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A double-chamber microbial electrolysis cell improved the anaerobic digestion efficiency and elucidated the underlying bio-electrochemical mechanism

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ABSTRACT

Bioelectrochemical regulation has been proven to enhance the traditional anaerobic digestion (AD) of biowastes. However, most of the research has been conducted using a single-chamber reactor that could not explain the underlying mechanism and/or role of electrodes in CH_4 production. Herein, a double-chamber microbial electrolysis cell integrated anaerobic digestion system (MEC-AD) was fed with corn stover (CS) and was exposed to 0.0 V to 1.5 V to elucidate the possible effects of cathode and anode on the AD performance. It was shown that CH_4 yield was enhanced by 80.41% at 0.6 V, however, there was a sharp decline in methanogens at 0.9 V, which finally resulted in acidification. Interestingly, electroactive bacteria of *Enterococcus* were enriched on the electrode at 0.6 V which accelerated the degradation of CS, and thus, enhanced the subsequent mixotrophic methanogenesis dominated by *Methanosarcina* in the bulk solution of the anode chamber. While, *Enterococcus* and *Methanobacterium* were shown to be the dominating microbes in the cathode chamber, which were found syntrophically associated to enhance hydrogenotrophic methanogenesis via the direct interspecies electron transfer process. The synergistic stabilization of both anode and cathode chambers resulted in the final performance improvement in the MEC-AD system. This MEC-AD system provided a deeper understanding for methane enhancement, which will facilitate the scale-up application of the double-chamber systems.

1. Introduction

Rapid progress in global modernization has led to foster the rate of global warming, which is why it has become an emergent concern that needs to be addressed on immediate basis. Thus, significant efforts and innovative measures are being made to reduce fossil fuel dependence and explore renewable energy alternatives [1]. Anaerobic digestion (AD) has been extensively applied as a favorable technology to produce renewable energy and is believed to lower the carbon footprint [2]. Although conventional AD technology owns various advantages, its drawbacks remain unsolved [3]. When treating the lignocellulosic substrates such as corn stover (CS), the conventional AD system requires an extended time to establish a new stable microbial community to hydrolyze the refractory compounds (cellulose, hemicellulose, and lignin), which prolongs the retention time of hydrolysis, and consequently, results in a compromised treatment efficiency [4]. Thus, enhancing the hydrolysis of the lignocellulosic substrates has proven to be a prerequisite for improving AD performance.

Recently, microbial electrolysis cell (MEC) technology has obtained increasing attention when it was combined with AD to enhance substrate degradation and anaerobic performance. A typical MEC is comprised of an anode and a cathode with an additional external power supply, which are separated by ion-exchange membranes to facilitate the transport of ions [5]. The anode accepts electrons while organics are oxidized by electroactive microorganisms to produce electrons, H^+, and CO_2 [6]. The harnessed electrons are then delivered to cathode via an external circuit in which the electrons are utilized by electroactive microorganisms to reduce the targeted compounds to value-added

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products (i.e., CH_4 and H_2) [7]. There exist various advantages when the MEC was integrated with the AD (MEC-AD) [8]. Specifically, the electroactive bacteria can be enriched at the surface of electrodes and in the bulk solution of the MEC-AD system with the external voltage applied, which can enhance the direct interspecies electron transfer (DIET) process and hydrolysis of the recalcitrant substrates, simultaneously [9]. The anaerobic performance can thus be improved via the MEC-AD system.

The voltages had a substantial impact on the anaerobic digestion efficiency of the MEC-AD system. Applying voltage in a suitable range can improve the DIET process of the microbial community, subsequently, leading to the enhancement of the CH_4 yield and production [6]. Besides, the electroactive bacteria is enriched in the MEC-AD system when voltage is applied, which accelerates the degradation of volatile fatty acids (VFA) and other organic compounds [10]. Generally, the suitable voltage applied to MEC-AD ranges from 0.1 to 1.8 V [11]. Flores-Rodriguez et al. investigated the anaerobic performance of acetate at the applied voltage of 0.5–1.5 V, and obtained the maximum CH_4 yield of 0.351 L CH_4 g^{-1} COD at an applied voltage of 1.0 V [12]. Choi et al. also found that the applied voltage of 1.0 V was optimal for CH_4 yields (408.3 mL/g COD glucose) among various voltages (0.5, 0.7, 1.0, and 1.5 V) [13]. The optimum voltages reported by researchers are different, which mainly could be due to variations of inoculum, substrates, electrode materials, and reactor configuration [14].

Most of the MEC-AD experiments have been conducted in a single-chamber reactor, which often decreases the internal resistance and increases the mass transfer ability [11]. Nonetheless, it can only exhibit the global effect on the improvement of AD performance. The specific improving mechanism of cathode and anode on CH_4 production in the MEC-AD system is still unknown. Thus, in this study, a double-chamber MEC-AD system fed with CS was applied to elucidate the respective effects of cathode and anode on anaerobic performance for the very first time. Besides, different voltages were also applied to the reactor to find an optimal voltage for the best anaerobic performance. The profile of the electroactive microorganisms and the functional microbial flora under different applied voltage conditions were also unveiled using 16S rDNA sequencing.

2. Materials and methods

2.1. Feedstock and inoculum

Corn stover (CS) was oven-dried, finely grounded to a 40-size mesh, and stored at room temperature in plastic bags. The characteristics of the CS and inoculum were measured and are shown in Table 1. The total solids (TS) and volatile solids (VS) of CS are 877.50 ± 6.7 g/kg and 90.64 ± 0.41% TS, respectively. The dominant components of CS are glucan (37.28 ± 3.34%), xylan (21.25 ± 0.93%), and lignin (19.6 ± 0.5%). Inoculum with a pH value of 8.21 ± 0.12 was obtained from an industrial-scale anaerobic mesophilic (35 °C) reactor treated with chicken manure and CS in Zhucheng Shunwo Agricultural Technology Co. LTD. Before use, the collected inoculum was acclimatized in a 20 L anaerobic reactor for 30 d by feeding CS at 35 °C until the CH_4 content in the biogas exceeded by 60%. The TS and VS of inoculum were 4.30 ± 0.30 g/kg and 57.27 ± 3.12% TS, respectively. Before the batch experiments, the inoculum was well mixed and passed through a 40-mesh screen to remove large particles.

Table 1
Characteristics of corn stover (CS) and inoculum.

Parameters	Corn stover	Inoculum
pH	/	8.21 ± 0.12
TS (g/kg)	877.50 ± 6.70	4.30 ± 0.30
VS (% TS)	90.64 ± 0.41	57.27 ± 3.12
Moisture content (%)	12.24 ± 0.67	99.56 ± 0.03
Glucan (%)	37.28 ± 3.34	NA
Xylan (%)	21.25 ± 0.93	NA
Lignin (%)	19.60 ± 0.50	NA

Note: NA represents not available

2.2. Experimental setup and design

The batch of anaerobic digestion was performed in a self-designed double-chamber MEC-AD reactor (Fig. S1). The total volume of each chamber was 250 mL with a working volume of 200 mL. Two identical carbon cloths (4 cm × 3 cm) were placed in each chamber and served as anode and cathode. The produced biogas was collected via the gas bag with a total volume of 200 mL. A potentiostat (MPS-3003H, China) was applied to the MEC-AD system to set the voltage of the two electrodes. The nitrogen was allowed to mix into the MEC-AD reactor for 15 min after feeding and was incubated at 35 °C. The content of each chamber included 6 g (dry weight) of CS and 194 g of activated inoculum. The control test was conducted with 194 g activated inoculum only. A series of voltages (0, 0.6, 0.9, 1.2, and 1.5 V) were applied to the system after the overall anaerobic process was set up. The biogas production was measured by collecting biogas in the gas bag daily. The chamber mixture was mixed twice a day. All the experiments were run in triplicate.

2.3. Analytical methods

TS and VS were analyzed based on the previously published methods [15]. The main components of CS were analyzed via two-step acid hydrolysis [16]. Biogas composition was analyzed by a gas chromatograph (Agilent Technologies, USA), equipped with a thermal conductivity detector. Biogas was collected in a 50 mL disposable syringe. The liquid digestate was also periodically collected, centrifuged (10,000 rpm, 10 min), filtered (0.2-μm filter), and analyzed for the liquid parameters, i.e., pH, total ammonium nitrogen (TAN), and volatile fatty acids (VFAs). The pH was measured using a pH meter (Leici, PHS-3E, China). Nessler's reagent spectrophotometry was used for the measurement of TAN concentration. The VFAs including acetic acid, propionic acid, butyric acids (n and i), and valeric acid (n and i) were estimated using the same gas chromatograph (Agilent Technologies) using a flame ionization detector. A digital multimeter (17B+, FLUKE, China) was used to monitor the current variation daily. An electrochemical instrument (CHI1000C, Chenhua Co. Ltd., China) was used to conduct the cyclic voltammetry (CV) analysis in the potential range between −1.0 V and 1.0 V (vs. Ag/AgCl reference electrode) with a scan rate of 0.04 V/s. The morphology of the surface of the electrode was observed using scanning electron microscopy (SEM, S-3400II, Hitachi, Japan).

