed MFC, a considerably high Coulombic efficiency up to 93% was attained [37], and the H_2 saturation inhibited both the propionate degradation (by 18%) and current generation (by 19%). Contrasted with an acetate-enriched community, it was deduced that propionate was oxidized directly in electroactive biofilm instead of a syntrophic manner thus retarded by H_2. However, this verdict is problematic because (i) the propionate to H_2 oxidation is endergonic demanding low H_2 pressure, thus H_2 saturation would certainly inhibited it regardless of whether involving in syntrophic association [55]; (ii) electron mediators between syntrophic partners include not only H_2, but also formate, acetate and even DIET probably [55,56], which could also happen in the electroactive consortia. Therefore, to rule out the syntrophic propionate oxidation in BES would ask for way more evidence.

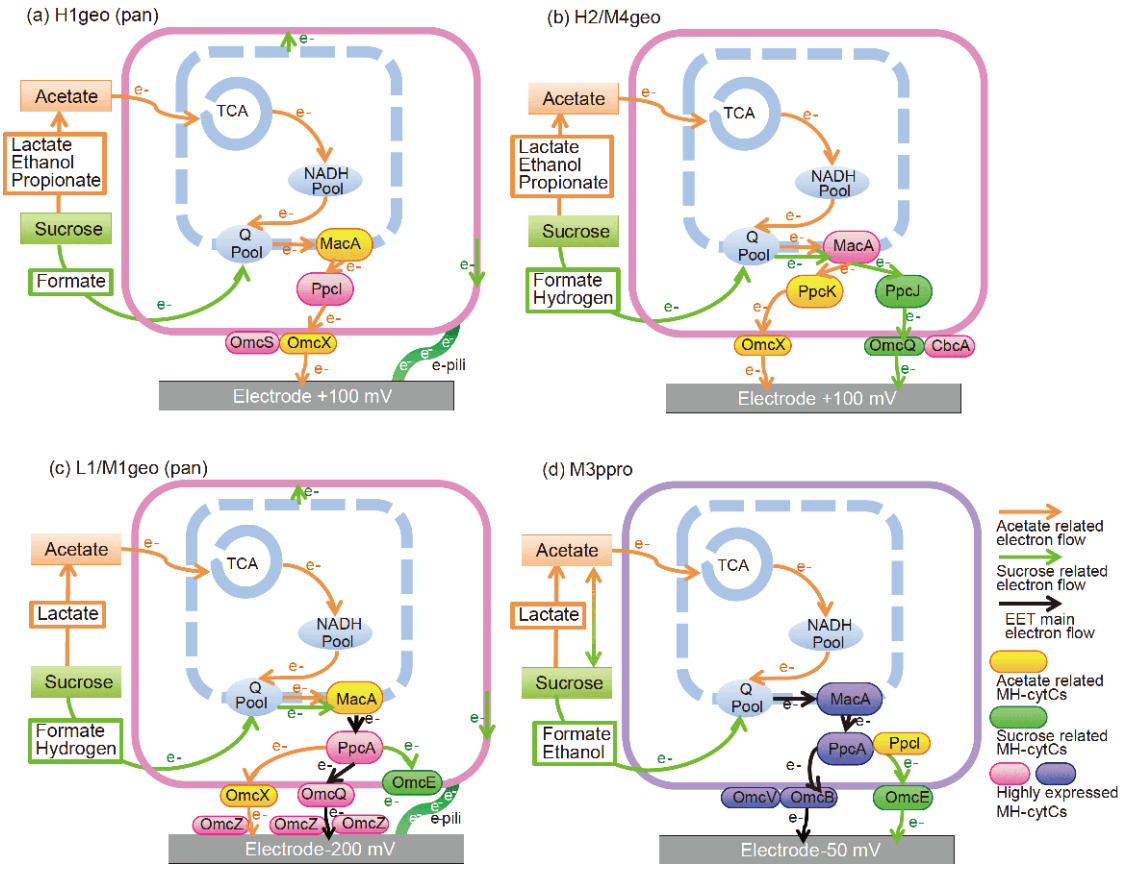

Figure 1 (Color online) Metabolic pathways in four *Geobacteraceae* OCSs related to applied substrate. (a) The OCS represented by a pan-genome H1geo, afflicted to *Geobacter* Subsurface clade 1; (b) the OCS H2/M4geo, an unknown *Geobacter* clade; (c) the OCS represented by a pan-genome L1/M1geo, afflicted to *Geobacter* Subsurface clade 2; (d) the OCS M3ppro, closely related to *Pelobacter propionicus*. Excellular electron transfer pathways originated from sucrose or acetate are shown as yellow or green arrows, respectively. Black arrows represent the main electron flow. MH-cytC proteins for different reactions are shown as ovals with their names inside.

Under some circumstances, unexpected microorganisms turn up and even dominate in electroactive microbial consortium. For example, *Pelobacter*, *Xanthomonas*, *Comamonas*, etc., are usually found instead of typical exoelectrogens such as *Shewanella* in systems fed with their preferred substrates, but their roles in the excellular electron transfer and/or pollution degradation are not clear. A strain isolated from marine sediments, *Desulfuromonas acetoxidans*, was reported to have the ability of oxidizing acetate and benzoate with electrodes as the electron acceptor [57]. A pure culture of *Enterobacter cloacae* was verified to have the ability of converting cellulose into current without exogenous mediators as a sole microorganism [32]. *Xanthomonas sp.