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1 **Exoelectrogenic anaerobic granular sludge**
2 **for simultaneous electricity generation and**
3 **wastewater treatment**

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Abstract

Thick and electroactive biofilm is the key for successful development of microbial electrochemical technologies and systems (METs). In this study, intact anaerobic granular sludge (AGS), which are spherical and dense microbial associations, was successfully demonstrated as novel and efficient biocatalysts in METs such as microbial fuel cell (MFC). Three different strategies were explored to shift the microbial composition of AGS from methanogenic into exoelectrogenic microbes, including varying external resistance, organic loading, and manipulating anode potential. Among other strategies, only with positive anode potential, AGS was successfully shifted from methanogenic to exoelectrogenic conditions, as indicated by the significantly high current response (10.32 A/m²) and 100% removal of organic carbon from wastewater. Moreover, AGS bioanode showed no significant decrease in current generation and organic removal at pH 5, indicating good tolerance of AGS to acidic conditions. Finally, 16S rRNA sequencing revealed the enrichment of exoelectrogens and inhibition of methanogens in the microbial community of AGS after anode potential control. This study provides a proof-in-concept of extracting electrical energy from organic wastes by exoelectrogenic AGS along with simultaneous wastewater treatment, and meanwhile opens up a new paradigm to create efficient and cost-effective exoelectrogenic biocatalyst for boosting the industrial application of METs.

Keywords: Anaerobic granular sludge; Exoelectrogenic biocatalyst; Electric energy; 16S rRNA analysis; Wastewater treatment.

39 **Introduction**

40 Growing concerns over the intensive energy consumption for conventional wastewater
41 treatment technologies has boosted interest in the development of energy-neutral treatment
42 technologies¹. Microbial electrochemical technologies and systems (METs) has shown
43 promising potential in several applications spanning from renewable electricity production
44 to biochemical and bioproducts production by using the electrons derived from waste
45 organic matters by bacteria to perform dedicated reduction reaction²⁻⁴. Though promising,
46 MFC technologies are still encountering a long-standing challenge to develop thick and
47 efficient electroactive biofilm on the anode electrode. On the one hand, the limited biomass
48 content and retention in biofilm would lead to the low capacity for organic carbon removal
49 compared to conventional biotechnologies, and thus, extra post-treatment processes are
50 always required^{5, 6}, which would greatly increase the operational and maintenance cost. On
51 the other hand, MFC reactors which rely on thin anodic biofilm can't produce substantial
52 quantities of power to offset the practical energy demands for wastewater treatment^{7, 8}.
53 Thus, the conventional ways of fabricating electroactive biocatalysts (as biofilm) on the
54 anode do limit the wide application of MFC technology for wastewater treatment and
55 energy generation^{6, 9}.

56 In the past decades, anaerobic granular sludge (AGS), as aggregates of microorganism, is
57 popular among anaerobic biocatalysts for simultaneous bioenergy production (i.e., biogas
58 through anaerobic digestion) and wastewater treatment, due to its high organic removal
59 capacity and good tolerance to extreme conditions (e.g., toxic compounds and acidic
60 shocks)¹⁰⁻¹². In a previous MFC study¹³, homogeneous bacterial suspension, derived from
61 grinded AGS using a mortar and pestle followed by filtration (0.25-mm pore size sieve),

has even been demonstrated as efficient inoculum for cultivating anodic biofilm. Thus, AGS could be a potential habitat of exoelectrogenic bacteria, in addition to methanogens. More recently, it has been found that the whole microbial aggregates can be electroactive if direct interspecies electron transfer occurs among the diverse microbial consortia^{14, 15}. Considering the essential properties of AGS with dense microbes, special channel morphology and potential conductivity, it is reasonable to hypothesize that intact AGS could function as an effective biocatalyst for an MFC if electroactive bacteria are enriched inside of granule. To date, intact AGS has never been tested as electroactive biocatalyst in the field of METs. Integration of intact AGS into anode could address the key challenge of MFC and greatly boost its capacity for electricity generation and wastewater treatment. Such combination could further strength the advantages of MFC over conventional anaerobic treatment processes, in addition to the inherent merits of mild operating conditions, high removal and energy efficiency for low strength wastewater, and easy use and transport of end product (electricity in this case)^{16, 17}.