The experimental data of the cumulative CH_4 yield was fitted by the modified Gompertz equation (Eq. (1) [17].

$$M(t) = P\cdot\exp\left\{-\exp\left[\frac{Rm^{*}e}{P}\cdot(\lambda - t) + 1\right]\right\} \quad (1)$$

$$V(t) = \frac{dM(t)}{d(t)} = R_m\cdot\exp\left\{2 + \frac{Rm\cdot e}{P}(\lambda - t) - \exp\left[\frac{Rm\cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (2)$$

$$t_{max} = \lambda + \frac{P}{Rm^{*}e} \quad (3)$$

Where;

M(t) = cumulative CH_4 yield (L/Kg TS), t = fermentative time (d), P = CH_4 production potential (L/Kg TS), R_m = maximum CH_4 production rate (L/(kg TS·d), and λ = lag phase time (d). Eq. (2) showed the CH_4 production rate (V) equation, which was gained via differentiating Eq. (1). t_{max} equation was obtained from equation V(t) when V = R_m (Eq. (3). The results of P, R_m, t, and λ were fitted by the Curve Expert software version 1.4 after input the daily experimental data. Then, the empirical and the fitted data were plotted by the Origin software version 2018 (Origin Software, Inc, USA).

2.4. Sequencing of 16S rRNA gene

The digestate samples were collected at the start-up phase (day 3), exponential phase (day 9), and the end of the fermentation (day 22) during the batch MEC-AD fermentation process. The DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, USA). The quality and the concentration of DNA were measured by NanoDrop spectrophotometer. After that, the PCR amplification was conducted using the universal primers 515FmodF (GTGYCAGCMGCCGCGGTAA) and 806RmodR (GGACTACNVGGGTWTCTAAT) for bacteria and archaea. Purified amplicons were commercially sequenced by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The shifting of the microbial community structure under different applied voltages was analyzed using the online tool of the Majorbio ISanger Cloud Platform.

3. Results and discussion

3.1. Effect of the voltage on biogas production

3.1.1. Daily biogas production

Daily biogas production, including CH_4 and CO_2, reflected the anaerobic performances (Fig. 1), where the highest values were obtained at 0.6 V, followed by 1.2 V. Considering the highest daily CH_4 production for each chamber, 0.6 V-A (142 mL) and 0.6 V-C (125 mL) were 3.7-fold and 3.3-fold higher than 0 V (38 mL), respectively. The number of days when daily CH_4 production of 0.6 V-A and 0.6 V-C exceeded the highest daily CH_4 production of 0 V were 7 and 8, respectively, while for 1.2 V-A and 1.2 V-C, the number were 5 and 6, respectively. The system continuously produced biogas until day 20th (0.6 V-A and 1.2 V-C) and day 21st (0 V, 0.6 V-C, and 1.2 V-A), which were longer than other groups (5–10 days). These results showed that employing 0.6 V and 1.2 V not only maintained but enhanced CH_4 production in both chambers, while the highest daily CH_4 production was obtained at 0.6 V.

3.1.2. Methane production

We further focused on the methane production potential of each chamber through the accumulative CH_4 yield and CH_4 production rate in the MEC-AD system in response to different voltages (Fig. 2 and Table 2). It was found that 0.6 V was shown to enhance the accumulative CH_4 yield in both anode and cathode chambers by 1.90-fold and 1.70-fold, respectively, and the maximum CH_4 production rates (R_m) were improved by 3.26-fold and 2.92-fold, respectively. Alternatively, 1.2 V conferred a slight improvement for the accumulative CH_4 yield in both anode and cathode chambers (1.40-fold and 1.25-fold, respectively). Although the R_m was marginally improved in the anode chamber (1.35-fold), it was noticeably increased in the cathode chamber (3.19-fold). The accumulative CH_4 yields were inhibited at 0.9 V conditions in both chambers, leading to the lowest CH_4 production. The accumulative CH_4 yield was increased slightly in the anode chamber (1.41-fold) but inhibited in the cathode chamber of 1.5 V. Thus, the sum of the final CH_4 yield was slightly inhibited at 1.5 V. The sum of the final CH_4 yield in the cathode and anode chambers was enhanced at 0.6 V (248.74 L/Kg TS) and 1.2 V (183.23 L/Kg TS). The optimal applied voltage for the sum of

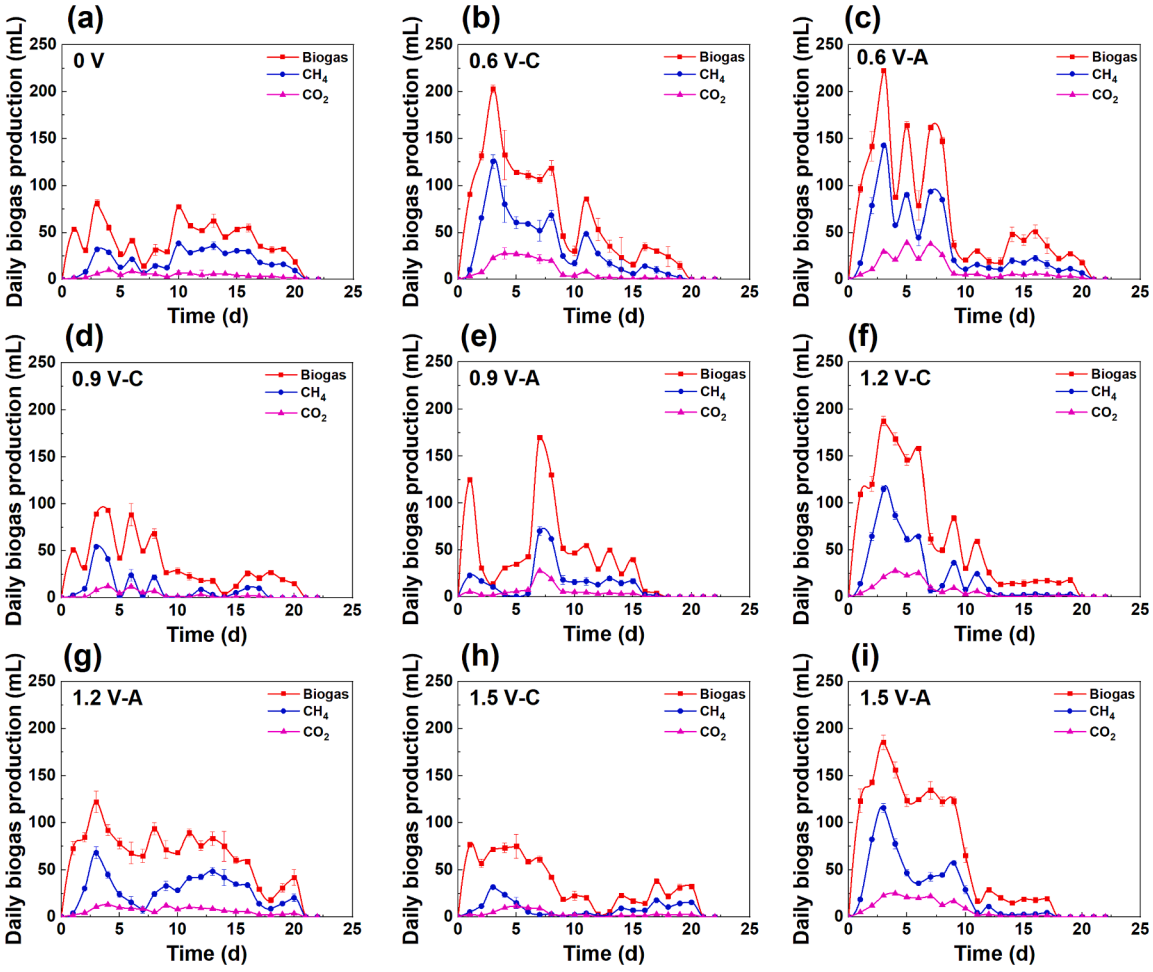

Fig. 1. The daily biogas production in the microbial electrolysis cell integrated anaerobic digestion (MEC-AD) system. (a): 0 V, (b): 0.6 V-C, (c): 0.6 V-A, (d): 0.9 V-C, (e): 0.9 V-A, (f): 1.2 V-C, (g): 1.2 V-A, (h): 1.5 V-C, and (i): 0.6 V-A. C: Cathode chamber; A: Anode chamber.