* was found to dominate the anodic biofilm of MFCs fed with volatile fatty acids (VFAs) by 16S rRNA gene analysis [21]. Glycerol consumer *Actinomyces* [58] occupied a dominant percentage of 29% in a glycerol fed MFC [58]. *Pelobacter propionicus* dominated the microbial communities in acetic acid, lactic acid, ethanol, and succinic acid fed MFCs (63%, 39%, 21% and 15%, respectively) unexpectedly, lacking the cytochromes required for anodic electron transfer such as MacA and OmcB [26]. Another report also deduced that *P. propionicus* (39%) was responsible to the primary fermentation (e.g., ethanol) and produce acetate readily used by *Geobacter sulferreducens* (7%) for anodic respiration in a lactate maintaining MFC [31]. These species might possess a significant position in electroactive microbial communities.

In a substrate switching experiment, *Anaeromusa* species were remarkably enriched by lactate, and they dominated again when lactate fed secondly, thus *Pseudomonas spp.* was thought to play important roles in lactate degradation [54]. *Pseudomonas* species are on the other hand known to perform electron transfer mediated by its endogenous secondary metabolites phenazines as electron shuttles [59], thus the phenazine concentration is supposed to correlate with high current. The electricity production by *Pseudomonas aeruginosa* was revealed to be substrate-dependent by Bosire et al. [60]. Strain PA14 produced 3-fold-higher current density with 2,3-butanediol over glucose, while strain KRP1 produced its highest current with glucose (19 μA cm^{-2}). Via the distinguishing of redox peak in CV tests, different phenazines responsible to the electron shuttle were also identified. Results showed that the switch in the phenazine spectrum toward pyocyanin (PYO) and the production *per se* were

stimulated by 2,3-butanediol, and confirmed the assumption that 2,3-butanediol interacts with the quorum-sensing regulatory system and then influences the phenazine production, and eventually stimulates the current generation [59].

3 Substrate-dependent inter-species relationships

As mentioned in context, exoelectrogens need syntrophic partners to convert unsuitable substrates into current, and share the same ecological niche with many competitors such as methanogens. These negative or positive relationships are key factors to understand the ecology of this microbial consortium, as well as the inherent mechanism of the electricity generating fluctuation under different circumstances in reality. In this section, the substrate-dependent inter-species relationships are summarized, including the competition between biological processes in BESs and the cooperation of microorganisms with diverse functions.