In this context, switching intact AGS from methanogenic to exoelectrogenic is a key to achieve a successful integration. Thus, in this study, intact AGS was for the first time manipulated and explored as biocatalyst in MFC for wastewater treatment and bioelectricity generation. Several strategies to transform the intact AGS from methanogenic to exoelectrogenic, were employed, and the outcomes were evaluated in terms of organic removal, current response, and coulombic efficiency. Finally, the microbial dynamics during manipulation of anode potential and microbial composition at different sites of anode electrode were analyzed. To the best of our knowledge, it is the first time to investigate the feasibility of tailoring intact granular sludge as biocatalyst for

bioelectricity generation, which offers new insights in development of viable and sustainable technology for cost-effective and efficient wastewater treatment.

Materials and methods

MFC set up

An MFC, made of nonconductive polycarbonate plates was constructed. The anode and cathode chambers with the same dimension size ($4 \times 5 \times 5$ cm) were separated by a cation exchange membrane (CEM, CMI 7000, Membrane international, NJ). Rubbers and screws were used to tighten the reactor to avoid leakage. The anode electrode was made of a carbon fiber brush wound into two twisted titanium wires (5.0 cm diameter, 5.0 cm length, Mill-Rose, USA), which were heated at 450 °C for 15 minutes before use as reported previously¹⁸. A reference electrode of Ag/AgCl electrode (+0.197 V vs SHE) was placed ~ 0.3 cm close to the anode for accurate control of anode potential. The anodic chamber was filled with 80 g wet AGS, which was collected from a mesophilic upflow anaerobic sludge blanket reactor fed with potato wastewater (Colsen, Netherland). A stainless-steel mesh was used to avoid the washing out of AGS and possible blocking issues. The total volume of anode chamber was 100 ml, while the working volume was 50 ml. An external recirculation bottle (filled with 500 ml synthetic wastewater) was connected to anode chamber with a recirculation flow rate of 50 ml/min. To maintain a sufficient mixing, the recirculation bottle was stirred at 400 rpm. A titanium woven wire mesh (4×4 cm, 0.15 mm aperture, William Gregor Limited, London) coated with 0.5 mg/cm² Pt was used as cathode electrode. In closed circuit, the anode and cathode electrodes were connected through an external resistance (1000 Ω, unless otherwise stated). During anode potential control by

potentiostat (Ivium-n-Stat, Ivium Technologies, Eindhoven, The Netherlands), three-electrode cell mode was adopted; anode as working electrode, cathode as counter electrode and Ag/AgCl as reference electrode.

Inoculation and operational strategies

AGS was directly used as the inoculum for MFC start-up. The synthetic wastewater contained (in g/L of distilled water): sodium acetate, 1 (unless otherwise stated); NH_4Cl , 0.31; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2.69; Na_2HPO_4 , 4.33; KCl , 0.13; 12.5 ml mineral solution and 12.5 ml vitamin solution as described before¹⁹. The final pH of synthetic wastewater always kept 7.0 ± 0.2 . The anode chamber and external bottle was filled with the aforementioned synthetic wastewater, reaching a total volume of 550 ml. The cathode chamber was filled with 100 ml ferricyanide solution (50 mM, pH 7) to exclude the instability of cathodic reaction. The catholyte was 50 mM phosphate buffer solution containing 50 mM ferricyanide.

Multiple reactors including duplicate set-ups have been operated for the tests according to different purposes. Three strategies were employed successively in the same reactor. As summarized in Table S1 in supporting information, strategy 1 referred to the effect of external resistance on MFC performance under closed circuit. During strategy 1 operation, the sodium acetate concentration was controlled at 1500 mg/L. Thereafter, the influence of different organic loading (1000, 1500 and 3000 mg/L) on system performance was evaluated in strategy 2, during which the resistance was selected as 10 Ω . Subsequently, in strategy 3, MFC was connected to the potentiostat and chronoamperometry measurement was used to control anode potential at +20 mV (VS Ag/AgCl). During strategy 3, the