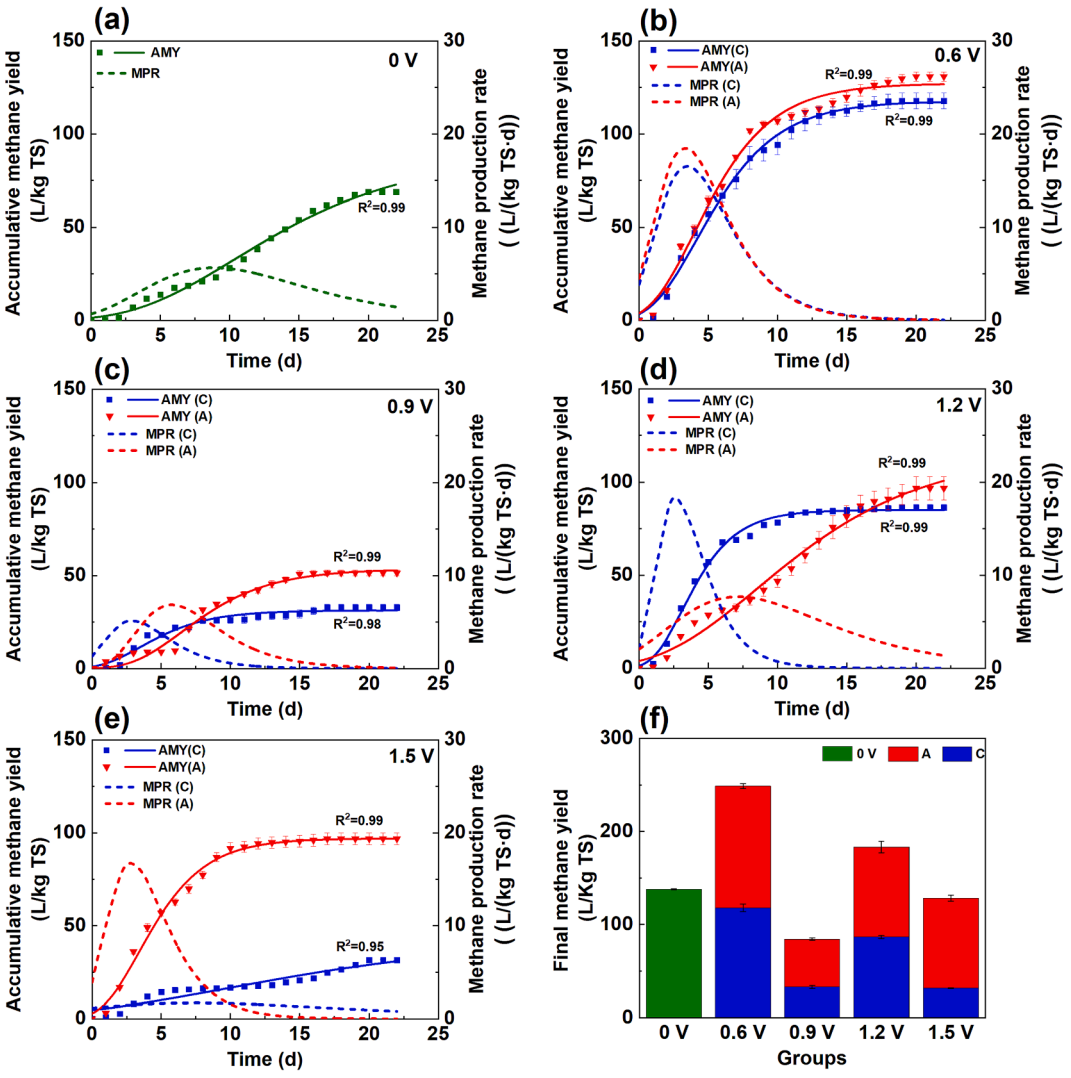

Fig. 2. Accumulative CH_4 yield and the CH_4 production rate for the MEC-AD system. (a): 0 V, (b): 0.6 V, (c): 0.9 V, (d): 1.2 V, (e): 1.5 V, and (f): the final CH_4 yield. Solid dots: experimental data of accumulative methane yield (AMY); Solid lines: fitted curves of AMY; Dash line: methane production rate (MPR); C: Cathode chamber; A: Anode chamber.

Table 2
Modified Gompertz equation parameters of different voltages condition under batch trial.

Groups	P (L/Kg TS)	R_m (L/Kg TS·d)	λ (d)	t_{max} (d)
0 V	88.72	5.65	3.12	8.89
0.6 V-C	117.67	16.49	0.87	3.49
0.6 V-A	127.16	18.43	0.80	3.33
0.9 V-C	31.26	5.12	0.68	2.92
0.9 V-A	53.57	6.84	2.89	5.77
1.2 V-C	85.00	18.01	0.95	2.68
1.2 V-A	116.39	7.68	1.57	7.14
1.5 V-C	43.83	1.70	0.02	7.31
1.5 V-A	96.96	16.63	0.71	2.85

Note: P: CH_4 production potential, R_m = maximum CH_4 production rate, λ: lag phase time, t_{max}: fermentative time at R_m.

the final CH_4 yield was 0.6 V with an 80.41% increase compared to 0 V.

The lag time (λ) was elucidated by the fitted Gompertz equation, which reflected the microbial activity and substrate degradability (Table 2). Interestingly, the lag time was shortened in all voltage conditions when compared to 0 V, indicating that applying voltage accelerated the start-up process. Long start-up time is deemed as a prevalent bottleneck for the cost-effective development of the traditional AD system [6]. Applying voltage might have enhanced the activity of microorganisms that accelerated the degradation of CS, thus, shorting lag time in the MEC-AD system. Applying voltage has proven to shorten the start-up process for both single and double-chamber AD systems. It has also been observed that voltage accelerates the acidification of waste-activated sludge in single chamber MEC-AD, and reduces the start-up phase [18].

Interestingly, there was no regular single peak in the single-factor test for optimizing the final methane production under 0.6–1.5 V conditions (Fig. 2f). Strictly, the voltage was not the sole factor, because the microbial community structure in each reactor was not identical [19]. Therefore, the different optimal voltages (0.6 V and 1.2 V) were found suitable for different microbial communities to produce multiple CH_4 production peaks. Besides, the optimal voltage for the MEC-AD system was also shown to be differing for the inoculum, substrates, electoral materials, and reactor design [6].

Flores-Rodriguez et al. observed that CH_4 production was improved by applying voltage when acetate was used as the sole substrate [12]. Instead, Choi et al. found that the applying voltage enhanced the CH_4 production when glucose was used as a substrate [13]. However, all these observations are based on the studies conducted in a single-chamber reactor, which could only exhibit the co-effect of anode and

cathode on the AD performance. While the overall AD performance can be enhanced via a single-chamber reactor, the improving mechanism of the anode and cathode cannot be well clarified [20]. In addition, the applying voltage may not be suitable for both electrodes to accelerate CH_4 production. For example, applying 1.5 V improved CH_4 production in the anode chamber but inhibited on cathode chamber. The double-chamber MEC-AD reactor divides the anode and cathode into independent spaces that does not allow the exchange of substrates and microorganisms except for protons. Thus, the respective effect of anode and cathode on anaerobic performance can be elucidated in the double-chamber only. In this study, an appropriate voltage of 0.6 V was shown to accelerate the anaerobic start-up process and enhance the CH_4 production for both the cathode and anode chambers in the MEC-AD system.

3.2. Process stability of the MEC-AD system

The anaerobic system is often prone to collapse when the key process parameters are disturbed by external environmental factors [21]. In this study, the external voltages ranging from 0 V to 1.5 V caused variations of the process parameters such as pH, TAN, and VFAs that might disturb the stability of the AD system.

3.2.1. pH and the total ammonia nitrogen

pH usually plays a decisive role in MEC-AD performance. Both the accumulation of VFA and TAN could lead to pH fluctuation [22]. Normally, the suitable pH for the stable operation of the AD system ranges between 6.5 and 8.0 because methanogens are sensitive to pH fluctuation and most electroactive bacteria and methanogens prefer to thrive in the neutral pH environment [23]. The pH was maintained at 8.22 ± 0.03 in the overall process at all applied voltage conditions (Fig. S2) because the inoculum was sampled from the reactor fed with chicken manure, which owns a high buffering capacity that can barely result in obvious disturbance even under severe acidic or ammonia inhibition conditions [24].