3.1 Competition between EET respiration and other microbial processes

Methanogenic communities are similar to exoelectrogenic communities speaking of their downstream position of the metabolic interaction with fermentative partners utilizing complex substrates [43]. Methanogens respire at energy levels close to the thermodynamic limits with a limited range of organic substrates such as acetate. But they have evolved in specific mechanisms for energy conservation therefore can ultimately compete against the electrogenic activity. Acetate and hydrogen are preferred electron donors for exoelectrogens such as the model exoelectrogen *Geobacter suffereducens* [46], which are meanwhile the typical substrates for hydrogenotrophic and acetoclastic methanogens. So under fermentable substrates conditions, there will always exist the possibility of the competition for these two typical products of fermentation. Dynamic variations of the fermentation end products, different ratios of the output hydrogen and acetate, as well as other factors such as extraneous inhibitors, decide the victor between methanogens and exoelectrogens. The competition relationships with methanogens operated with different substrates and inhibition strategies are summarized in Figure 2.

The competition for substrate between exoelectrogens and methanogens was firstly implied by the phenomenon of Coulombic efficiency decrease and methane production in an up-flow MFC operated with high organic loading rates [61]. The same competition for electron donor caused an increase of fermentation rates in reverse in a glucose fed MFC [62]. Attempts to identify the direct electron donor for anodic respiration meanwhile the methane origin (i.e., acetate or hydrogen) have been performed. A hydrogenotrophic methanogen *Methanobacteriales* was illustrated to consume all the hydrogen generated from ethanol fermentation, with no acetoclastic methanogenic genera detected in an ethanol fed MEC, indicating hydrogen as the electron transfer carrier instead of acetate [63]. It was then observed that in glycerol, milk and starch-fed MECs, hydrogen accounted for 100% in the gas production without any addition of chemical methanogenesis inhibitor [44]. Further test of initially bubbling hydrogen and replacing the acetate with sodium bicarbonate in the open circuit MEC confirmed that methane production from hydrogen was much lower than from acetate or propionate. Homoacetogenic bacteria and/or hydrogen oxidizing exoelectrogens were replaced by methanogens gradually in long-term operation, and a rough contribution of hydrogen to methane formation was calculated to be 20%–25% with glycerol or milk. But the proliferation of hydrogentrophic methanogens was proved to be unavoidable with glycerol or starch as substrates even under low hydrogen pressure [44]. From the discussion above, the variation between hydrogen or acetate path in the competition is substrate-dependent, appealing for further investigation especially from the biochemical and thermodynamical point of view.

Inhibition on the methanogenesis has been a promising strategy for improving the BES performance especially the Coulombic efficiency, and the investigation of the competition relationship. A frequent chemical inhibitor, 2-bromoethane sulfonic acid (2-BES), was reported to lower the electron lose coursed by methanogen from 26% to nearly 0 and enhance the CE by 24% in an ethanol fed MEC [63]. It was then reported to increase notably the *Geobacter* population in an ethanol fed MEC community from 1% to 21%, evidencing the competition for partial substrate between exoelectrogens and methanogens. The inhibition of methanogensis by 2-BES yielded the replacement of homoacetogens (namely *Acetobacterium*) to methanogens as H_2 scavengers in a later report [64]. However, Kaur et al. [65] showed that the injection of 2-BES (concentration of 0.1–0.27 mM) increased CE from 35% to 70% by suppressing methanogens and the introduction of oxygen to the anodic chamber also suppressed methanogens whilst slightly reducing the exoelectrogens activity. These inhibitors suppress methanogens with potential inhibition or toxicity on exoelectrogens. Green technologies without chemical addition and impact on exoelectrogens are still need to be investigated.

