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Microbial electrolysis contribution to anaerobic digestion of waste activated sludge, leading to accelerated methane production

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Abstract

Methane production rate (MPR) in waste activated sludge (WAS) digestion processes is typically limited by the initial steps of complex organic matter degradation, leading to a limited MPR due to sludge fermentation speed of solid particles. In this study, a novel microbial electrolysis AD reactor (ME-AD) was used to accelerate methane production for energy recovery from WAS. Carbon bioconversion was accelerated by ME producing H_2 at the cathode. MPR was enhanced to 91.8 gCH_4/m^3 reactor/d in the microbial electrolysis ME-AD reactor, thus improving the rate by 3 times compared to control conditions (30.6 gCH_4/m^3 reactor/d in AD). The methane production yield reached 116.2 mg/g VSS in the ME-AD reactor. According to balance calculation on electron transfer and methane yield, the increased methane production was mostly dependent on electron contribution through the ME system. Thus, the use of the novel ME-AD reactor allowed to significantly enhance carbon degradation and methane production from WAS.

Keywords: microbial electrolysis AD reactor; waste activated sludge; energy recovery; bio-electron; methanogenesis

1. Introduction

The large amount of activated sludge generated during wastewater treatment poses a critical threat (when not properly disposed) to ecological systems [1], while proper treatment and disposal of excess sludge is quite expensive (Wei et al. 2003). On the other hand, anaerobic digestion (AD) is widely used for sludge reduction as an energy saving and recovering method [2]. However, AD rate is substantially limited by the first two steps (hydrolysis and acidogenesis) to convert complex organic compounds into suitable substrates for methanogenesis, in raw sludge [3-5]. Commonly, it takes from 20 to 30 days to degrade 30-50% of the total COD or volatile solids (VS) of raw WAS, under mild environmental conditions [6]. The pressure of rapid human population growth and increasing energy demand have thus promoted further research on development and improvement of an rate-accelerating AD process, in order to enhance biogas production and achieve faster degradation rate from WAS [7, 8].

Recently, some researchers pointed out that bioelectrochemical systems have the ability to promote carbon oxidation on anode and in-site CO_2 capture and reduction on cathode, thus providing additional CH_4 formation in an integrated AD system [9, 10]. Recently a direct interspecies electron transfer for methanogenesis has been proved between *Geobacter* and *Methanosaeta* [11]. However, few efforts have been made to better understand bioelectrochemical contributions to organic conversion or methane promotion, which is very important to achieve viable reactor operations in the future. Lately, microbial electrolysis cells (MECs) have been tested for their ability to convert waste organic compounds from sludge fermentative liquid (SFL) to electrons and hydrogen, showing high efficiencies [12-14]. It seems thus possible to achieve a faster conversion of complex substrates and fermentation end-products into H_2, under an external voltage [15]. It is well-known that methane is synthesized by hydrogenotrophic and acetoclastic methanogenesis from simple carbon sources, including CO_2-type substrate, methyl-type and acid-type substrate (acetate) [16, 17]. More complex substrates can usually not be quickly (or directly) converted to methane. However, recovery products on cathode would trigger AD process in different energy-flow pathways, leading to methane production from CO_2 reduction [11]. Therefore, it is possible to stimulate a fast methane production with the contribution of microbial electrolysis process. On the other hand, the exact contribution of microbial electrolysis system in AD for sludge treatment still needs to be well understood, both in terms of its contribution to enhanced substrate degradation, as well as enhancement of methane production rate.

Therefore, in this study, a coupled system was tested, by putting a microbial electrolysis (ME) system into an AD system, for raw waste activated sludge treatment at mild environmental conditions. The microbial electrolysis system was enriched in MECs and the anodic biofilm was subsequently used to set up the ME-AD reactor. The performance of the methane production rate was evaluated, based on current electrons in the circuit of the ME-AD system. Moreover, functional communities (on key positions) were analyzed by means of high throughput sequencing, to illustrate microbial electrolysis stimulation.