The TAN ranged from 851.56 mg/L to 1,632.62 mg/L in all groups (Fig. 3), which was far below the ammonia inhibition threshold of 3,000 mg/L to 5,000 mg/L for an AD system [22]. Compared with 0 V, only 0.6 V showed an obvious difference in both cathode and anode at the end of fermentation which the TAN concentration decreased in the anode but increased in the cathode chamber. It has been reported that TAN could be removed and converted to N_2 via electrochemical oxidation on the anode [25]. However, the reducing environment in the cathode can only result in the formation of TAN by reducing nitrate or transamination from nitrogen-containing organic components [26]. The applied voltage of 0.6 V created an optimal electrochemical environment that might cause the oxidation of TAN to form N_2 in the anode and the reduction of nitrate that existed originally in the inoculum to form TAN in the cathode chamber. Nonetheless, neither the reduction nor the accumulation of TAN affected the overall process since the fluctuation was far below the ammonia inhibition threshold.

The accumulation of TAN often occurred in the AD system treated with high nitrogen content substrates such as chicken manure and food waste [27,28]. CS was the only substrate used in this study, such high carbon-containing and low nitrogen content substrate would mainly result in acidic accumulation rather than ammonia inhibition in an AD system. These results illustrated that neither pH nor TAN could markedly change MEC-AD performance due to stable pH and the relatively low value of TAN.

3.2.2. Volatile fatty acids

The accumulation of VFAs, particularly acetic acid and propionic acid, can cause a significant methanogens inhibition, which results in an imbalance between the syntrophic organic acid oxidation bacteria (SOB) and methanogens, ultimately leading to the souring and collapse of the AD system [29,30]. Details regarding the VFAs changes in the overall

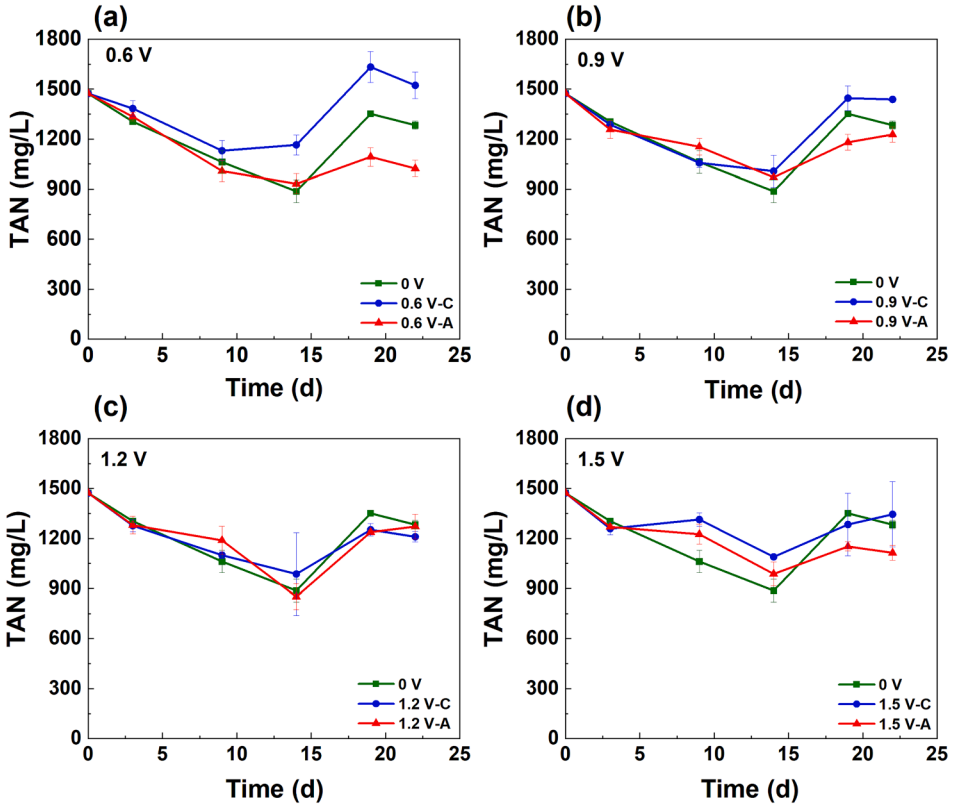

Fig. 3. Fluctuations in the total ammonia nitrogen (TAN) along with the fermentative time at different applied voltages; (a): 0.6 V, (b): 0.9 V, (c): 1.2 V, and (d): 1.5 V (C: cathode chamber; A: anode chamber).

fermentative process at different voltages are shown in Fig. 4.

For 0 V, the VFAs increased gradually from 3,318.81 mg/L to 4,026.72 mg/L, and then declined to 98.98 mg/L on day 19. The highest concentrations of propionic acid (1,915.25 mg/L) and acetic acid (1,690.82 mg/L) were much lower than the inhibition threshold of 3,000 mg/L [31]. The acidification did not occur at 0 V, and all the limited VFAs degraded from CS were consumed by the methanogens to produce CH_4. Compared with 0 V, the 0.6 V and 1.2 V exhibited lower VFA concentrations and rapid consumption within 19 days in both the cathode and anode chambers. A relatively faster VFA consumption was observed at the anode (0.6 V, 9 d) and the cathode (1.2 V, 14 d) when compared to other voltage conditions. This result was in line with the CH_4 production rate: a faster degradation of VFAs would lead to a higher CH_4 production rate.

Despite the complete consumption of acetic acid, notable concentrations of propionic acid were still present at the cathode (3,319.61 mg/L) and anode (3,660.58 mg/L) chambers at 0.9 V after fermentation. The accumulation of propionic acid with a concentration of 3,619.85 mg/L also occurred in the cathode chamber at 1.5 V. These results indicated that severe propionic acid inhibition occurred at both chambers at 0.9 V and at the cathode chamber at 1.5 V, which compromised the CH_4 production at 0.9 V and 1.5 V. Different types of functional microbes are enriched at a specific voltage condition [32]. The 0.9 V might be suitable for the prosperity of fermentative and acidic-producing bacteria (FAB) but not for methanogens, which led to the imbalance of the microbial community and resulted in the final collapse of the MEC-AD system at 0.9 V. Compared with the traditional AD system, the MEC-AD system was proven to have a higher process stability and faster biodegradability under an appropriate voltage of 0.6 V and 1.2 V because the produced VFAs from CS in both chambers were utilized quickly with the assistance of external electric energy.

3.3. Bio-electrochemical characteristics

The effect of electrical control on AD performance can be reflected by bio-electrochemical characteristics such as current and CV characteristic curve. Typically, a well-operated MEC-AD system exhibits a high current density, which is mainly because of the enrichment of electroactive bacteria and the formation of biofilm on the electrode that decreases the reactor's internal resistance [11]. Here, the fluctuation of the current was monitored daily, the cyclic voltammetry was conducted at the end of the fermentation, and the formation of biofilm on the electrodes was verified.

3.3.1. Current and equivalent resistance

As shown in Fig. 5a, the current was maintained at an average of 0.02 mA on the first four days for 0.6 V. Subsequently, its values sharply increased from 0.06 mA (day 5) to 1.02 mA (day 9), 51-fold of the initial value. Then the current was at a relatively higher value between 0.59 mA and 1.08 mA. The current slowly increased from 0.13 mA to 0.35 mA during the overall process at 1.2 V. There was an obvious current increase from 0.35 mA (day 4) to 0.64 mA (day 8) at 1.5 V. After that, its values gradually decreased to 0.29 mA at the end of the fermentation, which was even lower than the initial value. For 0.9 V, the current continuously decreased from 0.07 mA to 0.03 mA in the overall process. There existed no oxidation and reduction peaks for the MEC-AD system without voltage applied (0 V) in the CV curves (Fig. 5b). Nonetheless, two distinct redox peaks were observed at −20 mV for oxidation and −352 mV for reduction at the cathode chamber of 0.6 V. An oxidation peak and a reduction peak of 788 mV and 311 mV were found at the anode chamber of 0.6 V, respectively. The higher current of the oxidation peak implies a higher electron transfer efficiency, which indicated that the electroactive bacteria are enriched in both the cathode and anode chambers [33]. Additionally, the oxidation peak of −20 mV at the cathode of 0.6 V is fitted with the characteristic peak of cytochrome C-