The operation of open and closed circuit duty cycling was reported to inhibit the methanogenic community in MFCs [65]. Open circuit increased the methane percentage of the headspace by 8.8%, 3.2% and 4.7% for acetate, propionate and butyrate respectively. Methane produced in the MFC operated with maximum power point tracking experienced an increase with the acetate concentration, but nearly dis-

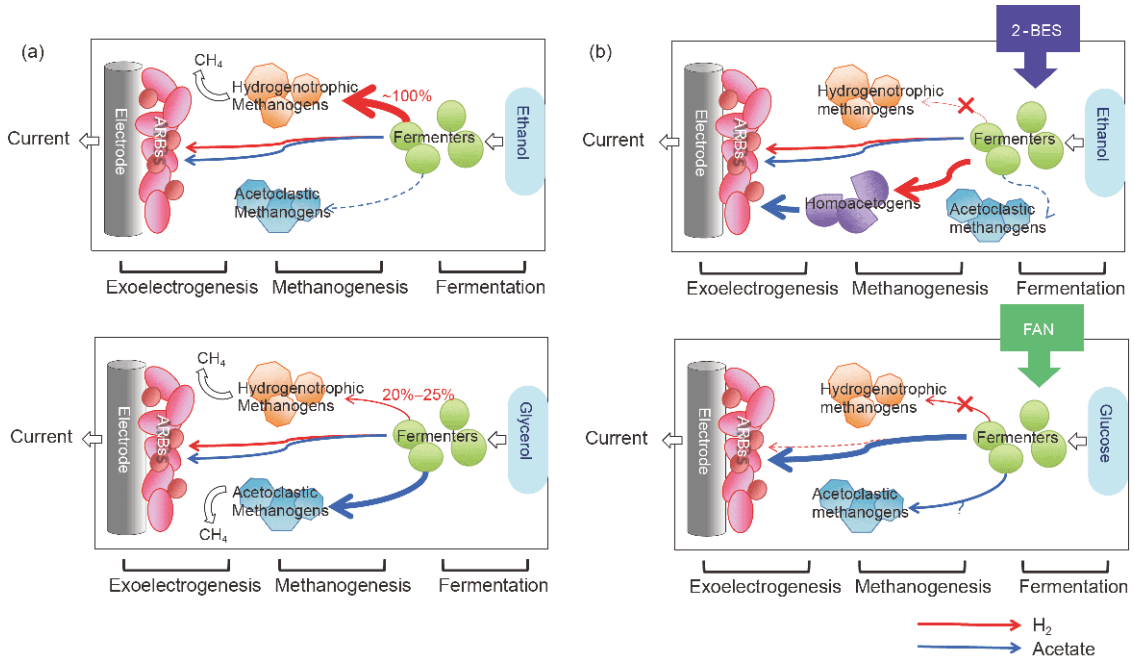

Figure 2 (Color online) Competition between methanogens and exoelectrogens dependent on initial substrates and inhibitors. (a) The different metabolic routes of exoelectrogenesis and methanogenesis in ethanol and glycerol fed BESs; (b) the variation stimulated by inhibiting factors such as the addition of 2-BES (2-bromoethane sulfonic acid) and FAN (free ammonia-nitrogen) with ethanol and glucose as the initial substrate, respectively. The red arrows represent the flux of hydrogen, while the blue arrows represent the flux of acetate. And the bandwidth of arrows represents the conceptual quantity of metabolic flux, with dotted lines meaning the least possible flux.

appeared after 12 days' starvation and was not detected even after sufficient substrate provision. It was then deduced that the methanogens could be eliminated simply by depriving them of substrate without affecting the activity of exoelectrogens, evidenced by the evenness of microbial community between open and close circuit [65], nevertheless the electroactivity was neither characterized nor discussed in this article. In addition, free ammonia-nitrogen (FAN, i.e., NH_3) was reported to alter the glucose fermentation pathways in MECs recently [66], by minimizing the production of H_2 thus suppressing the activity of hydrogenotrophic methanogens. On the other hand, such suppression also yielded the accumulation of organic acids such as lactate and propionate, thus selected phylotypes related to anode respiration (*Geobacteraceae*), lactic-acid production (*Lactobacillales*), and syntrophic acetate oxidation (*Clostridiaceae*). As a result, the CE increased consequently with the concentration of FAN (57% for 0.02 g of FAN/L of fed-MEC, compared to 76% for 0.18 g of FAN/L of fed-MECs and 62% for 0.37 g of FAN/L) [66]. But the absence of acetoclastic methanogens conflict to the existence of acetate syntrophic oxidizing bacteria was not explained.