2. Material and methods

2.1 Microbial electrolysis system setup

Microbial electrolysis cells were set up to enrich functional anodic communities, using single chamber reactors made of polycarbonate (45 mm diameter, 80 mm length; volume 130 mL) [18]. The anode was a graphite brush (40 mm diameter, 80 mm length; 1.01 m^2 surface area). The cathode was made from carbon cloth (40 mm diameter, YW-50 YiBang; China), covered with a Pt catalyst layer (0.5 mg Pt /cm^2 inner side). Eight single-chamber MEC reactors were inoculated, using aeration tank effluent from the Wenchang municipal WWTP in Harbin, China. All reactors were started up as replicates, at a fixed applied voltage of 0.8 V (FDPS-150, Fudan Tianxin Inc. China). Acetate (1500 mg L^{-1}) was used as carbon source in a phosphate buffer medium (50 mM; pH = 7.0) [15]. The replicates were operated in 48 h batches, until reaching stable (and similar) performance. Subsequently, three MEC reactors were randomly taken from the replicates, and kept running, using sludge fermentative liquid as carbon source, to test the biodegradation and energy recovery. Four anode brushes with functional biofilms were taken out from the remaining replicates and used as bioanode to set up hybrid ME-AD reactors.

2.2 ME-AD reactor operation and performance test

The novel ME-AD reactor consisted of a glass cylinder of 70 mm inner diameter x 180 mm height, with an effective volume of 650 mL. The anode brush with its biofilm (previously enriched in the MECs) and a new cathode were put into the cylinder. The distance between downside cathode and upside anode brush was 1 cm. The working volume was ~500 mL, with a headspace of ~150 mL, when the ME-AD reactor was operated in batch mode with 0.8 V external voltage at the beginning (Fig. S1). Current of electron transfer was measured over a 10 ohm resister in series connection with reactor using a multimeter (model 2700; Keithley Instruments). The bioelectrochemical system can work well for hydrogen harvest if the current went up over 0.5 mA[12, 19]. Two ME-AD reactors were set as replicates. Two AD reactors were

also operated as control reactor, without anode brush. 500 mL pretreated waste activated sludge was thus put into the ME-AD reactor for anaerobic digestion at room temperature (20-25 ^{o}C). Batch operations were monitored over 45 days, and six microbial community samples were taken at different time points.

2.3 Characteristics of waste activated sludge

Waste sludge was collected from the secondary sedimentation tank of the same local WWTP. The sludge was concentrated by settling for 24 h and washing away the water layer. The large particles were separated by means of a 40 mesh sieve before used as feedstock. The main characteristics of concentrated WAS are reported in Table S1. Bi-frequency ultrasonic pretreatment was performed with 28+40 kHz ultrasonicator (Ningbo Scientz Biotechnology Co., China), by applying an ultrasonic energy density of 0.5 kW/L for 10 min, before addition to the ME-AD reactors. Ultrasonic-pretreated WAS was hydrolyzed and acidified in bench-scale batch experiments for 4 days, at room temperature of 20-25ºC[12]. The sludge fermentative liquid was centrifuged and collected for single chamber MEC tests.

2.4 Sample collection, DNA extraction and 16S rRNA gene pyrosequencing

Biofilm samples were taken from graphite fibers, which were cut from anodes or cathode cloth and fragmented, using sterile scissors. Biofilm samples were taken in three different locations of the targeted electrode and combined together for DNA extraction. Before DNA extraction, fibers were gently rinsed with deionized water to remove the residual sludge [13]. Liquid samples were taken and centrifuged at 8000 g to remove supernatant; approximately 0.25 g pellet were used for DNA extraction. A rapid soil DNA isolation Kit (SK8234, Sangon Biotech, Shanghai) was used to extract DNA, according to the manufacturer's instructions. DNA was quantified by Qubit 2.0 DNA Kit for PCR amplification. PCR amplicons were visualized by using gel electrophoresis to confirm amplification of properly-sized products. Purified PCR products were quantified as described for the DNA extracts, then stored at -20 ℃ before pooling for sequencing.