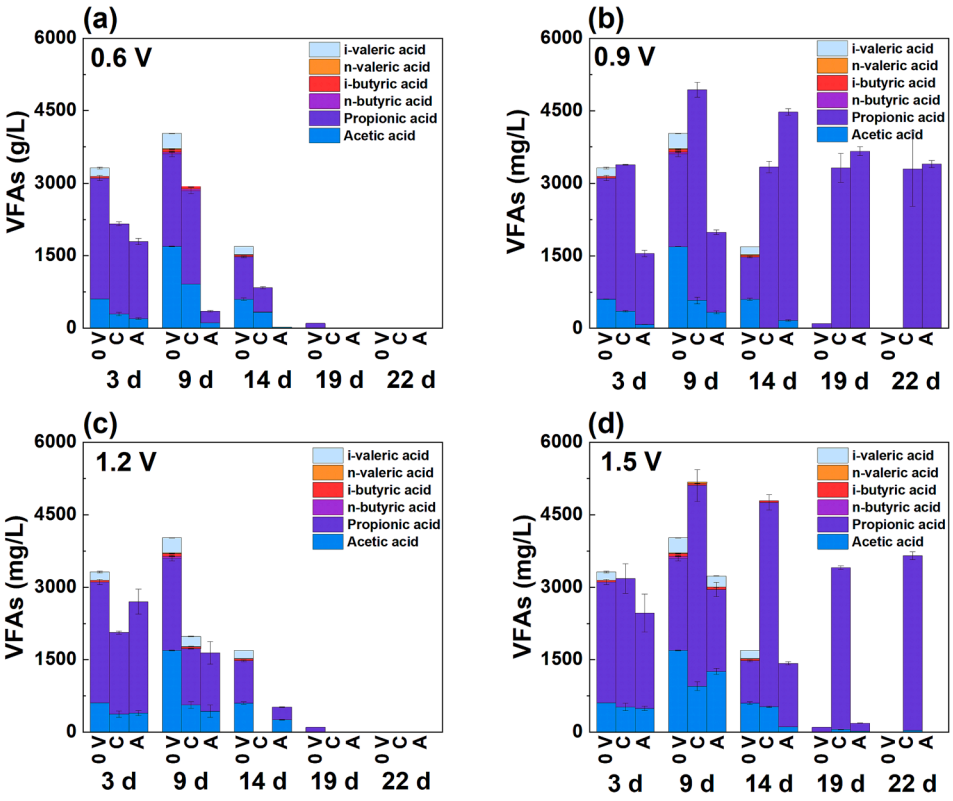

Fig. 4. Fluctuations of the volatile fatty acids (VFAs) along with the fermentative time at different applied voltages; (a): 0.6 V, (b): 0.9 V, (c): 1.2 V, and (d): 1.5 V (C: cathode chamber; A: anode chamber).

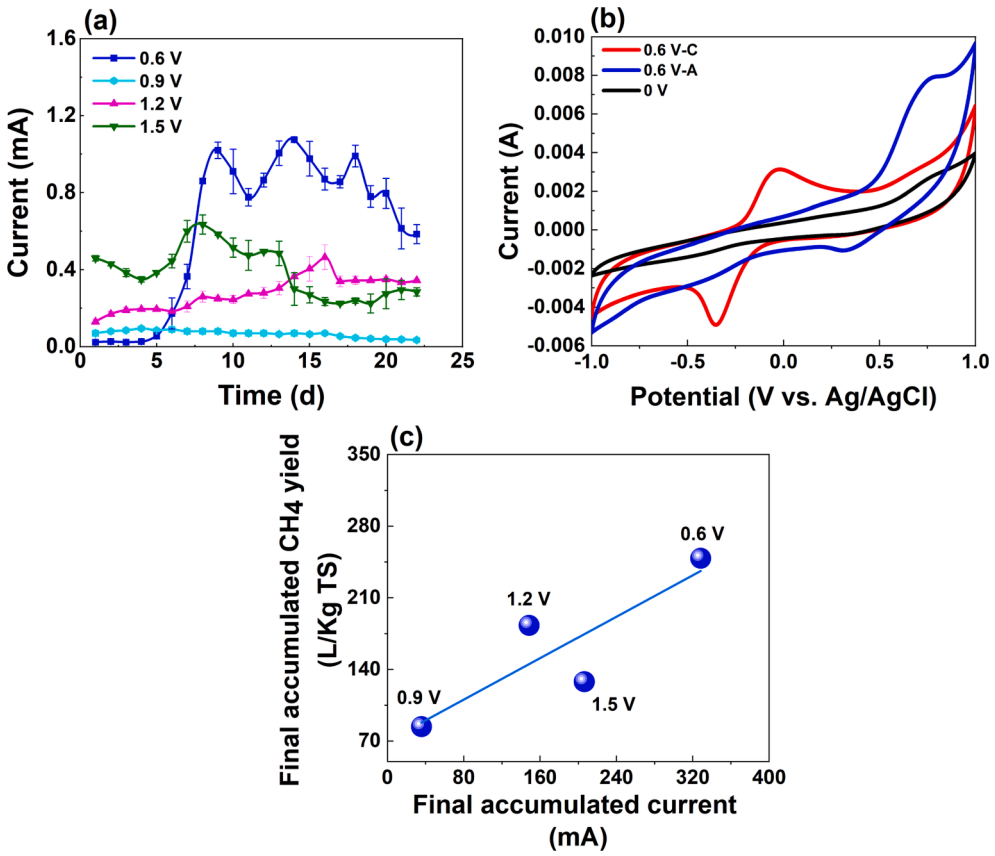

Fig. 5. Daily changes of the current (a) at different applied voltages; (b) Cyclic voltammetry comparison of 0 V and 0.6 V (C: cathode chamber; A: anode chamber); (c) The relationship between the final accumulated CH_4 yield and current.

550 at −20 ± 1 mV vs. Ag/AgCl in the membrane of the new electroactive bacteria of SCS5 [34]. This evidence indicated that the DIET process might occur in the cathode chamber of 0.6 V. As shown in Fig. 5c, there was a positive correlation between the final accumulative CH_4 yield and the final accumulative current, which reflected that the higher current referred to the higher CH_4 production. However, CH_4 yield was not shown to have a regular relationship with voltages. It has been reported that the electroactive bacteria are activated and enriched under an appropriate voltage condition which can accelerate the organic compounds degradation and the VFAs generation [6,35]. The electrotrophic methanogens can also be enriched to participate in the DIET with electroactive bacteria, leading to an increase of the current and a decrease of the equivalent resistance for the closed-circuit MEC-AD system [36].

In this study, a significant increase of the current at 0.6 V implied an enrichment of electroactive bacteria and archaea, which could be well-associated to transform the VFAs produced from CS to CH_4 via DIET in the biofilm on the electrode [37]. Additionally, biofilm can enhance electron transfer via direct cell–cell and cell-electrode contact by c-type cytochromes and conductive pills [38].

3.3.2. *Microbial morphology at the electrodes*

To further confirm the formation of biofilm on the surface of the electrodes, the morphology of carbon cloth was characterized by SEM (Fig. S3). Pure carbon cloth was smooth and clean without any attachment, while biofilm was sporadically attached to the filament of the carbon cloth at 0 V. Nonetheless, the biofilm with high density was observed at the electrode surface for both the anode and cathode at 0.6 V. Results showed that the electroactive microorganisms may be enriched at both electrodes to produce biofilm at the selected voltage of 0.6 V, which could explain the high current and low equivalent resistance to some extent. The CS would be oxidized by the electroactive bacteria attached on the anode surface to release electron, H^+, VFAs, and CO_2, and subsequently, the released electron would be transferred to the cathode via an external electric circuit to reduce CO_2 into CH_4 [3]. The higher enrichment of electroactive bacteria in the MEC-AD system resulted in a higher electron transfer efficiency, which finally led to improved CH_4 production. In this study, a comprehensive microbial community analysis was conducted to further confirm the contribution of electroactive bacteria and other functional microorganisms to the MEC-AD system.

3.4. *Structure of microbial community*

The microbial community plays a key role in the MEC-AD system. The system instability is directly caused by the destruction of the microbial community structure. Generally, there are three types of functional microbes in the MEC-AD system: FAB (fermentative and acidic-producing bacteria), SOB (syntrophic organic acid oxidation bacteria), and MEA (methanogenic archaea, also called methanogens). The organic compounds are initially degraded by FAB to produce organic acids, H_2, and CO_2. Then, these intermediates are further converted to CH_4 by methanogenesis through a syntrophic association between SOB and methanogens [21]. Here, structure of the microbial community was thoroughly investigated which elucidated the impact of applied voltages on the functional microbes at the electrode and in the bulk solution.

3.4.1. *Shifting of the functional microbial community*

The shifts of the top 30 microbial genera in the chamber are exhibited in Fig. 6.