Other competition relationships such as nitrate reduction in anodic environment were also investigated but still limit in a small range. It was once observed that nitrate respiration could influence anodic respiration by consuming organic carbon [67]. The kinetics of Nernst-Monod was afterwards applied to describe the electron transfer in the competition relationship between anode respiration and nitrate respiration recently [68]. Fitting results of the model indicated that nitrate respiration could indirectly affect the microbial anode respiration by altering the available substrate concentration. However, more experimental evidence is necessary to support this conclusion.

3.2 Cooperation between exoelectrogens and other microorganisms

Exoelectrogens and their fermentative or hydrolyzing cooperators formed a hierarchical community for the degradation of a wide range of substrates [43]. The degradation of complex or fermentable substrates such as cellulose, sucrose and glucose demand a cooperating relationship between exoelectrogens, fermenters (for substrate conversion), homoacetogens (for acetate provision and H_2 scavengers) and even aerobes on some conditions for oxygen consuming. Exoelectrogens were verified to accelerate the fermentation of glucose by means of consuming acetate, propionate and hydrogen consistently. The removal of these end products of fermentation eliminates the feedback inhibition and thus makes the process thermodynamically feasible [62]. On the other hand, the fermenters feed exoelectrogens with small-molecule electron donors by degrading complex compounds. The inherent driving forces of several substrates fermentation in BESs have been reviewed here. Furthermore, the one-to-one relationships between functional microorganisms

studied with defined co-culture have thrown light on the in-depth mechanism of the cooperation in microbial communities, and are also reviewed here. Possible syntrophic interactions demand by the fermentable substrates conversion in BESs are illustrated in Figure 3.

3.2.1 *Associations with fermenters in mixed culture*

The phylum *Firmicutes* including *Clostridium* and *Bacilli* has been known as common fermenters in BES. They were previously found in the glucose fed MFCs rather than those fed with acetate and lactate, considered to ferment glucose to small molecules and scavenge oxygen [22]. Denaturing gradient gel electrophoresis (DGGE) analysis also showed that *Clostridium* was a key component in the anodic community of MFCs fed with corn stover, indicating the importance of this genus in complex substance fermentation [41]. *Clostridium thermocellum* (62%) were later found to dominate in a cellulose fed MFC with a proportion of 17% for *Geobacter sulferreducens* (achieved a relatively high current density of 1070 mW/m^2 as aforementioned) by 16S rRNA clone analysis [31], implying an efficient syntrophic relationship between them.

Besides, a three-way syntrophic interaction among exoelectrogens, fermenters and methanogens was reported by Parameswaran et al. [64] in an ethanol fed MEC, which was dominated by the ethanol fermenter, *Pelobacter*. This genus was elucidated to transfer H_2 as fermentation product to a homoacetogens *Acetobacterium* who provides acetate to exoelectrogens as fuel for current generation [64]. Fermenters in more complicated conditions hold even more indispensable positions. The glycerol consumer *Actinomyces sp.* occupied accounted for 29% in glycerol fed electroactive microbial community, and 13% in milk fed system responsible of the convert from lactose to lactic acid [58]. The sugar and organic acids fermenter *Petrimonas* were detected as 7% in a milk-fed MFC. Two glucose fermenter *Dysgonomonas* and *Proteiniphilum* were clearly identified in starch fed MFC besides *Geobacter* [44].