Miseq sequencing was constructed for Illumina, using bacterial primers 341F: CCTACACGACGCTCTTCCGATCTN (barcode) CCTACGGGNGGCWGCAG and 805R: GACTGGAGTTCCTTGGCACCCGAGAATTCCAGACTACHVGGGTATCTAATCC） for the V3-V4 region of the 16S rRNA gene. Raw sequencing data obtained from this study were deposited in the NCBI Sequence Read Archive. To minimize the effects of random sequencing errors, low-quality sequences were removed, by eliminating those without an exact match with the forward primer, those without a

recognizable reverse primer, length shorter than 200 nucleotides, or containing any ambiguous base calls (Ns).

2.5 Analysis and calculation method

Voltages were measured over a 10 ohm resister in each circuit, using a multimeter (model 2700; Keithley Instruments). The electron production and coulombic contribution were calculated in order to characterize the performance of the ME system [20]. The gas was collected in a gas bag (500 mL; Cali5-Bond; Calibrated Instrument Inc) and the volume measured by means of a glass syringe. Gas composition (methane, hydrogen, carbon dioxide) was analyzed by a gas chromatograph (Fuli, GC9790; Zhengjiang instrument Inc, China), with a packed column [12] (TDX-01; 2 m length) and a TCD detector. VFAs were analyzed by a gas chromatograph (Agilent, 4890; J&W Scientific, USA), with a capillary column (19095N-123HP-INNOWAX; 30×0.530 mm×1.00 μm; J&W Scientific, USA) [20], equipped with an FID. Liquid samples were centrifuged at 10,000 rpm min^{-1} and filtered through 0.45 μm membrane filters, before GC analysis. The sludge was characterized according to standard methods, including TSS, VSS[21].

The coulombic efficiency were calculated to characterize the performance of MEC reactor. Columbic efficiency indicated the recovery ability of electron, defined by the ratio of coulombs recovery to the total coulombs in substrate, which is integrated by current and time according to the equation $Q=\int I\times t$. The coulombs recovery can be calculated by the equation $Q=\int i\cdot\Delta t$, where i is the current of the external circuit. The total coulombs can be calculated by the equation $Q_t=(CODin-CODout)\cdot V\cdot F\cdot b/M_{O2}$, where F represents the Faraday constant, 96485 C/mol; M_{O2} is the molar mass of oxygen, 32 g/mol; b is the complete oxidation requirement of electron per mole oxygen and b is 4 mol-e^-/mol. The current to theoretical methane yield was calculated by $CO_2 + 8H^+ + 8e^- = CH_4 + 2H_2O$ [22], where the electrons were determined by the integration of current and time.

DNA sequences were clustered into operational taxonomic units (OTUs) by setting a 0.03 or 0.05 distance limit (equivalent to 97% or 95% similarity), using the MOTHUR program. Sequences were phylogenetically assigned to taxonomic classifications, using an RDP naïve Bayesian rRNA classifier with a confidence threshold of 80% (http://rdp.cme.msu.edu/classifier/classifier.jsp). After phylogenetic allocation of the sequences down to the phylum, class and genus level, relative abundance of a given phylogenetic group was set as the number of sequences affiliated to that group, divided by the total number of sequences per sample.