1) Twenty genera belonged to FAB

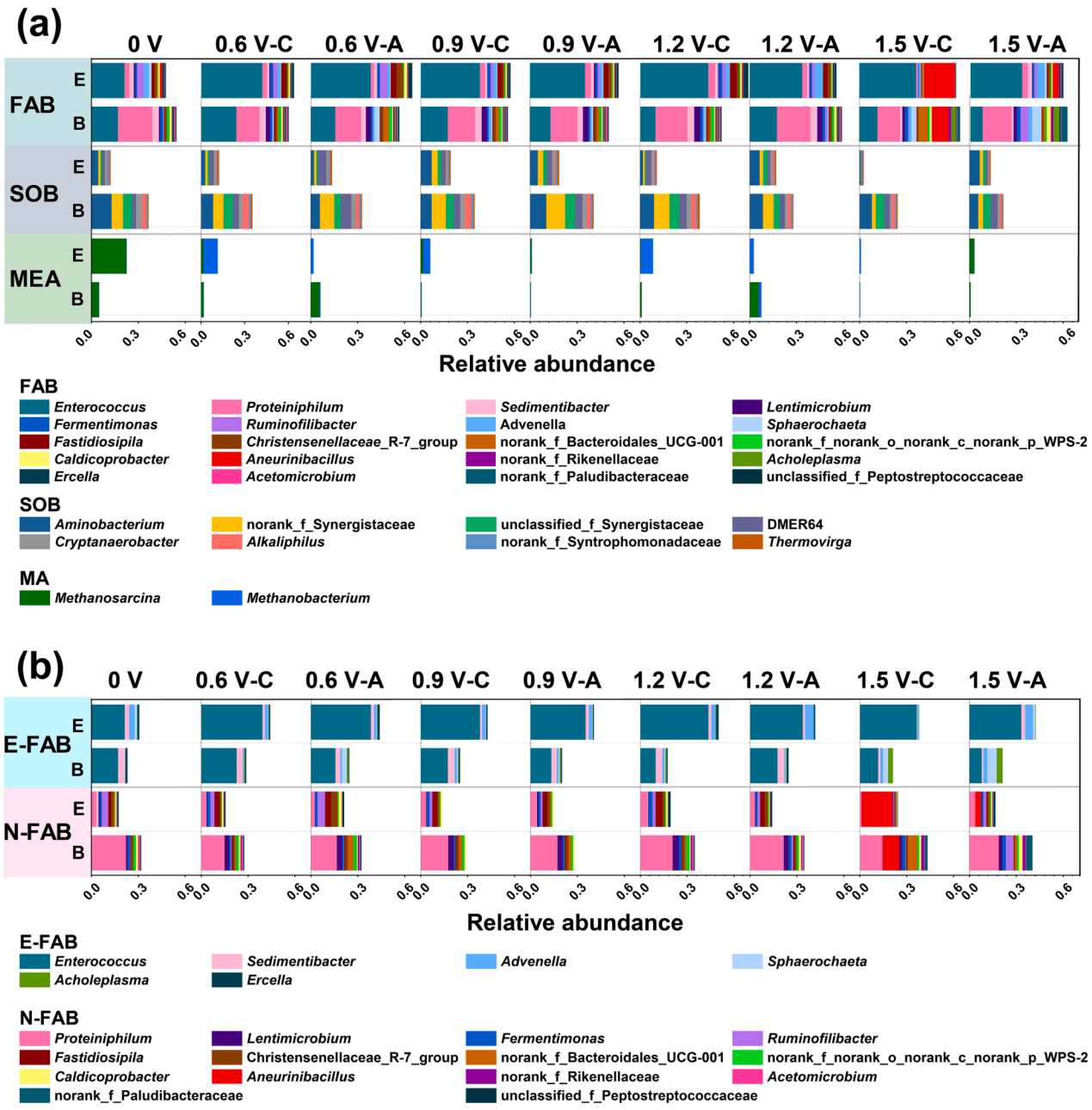

Fig. 6. The overall profile of the functional microbial community; (a) The shifts of the functional microbial community on genus level at different applied voltages; (b) the shifts of the FAB that were classified as E-FAB and N-FAB (FAB: fermentative and acidic-producing bacteria; SOB: syntrophic organic acid oxidation bacteria; MEA: methanogenic archaea; E-FAB: FAB with electroactivity; N-FAB: FAB without electroactivity; E: electrode; B: bulk solution; C: cathode chamber; A: anode chamber).

The FAB were far more abundant than the other two types of functional microorganisms in bulk solution and electrodes because the recalcitrant CS first needs to be degraded to organic acids that can further be utilized by SOB and methanogens. The relative abundance of FAB showed barely difference between the bulk solution and electrodes across all the voltage conditions. However, the abundance of different genera in FAB varied obviously between the bulk solution and electrode (Fig. 6a). In addition, some of the genera in the FAB have been reported to own the electroactivity. Thus, the FAB with electroactivity (E-FAB) and the FAB without electroactivity (N-FAB) were analyzed separately to deepen understanding of their respective effect on the MEC-AD performance (Fig. 6b).

E-FAB contained 6 types of genera including *Enterococcus*, *Sedimentibacter*, *Advenella*, *Sphaerochaeta*, *Acholeplasma*, and *Ercella*, with *Enterococcus* being the dominant genus [35,39–43]. *Enterococcus* is known for its ability to decompose recalcitrant substrates into lactic acid and other organic acids [35]. In the bulk solution, the boom of *Enterococcus* was not observed among all applied voltages. On the electrodes, the relative abundance of *Enterococcus* was found to be significantly higher in the voltage-applied groups, with an average relative abundance of 37.17%, when compared to 0 V (21.18%). This represented a 1.75-fold increase in *Enterococcus* abundance on the electrode surface in the voltage-exposed groups. This suggested that the electroactive bacteria were preferentially enriched on the surface of the electrode when compared to the bulk solution. Despite observing a significant increase in *Enterococcus* abundance on the electrode surface in the voltage-exposed groups, the differences in relative abundance among the different voltage conditions were not perceptible. Results suggested that

the applied voltage significantly stimulated the enrichment of *Enterococcus* on both the cathode and anode surfaces, with little effect on its relative abundance across different voltage conditions.

For the N-FAB in the bulk solution, the relative abundance was consistent across all voltage conditions, accounting for a proportion ranging from 14.48% to 21.99%, with *Proteiniphilum* being the dominant genus. The relative abundance of *Proteiniphilum* was slightly decreased in response to voltage conditions in comparison to 0 V. In addition to *Proteiniphilum*, other genera's abundance showed differences at applied voltage conditions. For instance, the dominant genera shifted from *Fermentimonas* (1.75%) at 0 V to *Lentimicrobium* (2.78%) at the cathode chamber of 0.6 V, and norank_f_Bacteroidales_UCG-001 (3.55%) at the anode chamber of 0.6 V [44,45]. It has been reported that *Proteiniphilum* can degrade nitrogen-containing substrates to produce acetic acid and propionic acid [46]. In this study, the initial inoculum was collected from the anaerobic reactor fed with the high nitrogen-containing substrate (chicken manure), leading to the enrichment of *Proteiniphilum*. The relative abundance of *Proteiniphilum* did not decrease in the MEC-AD system fed with CS as the only substrate, which indicated that *Proteiniphilum* might also thrive in the reactor fed with substrates with the high carbon-containing substrate. At 0.6 V, the relative abundance of *Proteiniphilum* showed a slight decrease from 21.99% to 14.89% at the cathode chamber and 16.53% at the anode chamber, which illustrated that its activity would be suppressed under a well-operated MEC-AD system.

For the N-FAB on the electrodes, the relative abundance of N-FAB was lower than that in the bulk solution, indicating that N-FAB was mainly enriched in the bulk solution. The relative abundance of N-FAB also showed an insignificant fluctuation among different voltage groups, which showed that applied voltage did not bring significant changes of N-FAB in both the bulk solution and the electrodes. Nonetheless, the specific genus in N-FAB were changed among each group at the electrodes. For example, *Ruminofilibacter* was the dominant genus (4.10%) at 0 V and the anode of 0.6 V, but it was changed to *Fastidiosipila* (3.39%) at the cathode of 0.6 V. While, *Proteiniphilum* was the dominant genus at 0.9 V and 1.2 V, which was changed to *Aneurinibacillus* at 1.5 V. *Ruminofilibacter* was identified as a FAB that can degrade lignocellulose by secreting hemicellulose [47]. Species from *Aneurinibacillus* can degrade lignin from lignocellulosic substrates, which had been used as the microbial agent for composting [48,49]. The enrichment of these cellulolytic bacteria at the electrodes of 1.5 V and anode of 0.6 V implied that the CS was well degraded in these reactors.