Fermentation routes and the influence factors have been investigated with various means till now. Via electron flux calculations, glucose as substrate in MFCs was verified to convert to acetate and hydrogen first consumed by exoelectrogens readily for electricity conversion, and anodic hydrogen oxidation was shown to be a major path rather than acetate [62]. By means of monitoring the fermentation process in glucose/sucrose fed MFCs and MECs poised at different potentials, it was further found that the constitute of primary sugar fermentation products varied among lactate, propionate and acetate and that the fermentation processes could be affected by the EET operating conditions [34]. This hypothesis was confirmed by the 16S rRNA gene clone libraries and the subsequent analysis. Canonical correspondence analysis (CCA) diagrams showed a clear trend that fermenting *Lactococcus, Anaeroarcus* and *Aeromonas* strains preferred higher EET rate under positive anode potentials while *Tolumonas* and *Trichococcus* strains correlated

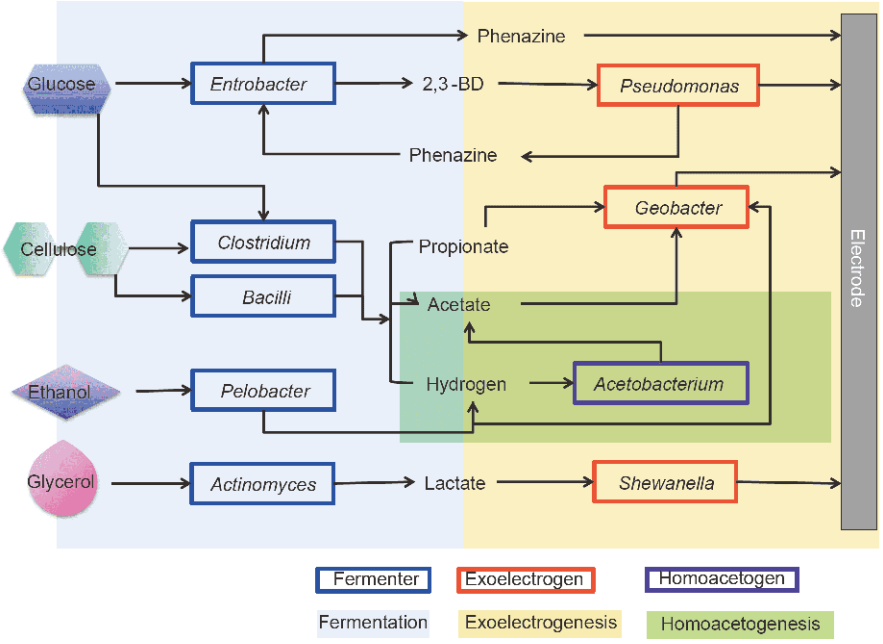

Figure 3 (Color online) The syntrophic association of fermenters, exoelectrogens and homoacetogens in BESs utilizing glucose, cellulose, ethanol and glycerol. Fermenters represented by the blue frames convert initial substrates into suitable electron donors utilized by exoelectrogens (represented by the red frames). On some occasions, hydrogen produced by fermentation is converted into acetate by homoacetogens represented by purple frames. Reactions included in the blue background are fermentation, and those included in the yellow background are exoelectrogenesis with green background representing the homoacetogenesis.

with lower EET rate, which implied a correlation between EET activity and the fermentation course. Moreover, the impact of substrates showed diversity within the genus *Geobacter* in phylogenetic analyses. Phylotypes classified within *G. metallireducens* clade were mainly observed in MFCs fed with fatty acids and low potential condition in sucrose fed MEC, while Geobacter subsurface clade 1 and 2 clearly associated with fermenters such as *Lactococcus* and *Anaeroarcus* under higher potentials, suggesting a symbiotic relationships towards complex substrates degradation at higher EET rates [34]. Another research with comparative metatranscriptomics revealed that sucrose fermenters from the phylum *Tolumonas*, *Lactococcus* and *Firmicutes* were positively responding to the stimulus of open circuit. Highly downregulated genes stimulated for the fermenters included those encoding sugar transporters, key genes for glycolysis and translation-related ribosomal proteins, supporting their metabolic role of sucrose fermentation [48].