3. Result and discussion

3.1 Fermentation products and enhanced organic removal in ME-AD system.

The ME-AD and AD control reactors were directly filled with ultrasonic-pretreated WAS and operated under batch operation (1 day). The highest VFAs accumulation in the AD reactor was 5100 mg COD/L (from the 3rd to the 10th day), while it increased to 4300 mg COD/L in the ME-AD reactor (Fig. S3). Methane production was detected after 4 days operation in all reactors. The current was below 2 mA in the first 5 days, under a supplied voltage of 0.8 V. Subsequently, current in the ME-AD reactor started to increase sensibly, going from 2.2 mA, (the 6th day) up to 11.8 mA on the 10th day (Fig. S2). Methane production was simultaneously increased from 100 mL to 1200 mL during 10-35 day (Fig. 1). After 12 days, the obtained methane production rate was 91.8 gCH_4/m^3 reactor/d (138 mL CH_4/ L reactor/d) (R^2=0.981) in the ME-AD, thus resulting in a significant improvement, compared to 30.6 gCH_4/m^3 reactor/d (46 mL CH_4/L reactor/d) (R^2=0.967) observed in the AD reactor. The degradation of polysaccharides and proteins were enhanced in ME-AD reactor. Compared to AD control, a lower accumulation was detected both on polysaccharides and protein in ME-AD (Fig. S4-5).

During enhancement of microbial electrolysis, the MECs achieved high efficiency performances with coulombic efficiency of 102.7±4.5% in the direct-MEC start-up mode, using acetate as carbon source with a COD removal of 87.5±3.3%, at an applied voltage of 0.8 V. Average hydrogen conversion yield reached 3.5±0.3 mol H_2/ mol acetate, with a hydrogen production rate of 1.7±0.1 L H_2/L reactor/d. Very little methane was also detected, after approximately one month operation. However, in ME-AD reactor hydrogen was only detected in the headspace on the 7th day, in one of ME-AD reactors out of four replicates, indicating that methane was quickly produced under the functions of microbial electrolysis. It is worth noting that acetotrophic methanogens can compete with anode respiring bacteria to degrade acetate [17]. However, besides acetate, much more propionate, butyrate and valerate were also degraded, in a short time, in the ME-AD reactor. A further enhancement was achieved after 26 days operation, with a methane production yield in ME-AD reactors reaching 116.2 mg/g VSS, which was twice that of the AD control (56.5 $mgCH_4$/g VSS). ME-AD showed an enhanced removal of polysaccharides and proteins (~30% and ~50% respectively). VSS removal increased from 38% to 48%. Bioelectrochemical systems were proved to have great potential to degrade complex carbons [23], with a high diversity of communities in electrode biofilm to enhance carbon degradation [15].

3.2 Methane production balance calculation based on electron transfer

When bioelectrochemical contribution to methane production was evaluated, based on electrons in current, the difference between the ME-AD and the AD control was quite close to the part of methane deriving from bioelectrochemical contribution, suggesting that the increased part of methane was contributed mostly by microbial electrolysis process. For instance, when methane production accelerated (between day 16 and 20) with a current of 11.08 ± 0.40 mA, the methane production averaged 66.98 mL per day in ME-AD. The calculated (average) methane generation (representing the biochemical contribution) was 27.8±1.0 mL per day, based on current, while methane production in the AD control averaged 33.56 mL per day.

In addition, it was also proved when ME-AD was operated in open circuit (Fig. 2). The methane production decreased from 55.9±9.7 mL to 25.3±6.9 mL when the applied voltage was removed (Table S2). Methane production rate was reduced by 54.8% in open circuit of ME-AD reactor. It also reasonably matched the part of reduced methane, which was calculated from current electrons (11.84 ± 0.55 mA) up to 29.7±1.4 mL. The enhanced methanogenesis was primarily caused by the hydrogen-utilizing process in single chamber MEC reactor [22]. Hydrogen consumption was also detected in other studies [9]. Furthermore, it has been pointed out that anodic respiring bacteria could compete with acetotrophic methanogenesis on organic oxidation [24]. Therefore, recovered hydrogen would be more feasible to hydrogen-utilizing microorganisms.