2) Eight genera belonged to SOB

The relative abundance of SOB was higher in the bulk solution than on the electrodes, indicating that the syntrophic oxidation of organic acid primarily occurred in the bulk solution (Fig. 6a). In the bulk solution, the dominant genus namely *Aminobacterium* was decreased at each applied voltage condition that relative abundance ranging from 4.49% to 8.18% when compared with that at 0 V (10.12%). Some species from *Aminobacterium* can ferment amino acid and other organic compounds to produce H_2, CO_2, and acetic acid, and thus, can be syntrophic with methanogens [3,50]. The decrease in *Aminobacterium* population showed that voltage posed a slight stress on these cells. On the electrode, the dominant genus was shifted from *Aminobacterium* to DMER64 when the voltage was shifted from 0 V to 0.6 V. Species within DMER64 might have enhanced the H_2 transfer between different species, which could be beneficial for the hydrogenotrophic methanogenesis [51].

3) Two genera belonged to MEA

Methanosarcina served as a mixotrophic methanogen that can produce CH_4 by acetotrophic, hydrogenotrophic, and methylotrophic methanogenesis [52]. *Methanobacterium* was reported as a hydrogenotrophic methanogen that can only use CO_2 and H_2 to produce CH_4, and additionally, it also functions as an electrographic methanogen that can receive electrons from electron donors [53]. In the bulk solution, *Methanosarcina* can only maintain a relatively high abundance at 0 V (4.91%) and at the anode of 0.6 V (5.83%) and 1.2 V (5.80%). On the electrode, a relatively high abundance of *Methanosarcina* (22.44%) was observed at 0 V which was sharply decreased at all the applied voltage conditions, suggesting a severe inhibition of *Methanosarcina* at the applied voltages (0.6–1.5 V). Compared with *Methanosarcina*, *Methanobacterium* was shown to be thriving at the cathode of 0.6 V, 0.9 V, and 1.2 V with relative abundances of 11.38%, 6.14%, and 7.86%, respectively. These results indicated that both *Methanosarcina* and *Methanobacterium* were sensitive to the varying voltages. *Methanosarcina* was inclined to thrive in a MEC-AD system without applied voltage. Carbon cloth can serve as a favorable habitat for the growth of *Methanosarcina* and thus lead to its enrichment on the electrode. Although methanogens both survived at 0.6 V and 1.2 V, the types for the dominant methanogenesis were different. Hydrogenotrophic methanogenesis between *Methanobacterium* and *Enterococcus* was preferred to thrive on the cathode at 0.6 and 1.2 V. The DIET process in the microbial community had been reported by many researchers in recent years. However, there are no methods for directly measuring the rates of interspecies electron transfer or the flux of H_2 and formate between species in methanogenic communities [54]. Some researchers observed the microbes attached via cell–cell and/or cell-pill-cell connection by SEM, which concluded that the DIET process happened in the system [53]. Other researchers proved the high expression of the genes relative to cytochrome C and enzymes required for DIET, which also reflected that the DIET process occurred [36]. Although none of these methods can truly prove the existence of DIET, this evidence showed that the DIET possibly happened in the AD system. In our study, we found both *Enterococcus* and *Methanobacterium* enriched on the cathode. Based on the research that *Methanobacterium* is capable of DIET [54], we assumed that the DIET process may occur between *Enterococcus* and *Methanobacterium*. In the bulk solution of the anode chamber, *Aminobacterium* was shown to have a syntrophic association with *Methanosarcina* to conduct the mixotrophic methanogenesis at 0.6 V and 1.2 V. The enrichment of *Enterococcus* on the electrode of the anode chamber might have contributed to a faster degradation of CS. A well-operated AD system depends on the balance among each functional microbial community. The collapse of the MEC-AD system in the voltage of 0.9 V and 1.5 V might have been caused by the depletion of methanogens stressed by the applied voltage.

3.4.2. Dominant functional microorganisms at 0.6 V

The 0.6 V accelerated the methanogenesis and enhanced the CH_4 production in the MEC-AD reactor fed with CS. Herein, the differences of the top microorganisms in each functional group between the bulk solution and the electrode at 0.6 V are unveiled (Fig. 7). *Proteiniphilum* was shown to thrive at the bulk solution for both 0 V and 0.6 V, which indicated the dominant fermentative and acid-producing process was accomplished in the bulk solution for both chambers. *Enterococcus* functioned as E-FAB are the most dominant genus in the MEC-AD system. It had a relatively higher abundance on both electrodes, which proposed that it mainly accelerated the degradation of CS at the anode, and it played an important role at the cathode where it was well associated with *Methanobacterium* for producing CH_4 via DIET. Both *Aminobacterium* and norank_f_Synergistaceae were more enriched in the bulk solution and barely affected by 0.6 V, illustrating that the SOB was well associated with *Methanosarcina* in the bulk solution of the anode chamber [55]. *Methanosarcina* can thrive in the MEC-AD reactor without applied voltage for both bulk solution and electrode, where a relatively higher abundance (22.44%) was observed on the electrode. It suffered a severe inhibition at the electrode of the anode chamber but boomed at the bulk solution of the anode chamber. *Methanobacterium* could only thrive on the electrode at 0.6 V with a higher relative abundance at the cathode chamber than the anode chamber, which indicated that it can be stimulated by the applied voltage and can thrive under the reducing

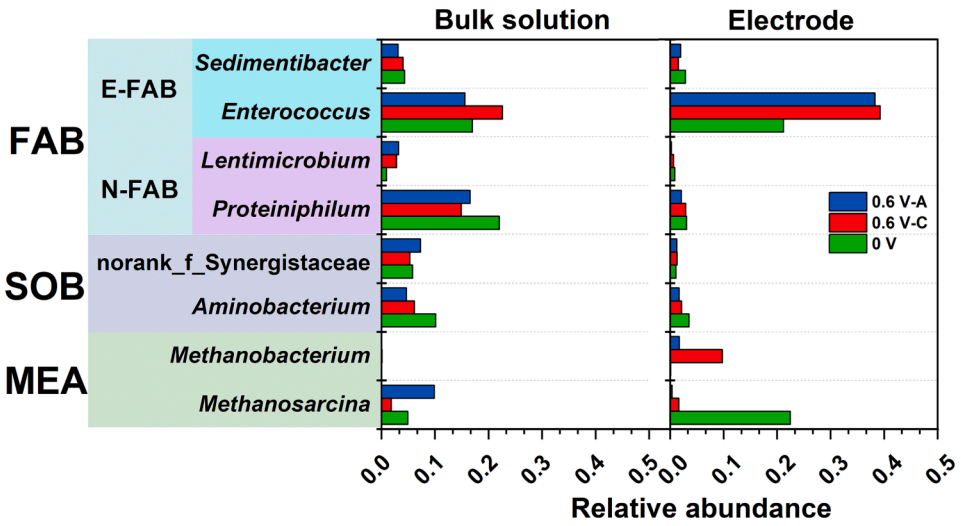

Fig. 7. Comparison of the relative abundance of the top two genera from each functional microorganism between the bulk solution and the electrode (FAB: fermentative and acidic-producing bacteria; SOB: syntrophic organic acid oxidation bacteria; MEA: methanogenic archaea; E-FAB: FAB with electroactivity; N-FAB: FAB without electroactivity; C: cathode chamber; A: anode chamber).

environment. In this study, a part of the released H^+ in the bulk solution of the anode chamber could pass through the proton membrane and transfer to the cathode chamber via the electric field. The released electrons at the anode could also transfer to the cathode via the external circuit [3]. Thus, *Methanobacterium*, which functioned as the strict hydrogenotrophic methanogen, can use electron and H^+ reducing CO_2 to form CH_4 on the cathode.

The correlation network reflected the stability and the relationship between the core functional microorganisms in the MEC-AD system before and after the voltage applied (Fig. S4). After the voltage of 0.6 V was applied to the MEC-AD system, the network of the bacterial community became more intensive. The dominant genera of *Enterococcus* showed no correlation with other microorganisms at 0 V but presented a positive correlation with electroactive bacteria of *Ercella* and SOB of DMER64 and norank_f_Synergistaceae at 0.6 V. The dominant methanogen of *Methanosarcina* has no or negative correlation with other methanogens at 0 V and *Methanobacterium* showed a negative correlation with *Methanosarcina at* 0.6 V, which was in line with the results mentioned before that *Methanosarcina* was more sensitive to the applied voltage in comparison with *Methanobacterium*.