Considering the spatial separation and differences in the most suitable environment between fermenters and exoelectrogens, the maximum enrichment of both groups in electrochemical environment could be challenging on various conditions. Montpart et al. [44] obtained a syntrophic consortium by a separate growing inoculation strategy to optimize the distribution of fermenters and exoelectrogens. They enriched an anodic biofilm for exoelectrogens and anaerobic digester sludge in culture flasks for fermenters respectively, and then combined them together in MFCs. The current density showed a high tolerance to the switch of substrate from acetate to complex substrates such as milk, indicating a mature biofilm of high tolerance [44]. This report implied the difficulty of the establishment of stable relationships and its probable strategies.

3.2.2 *Defined co-culture with syntrophic partners*

Defined co-culture experiments evident the associations between microorganisms directly. In BES treating specific substrates, the cooperation between exoelectrogens and their fermenting partners typically transit the substrates essential for the respiration with electrodes such as hydrogen and acetate as the end products of fermentation. In such association, the inter-species interaction is at substrate level without physiological adjustment, yet both sides involved benefit from it. In the aforementioned MFC constructed with defined co-culture of *Clostridium cellulolyticum* and *Geobacter sulferrenducens* [30], this typical syntrophic combination of fermenter and exoelectrogen provides essential substrates (i.e., hydrogen and acetate) for *Geobacter*, who enhances the cellulose removal compared to the monoculture of fermenter by 18% in return. In another aggregation, *S. oneidensis* showed approximate electricity production and gene expression levels with or without the homolactic fermenter *Lactococcus lactis* in BES [49], indicating such indirect cooperation was merely at the substrate level. *S. oneidensis* was then used in a well-designed three-species microbial consortium, along with an engineered *Escherichia coli* to convert glucose to lactate as its electron donor, and *Bacillus subtilis* to riboflavin as an electron shuttle. Such delicate consortium achieved a high energy conversion efficiency up to 55.7% and lasted for 15 d [69]. A glycerol fermenter, *Klebsiella pneumonia* was investigated as the syntrophic partner of *S. oneidensis* in glycerol fed MFCs. A current density of 10 mA/m^2 was obtained with acidic by-products consumed persistently with the co-culture compared by any of them. Spatial distribution of *S. oneidensis and K. pneumonia* was deduced to verify the selection driving force of electrodes as the electron acceptor [70].

Another example of case is the mutualism between the foregoing mediated exoelectrogen *Pseudomonas aeruginosa* and a glucose fermenter *Enterobacter aerogenes* in BES with glucose as the initial substrate, representing a tighter association in syntrophy. In the first report focusing on such relationship by Venkataraman et al. [71], it was observed that a co-culture of these two microbes generated 14-fold higher current density than either of the monocultures ((46.5±6.4) $\mu A/cm^2$ with the co-culture vs. (3.3±0.1) $\mu A/cm^2$ with *P. aeruginosa* and (2.5±1.3) $\mu A/cm^2$ with *E. aerogenes*). The deep seated mechanism was elucidated as that 2,3-butanediol as the product of glucose fermentation by *E. aerogenes* stimulates the phenazines production of *P. aeruginosa* especially towards pyocyanin than the three other phenazines, which can stimulate the non-electroactive *E. aerogenes* to respire with electrode thus enhance the electricity production. Schmitz and Rosenbaum [72] further inspected a linear dependency between maximum current densities and phenazine production. They also found that not only the oxygen concentration but also the substrate feeding scheme influence the electricity production. The fed-batch cultivation method enabled the co-culture to increase approximately 12-fold higher maximum current densities (17.1 $\mu A/cm^2$ to 212 $\mu A/cm^2$) than the initial co-culture and approximately 12-fold higher phenazine production (27 μg/mL to 320 μg/mL). Coulombic efficiencies up to 20.9% were attained, which was notably high considering the presence of another electron acceptor oxygen [72].