3.3 Microbial community structure detected on key locations of ME-AD reactors

Community structure showed prominent changes on functional groups on the family level (Fig. 3). A clear even distribution of anaerobic bacteria was detected at start-up phase (raw sludge). Specific community enrichment started on the anode biofilm in ME-AD system. Bacterial families belonging to the *Proteobacteria* and *Firmicutes* phyla dominated in the anode biofilm; however, typical anode respiring bacteria (like *Shewanella* and *Geobacter*) were not overwhelming on the 3rd day, at low current generation. Neither fermentative bacteria nor methanogens (*Archaea*) were enriched as dominant communities in anode biofilm. Only after methane production accelerated (with a current of ~10 mA), the bioelectrochemcial process showed a significantly enriched community of *Geobacteria* (from initial 0.04% to 21.86% of overall detected communities) in the anode biofilm. The two dominant communities belonged to the families of *Anaerolineaceae* [25] and *Coriobacteriaceae* (*Coriobacteriaceae* is a subclass of *Actinobacteria*), which are known to constitute a large part of anodic communities in bioelectrochemical systems [26].

Furthermore, there was a great contribution to extracellular electron transport, witnessed by the anode respiring communities found (Fig. 4), including *Geobacter* [27], *Shewanella* [28] and *Pseudomonas* [29]. *Geobacter* accounted for over 20% of total genus detected in the anode biofilm of ME-AD system (it was only 0.11% at the start-up) and was also detected in the cathode biofilm (0.33%). Probably, the great increase of *Geobacter* significantly enhanced organic oxidation and electron transferring, which occurred simultaneously with the arising of current and methane production. In fact, *Geobacter* represents one of the most important groups of exoelectrogens, showing high efficiency of electron transport between bacteria and electrode [27, 30]. Recently a direct interspecies electron transfer was proved between *Geobacter metallireducens* and *Methanosarcina barkeri* [31]. Thus, the development of a *Geobacter* community at the cathode is considered to have a great potential for the methane recovery in a ME system.

3.4 Hydrogenotrophic methanogens and *Acetobacterium* accumulation by ME.

Methanogens detected in the suspended solution were as low as 0.03% in AD and 0.05% in ME-AD, compared to 0.3% of the sludge start-up (Fig. 4). On the other hand, there was a remarkable ten-fold increase (0.56%) in anode biofilm (data not shown) of ME-AD system, at the end of the study. In addition, acetotrophic methanogens also increased (from initial 0.03% to 0.49%) in anode biofilm. Interestingly, they did not further increased in the ME system, despite the higher VFAs (i.e. acetate) concentration. *Methanosaeta* was the dominant class of acetotrophic methanogens in sludge start-up, with 0.27% out of total genus detected, while only 0.03% of total hydrogenotrophic methanogens were detected during sludge digestion. In the cathode biofilm, hydrogenotrophic methanogens were substantially boosted, which well supported the increased methane production rate. Few hydrogenotrophic methanogens were detected in suspended solution surrounding the electrodes.

Although it was inevitable to inhibit methanogens in the system[32], it was pointed out that anode respiring bacteria (ARB) can compete methanogens in anode biofilm because ARB have faster carbon degradation than methanogens[22]. Based on coulombic efficiency evaluation, which was calculated to characterize the recovery ability of bioanode from electrons of substrates, usually ~90% coulombic efficiency was achieved by ABR, which mean that ~10% loss may be caused by other microorganism (including acetotrophic methanogens) in single chamber MECs[22]. Therefore, acetotrophic methanogens would not easily become overwhelming communities over ARB on anode. Furthermore, it was very important to establish an ARB-dominant bioanode for integrated reactor firstly in order to enhance bioelectrochemical contributing methane production rate over conventional AD. Otherwise, an increased