3.4.3. *Mechanism of improved methanogenesis in double-chamber MEC-AD system*

Fig. 8 shows changes in the functional microbial community when the voltage of 0.6 V was applied to the MEC-AD system. In the bulk solution, the mixotrophic methanogenesis was dominated by *Methanosarcina* which was decreased at the cathode when the voltage was applied, but it still thrived at the anode. The FAB and SOB were barely affected by the applied voltage, which indicated that *Methanosarcina* played a key role to maintain the stability of the MEC-AD system. A large difference in methanogens was observed on the electrodes when the voltage was applied. The dominant methanogens changed from *Methanosarcina* to *Methanobacterium* on the cathode at applied voltage conditions. Additionally, the E-FAB, mainly *Enterococcus*, was significantly enriched on both cathode and anode when compared with that in the bulk solution at 0.6 V.

The mechanism that conferred an improved efficiency of the MEC-AD system is summarized in Fig. 9. In the bulk solution of both the cathode and anode chambers, the CS was mainly hydrolyzed by *Enterococcus* and *Proteiniphilum* (FAB) to produce VFAs, H^+, and CO_2. After that, *Aminobacterium* and norank_f_Synergistaceae (SOB) associated with *Methanosarcina* produced biogas via mixotrophic

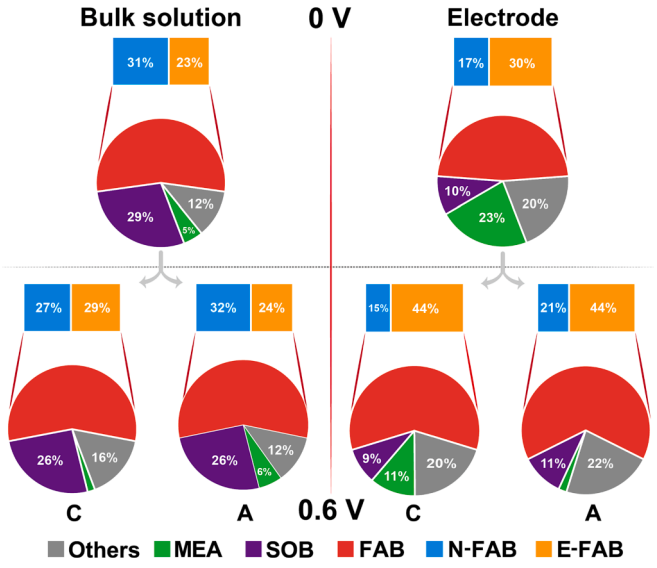

Fig. 8. The profiles of the functional microbial community at 0 V and 0.6 V (FAB: fermentative and acidic-producing bacteria; SOB: syntrophic organic acid oxidation bacteria; MEA: methanogenic archaea; E-FAB: FAB with electroactivity; N-FAB: FAB without electroactivity; C: cathode chamber; A: anode chamber).

methanogenesis. When the voltage of 0.6 V was applied, the enriched *Enterococcus* (E-FAB) accelerated the hydrolysis of CS on the anode. The released electron from CS was transferred to the cathode via an external circuit and a part of the released H^+ passed through the proton membrane to the cathode. On the cathode, the enriched *Enterococcus* and *Methanobacterium* could syntrophic associated with each other via DIET, and thus, the hydrogenotrophic methanogenesis could be accelerated to a large extent. In addition, *Methanobacterium* could also receive electrons from the cathode directly by reducing CO_2 to CH_4. Herein, the applied voltage enriched the *Enterococcus* on the surface of the anode, which led to an increased hydrolysis of CS. Subsequently, the mixotrophic methanogenesis stably proceeded in the bulk solution, which resulted in the final improvement of CH_4 production at the anode chamber. In contrast to that, the enrichment of *Methanobacterium* and *Enterococcus* led to an effective hydrogenotrophic methanogenesis via

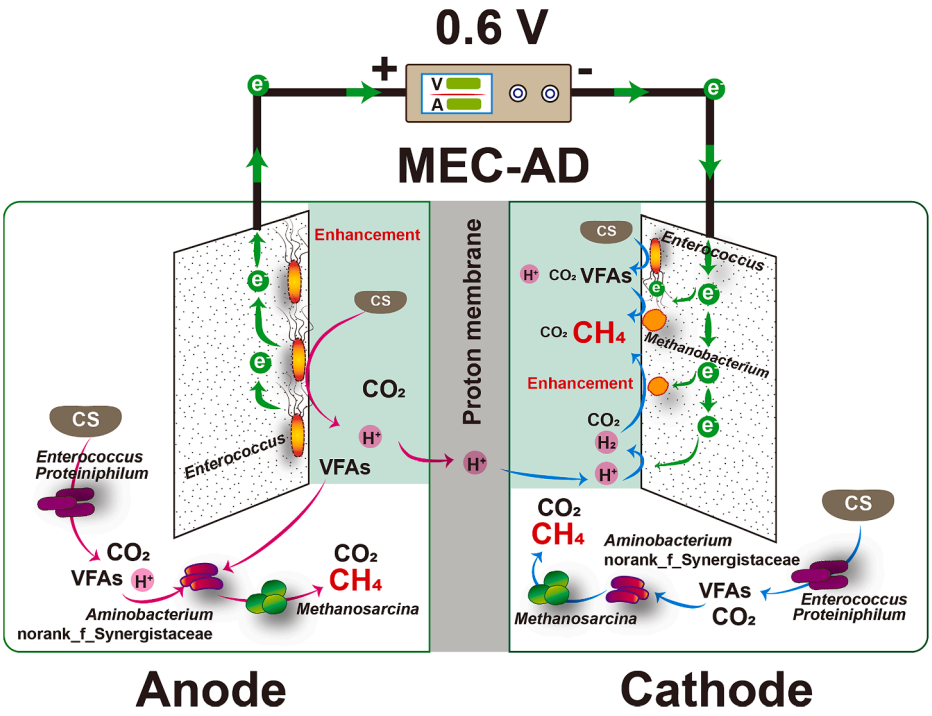

Fig. 9. The speculated fermentative process in the MEC-AD reactor at the selected voltage of 0.6 V (CS: corn stover; the light green areas represent the AD process enhanced by electrical control).

DIET on the surface of the cathode, and thus, resulted in the final enhancement of the CH_4 production at the cathode chamber. In a single-chamber MEC-AD system, researchers speculated that the anode oxidizes the organic compounds and releases electrons, H^+, and CO_2. After that, the cathode directly utilizes the released substrates for CH_4 production via mainly H_2-mediated methanogenesis [11]. Similar speculation has also been made for the reaction on the anode in the double-chamber MEC-AD system. However, the mixotrophic methanogenesis could still proceed in the bulk solution without further inhibition at the anode chamber of the double-chamber system, which has not been reported so far. In addition, the enhanced CH_4 production via DIET between electroactive bacteria and hydrogenotrophic methanogens on the cathode was also proposed in the double-chamber MEC-AD system.

4. Conclusion and prospects

The CH_4 yield of CS was enhanced by 80.41% in the MEC-AD system at a voltage of 0.6 V. Additionally, the anaerobic start-up process was also accelerated in cathode and anode chambers by 2.92-fold and 3.26-fold, respectively. A sharp suppression of methanogens was observed at the anode of 0.9 V, which finally resulted in the disassociation of microorganisms and caused the accumulation of propionic acid, subsequently compromising the CH_4 production. The enriched *Enterococcus* (E-FAB) on the anode accelerated the degradation of CS, and thus, enhanced the subsequent mixotrophic methanogenesis dominant by *Methanosarcina* in the bulk solution of the anode chamber. The enhanced syntrophic relationship between *Enterococcus* and *Methanobacterium* via DIET led to effective hydrogenotrophic methanogenesis on the electrode of the cathode chamber. Both the stable operation of the anode and cathode chambers led to the final improvement of CH_4 production in the MEC-AD system.

Therefore, the double-chamber MEC-AD system helped to develop a better understanding of methane production under specific voltages with CS as a substrate. This study will lead towards a better application of voltages and double-chamber systems for efficient AD-based methanogenesis.

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CRediT authorship contribution statement

Tian-Jie Ao: Investigation, Methodology, Writing – original draft. **Xin-Qing Zhao:** Writing – review & editing. **Muhammad Aamer Mehmood:** Conceptualization, Methodology, Writing – review & editing. **Ning Wang:** Writing – review & editing. **Hui Zhu:** Writing – review & editing. **Chen-Guang Liu:** Supervision, Funding acquisition, Project administration, Conceptualization, Writing – review & editing. **Feng-Wu Bai:** Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

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