Directly verified syntrophic consortia in BES like those mentioned above are still insufficient up to now, so the criterion to evaluate and classify the intimacy of the combination remains unclear, as well as the particular mechanisms behind some of these phenomena. Whether the interaction is based on the reaction kinetics of substrates and metabolites, or the direct physiological connection between the cooperators has a vital difference. For further understanding the syntrophic association participated by exoelectrogenesis, more investigations with defined co-culture are needed.

4 Methodology summary

The electrochemical performance characterized with current production, resistance and other indexes is the main criterion of BESs, as well as an indirect but powerful tool to represent the activity of functional microorganisms. Power density calculated with the current or voltage output and the area of electrodes, provides a standard index to measure the electricity generation capability of different systems with different substrates [20–26,28–30,32,35,38,54,66]. Coulombic efficiency measures the capability of electron recovery from the primary substrate in a BES, which is especially meaningful to represent the difference between substrates and whether a substrate can be directly utilized by the exoelectrogens [20,25,27–30,33,37,44,47,61,63,72]. Cyclic voltammery (CV) provides information of the microbial electrochemical activity by the absolute current values, and the electron-transferring paths by the potential of redox peaks [39,59,71], which was employed to rule out the possibility of hydrogen to reduce procyanin which could act as mediator for the respiration with electrodes [71]. Electrochemical impedance spectroscopy (EIS) measures the resistance of a BES and provides insight into the inherent differences among different electron transfer processes [39]. Polarization tests can characterize the maximum power density, the resistance via calculation and the limit factors of power production [25,28,32,58]. Other calculations such as electron sink [23,73], per-biomass electron donating rates [33] provide further information of the electricity generation and substrate usage efficiency. Overall, the electrochemical performance embodies the general result of complicated courses consist of biological and electrochemical reactions.

Taxonomic methods of 16S rRNA fingerprinting such as DGGE [21,22,25,26,29,32,35,37,39,43,54,58,65], terminal restriction fragment length polymorphisms (T-RFLP) [45], clone libraries [22–24,26,32–34,38,43,48,53,63,64,71] and high-throughput sequencing (HTS) [21,35,39,43,44,47,64,65,68] provide direct insight into the microbial community composition and structures in mixed cultures. Combination of T-RFLP and flow-cytometry DNA/scatter-plot distribution verified the dominance of *Geobacter sulferreducens* in acetate fed anodic biofilm [45]. Such cytometric approach method with traditional fingerprint methods provided the information of the dynamic structure of community and population, by clearly differentiating the sub-communities with regard to the cell size and DNA content, so the emergence and disappearance of distinct cell abundances could be traced [45]. A systematic stimulus induced meta-transcriptomic approach was employed to study the dynamics of the metabolic and transcriptional responses of electroactive biofilm mixed culture to changes in surrounding substrates [48], identifying metabolic networks within complex anodic microbial communities and the shift of the metabolic pathways without pure-culture experiments successfully.

5 Conclusion and implications

Beginning with the discrepancy of electrochemical performance in BESs using different substrates, the interaction between microbial current production and processes such as methanogenesis and fermentation was summarized. The different characteristics of substrates, such as the biodegradability, fermentability, solubility and even the cultivation and enrichment schemes, would lead to different terminal performance of BESs, via the selection upon different functional microorganisms and relationships among them.

In order to optimize the electron transfer and substrate degradation processes, the complete metabolic networks of pathways connected to different substrates in mixed culture need further and integrated exploration. Moreover, the ecological hierarchical structures of the electroactive biofilms might make a difference, such as the spatial distribution of microorganisms with separate functions of fermentation, methanogenesis, or electricity generation. One-to-one interspecies relationships especially functioning in the controversial substrates conversion are appealed for the identification of functional members in EABs and the oriented construction of microbial communities.

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