231 biomass with dominant anaerobic digestion functions will mainly contribute to bioreactor performance
232 improvement from biomass but not from current[33].
233 Among methanogens, the group that showed the highest increase was *Methanobacterium* (6.4% of total
234 genera detected), which belongs to the class *Methanobacteria,* and is known to grow on H_2/CO_2 and
235 formate as carbon source [17]. The second group was represented by *Methanosaeta* (accounting for 1.2%),
236 which functioned as acetotrophic methanogen. The third group was represented by *Methanospirillum*
237 (accounting for 0.56%), which is also known to be a hydrogenotrophic methanogen, belonging to the
238 family of *Methanospirillaceae*. The remaining detected genera were *Methanobrevibacter* (0.01%,
239 hydrogenotrophic) and *Methanosarcina* (0.01%, acetotrophic). *Methanosaeta* was the only genus also
240 found in initial raw sludge and solution communities in AD control. In the ME system, all
241 hydrogenotrophic processes were limited to the biofilm layer of cathode (i.e. *Methanospirillum, with* 0.01%)
242 and did not significantly spread to planktonic area. As reported by Rotaru and colleagues [11],
243 hydrogenotrophic methanogens are usually regulated by the hydrogen produced on the cathode surface or
244 by direct interspecies electron transport.
245 According to the balance calculation, it was reasonable to presume that electrons of current contributed to
246 final methane production. Noticeably, cathodic hydrogenotrophic methanogens contributed most to the
247 enhanced methane production, though anodic methanogens were slightly enriched. In any case, the detected
248 anodic methanogens were only 6% of those on the cathode, thus giving a far from predominant contribution
249 to total methane production. Actually, acetotrophic methanogens would not be competitive with
250 hydrogenotrophic methanogens in single chamber MEC reactor [22]. On the other hand, *Acetobacterium*
251 (family *Eubacteriaceae)*, a hydrogen scavenging bacterium, was substantially enriched in the cathode
252 biofilm. It was reported that homo-acetogenic processes only occur if methanogenesis is inhibited [34].
253 However, in the present study the hydrogen generation lead the coexistence of *Acetobacterium* and
254 hydrogenotrophic methanogens in cathode biofilm. This was witnessed by a stimulated increase of
255 *Methanosaeta* (1.2%) in the cathode biofilm, which was even higher than the acetotrophic methanogens in
256 anode biofilm (0.49%). The reactions were clearly limited to the cathode biofilm, because no noticeable
257 increase of acetate or acetotrophic methanogens were detected in the solution surrounding the cathode,
258 while the impact of this inner recycle is still poorly understood. Nonetheless, this phenomenon proved to
259 represent an important adaptation for biocathode communities to increase methane production rate in
260 sludge fermentation.

4. Conclusions

Methane production rate from waste activated sludge treatment were improved by 3 times (from 30.6 to 91.8 gCH_4/m^3 reactor/d) in a modified anaerobic digestion reactor, coupled with a microbial electrolysis system with a fixed external voltage of 0.8 V. Furthermore, the carbon degradation of VFAs, polysaccharides and proteins was accelerated by 22%, 43% and 48%, respectively, by the microbial electrolysis system. The VSS removal increased from 38% to 48%. Hydrogenotrophic methanogens were substantially enriched in cathode biofilm, which in turn lead to an increased methane production rate. The increased methane production was comparable to methane conversion from current electrons. Microbial communities in electrode biofilms shifted under application of an external voltage. Bioelectrochemcial function was enhanced by enrichment of *Geobacter* sp., thus favouring extracellular electron transport in anode biofilm. Moreover, hydrogenotrophic methanogens and *Acetobacterium* were substantially enriched in cathode biofilm, which was important for enhanced energy recovery and methane production. Based on electron transport, increased biogas production was primarily caused by a hydrogen-utilizing process, in ME-AD system.

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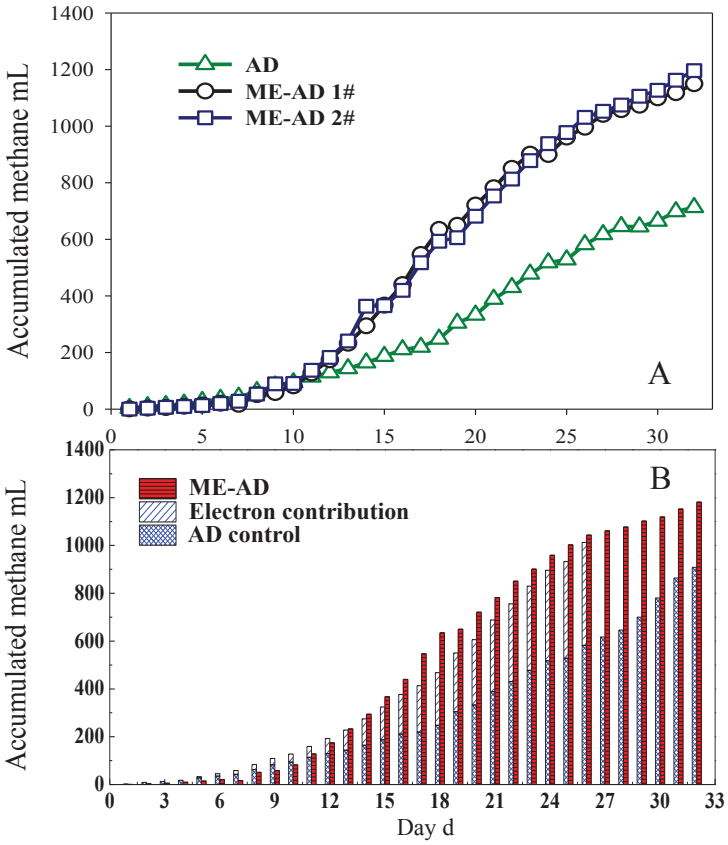

Figure 1 Methane production (A) and contribution of electrons (B) in ME-AD reactor (External voltage 0.8 V for ME-AD: 0-27 d; Voltage cut: 27-32 d)

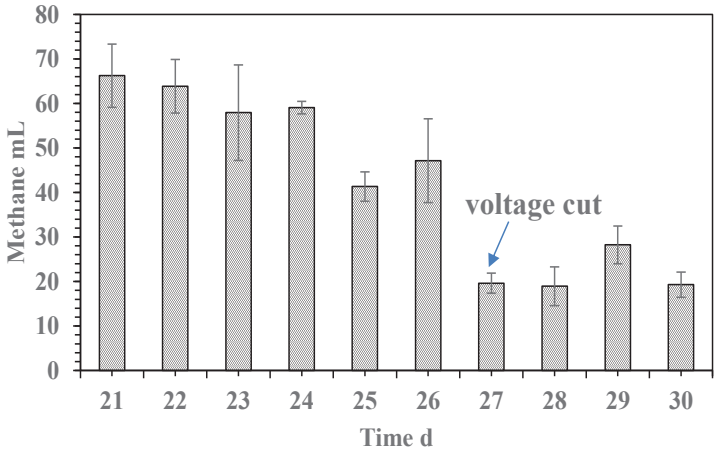

Figure 2 Methane production with and without applied voltage in ME-AD reactors

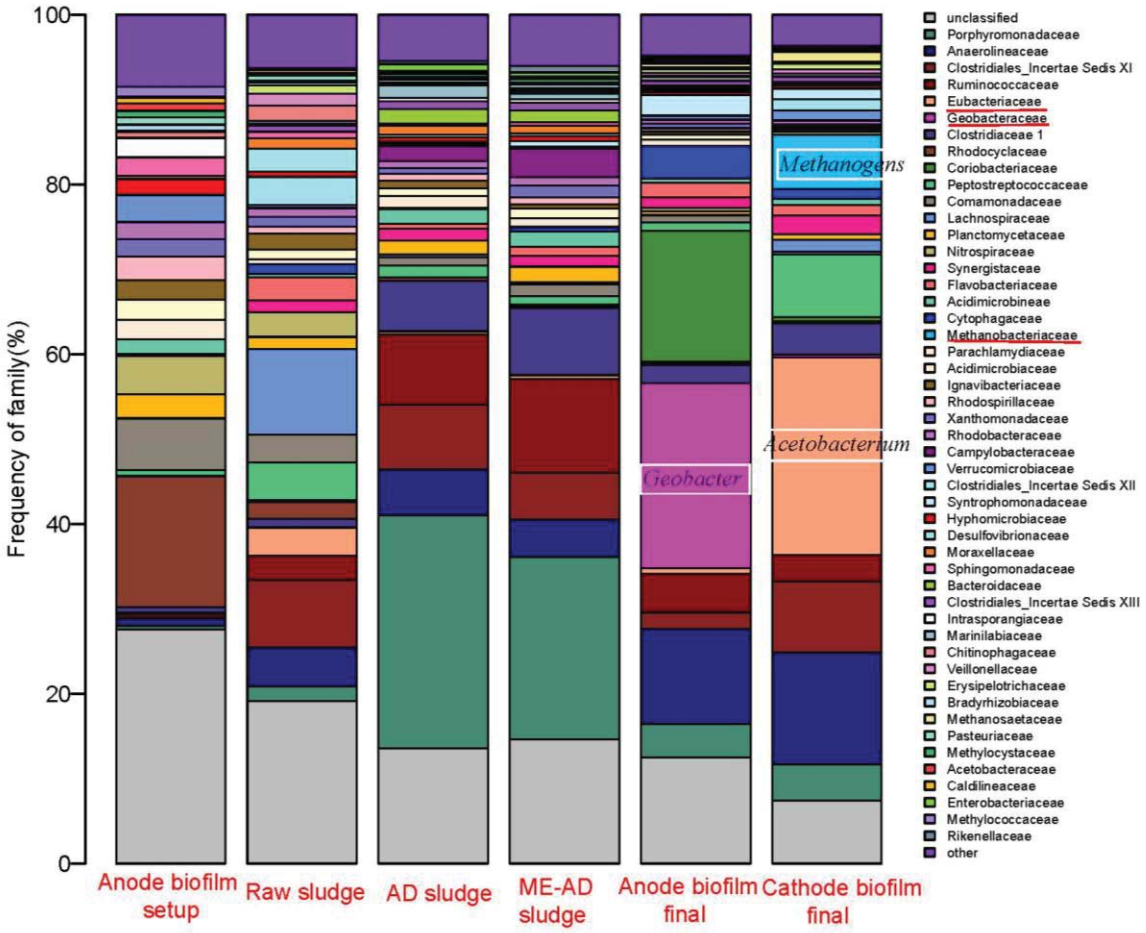

Figure 3 Community structure on classifier of family regulated by microbial electrolysis in anaerobic digestion

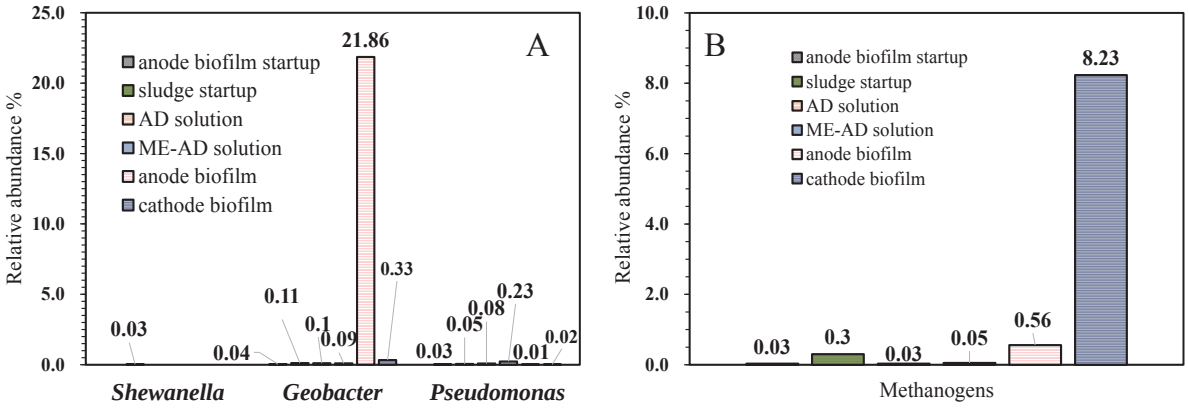

Figure 4 Dominant species of anode respiring bacteria (A) and methanogens (B) in different positions of ME-AD reactor

Supplementary Interactive Plot Data (CSV)

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