sodium acetate level was 1000 mg/L. Sequentially, to evaluate the persistence of positive effect by controlling anode potential, MFC was connected in a closed circuit with 10 Ω resistance, and fed with 1000 mg/L sodium acetate. Afterwards, the AGS were removed out of the anode chamber to evaluate the functions of AGS, denoted as Control 1. The cultivated AGS were transferred into another identical MFC with a completely new anode to explore the current generation of the removed AGS, denoted as Control 2. The transferring process was performed in an anaerobic box. The operational parameters (1000 mg/L sodium acetate and 10 Ω resistance) were employed for Control 1 and Control 2. At the end, to evaluate the robust resistance to low pH conditions, same AGS were placed back to the anode chamber and operated under different initial wastewater pH varying from 5 to 7. For comparison of AGS powered MFC with conventional MFC inoculated with domestic wastewater, one set of MFC reactors with same configuration was constructed (Control 3) and inoculated with domestic wastewater obtained from primary clarifier at Lundtofte Wastewater Treatment Plant (Lyngby, Denmark). For all the reactors, at the beginning of each batch, anode chamber and recirculation bottle was flushed with pure N₂ for 10 minutes to keep anaerobic conditions.

Analytical methods and calculations

During strategy 1 and 2, the voltage across an external resistance was recorded by a digital multimeter (model 2700, Keithley Instruments, Inc.; Cleveland, OH) every 30 minutes. Current was calculated according to ohm's law ($I=U/R$). Current density was normalized by the projected cathode area (16 cm²). During strategy 3, the current response was recorded by the potentiostat every 1 min. Coulombic efficiency (CE) was calculated as

151 $CE = \frac{C_t}{C_{th}} \times 100\%$, where C_t was the total coulombs calculated by integrating current
 152 response with time, calculated as $C_t = \int Idt$, C_{th} was the theoretical amount of coulombs
 153 based on the COD degradation, calculated as $C_{th} = \frac{Fb\Delta COD}{M}$, where F is Faraday's
 154 constant ($96485 \text{ C mol}^{-1} \text{ e}^{-}$), b is 4 referring to the transferred electrons per mole of oxygen,
 155 M is 32 representing the molecular weights of oxygen, ΔCOD is the removed COD amounts
 156 (unit gram)²⁰.

157 Total chemical oxygen demand (TCOD) was measured according to the standard method
 158 (APHA, 1999). COD removal rates were fitted assuming a first-order kinetic reaction with
 159 respect to substrate concentrations, and calculated according to the following equation:

160
$$\ln \frac{COD_t}{COD_0} = -kt \quad \text{Eq (1)}$$

161 where COD_0 is the initial COD concentration, COD_t is the COD concentration at time t,
 162 and k is the first-order kinetic rate coefficient. The coefficient k at varied pH was calculated
 163 and compared in section 3.2, in order to distinguish the optimal pH regarding to the fastest
 164 carbon utilization.

165 Acetate was measured via a GC with FID detection (Agilent 6890). The sample pH was
 166 immediately measured by using A PHM 210 pH meter (Radiometer). Produced gas was
 167 collected by connecting a gasbag to the headspace of recirculation bottle. The volume was
 168 measured by a 100 ml syringe. CO_2 and CH_4 were analyzed by a GC-TCD fitted with
 169 paralleled column of $1.1 \text{ m} \times 3/16$ 'Molsieve 137 and $0.7 \text{ m} \times 1/4$ ' with H_2 as the carrier
 170 gas (MGC 82-12, Microlab A/S, Denmark), and H_2 was determined by a GC-TCD fitted

with a 4.5 m × 3 mms-m stainless column packed with Molsieve SA (10/80), as previously described²¹.

Mastersizer 2000 (Malvern Instruments, UK) was employed to measure the particle size distribution of the raw AGS and cultivated AGS after strategy 3. Scanning electron microscopy (SEM - FEI Quanta 200 ESEM FEG equipped with energy dispersion spectroscopy, EDS - Oxford) was used for the observation of AGS morphology. For morphological characterization, the raw AGS and AGS after strategy 3 were sampled, washed with phosphorus buffer solution (50 mM, pH 7) and fixed by soaking into 4% formaldehyde for 24 hours at 4 °C. Subsequently, the samples were washed by gradient 25%, 50%, 75%, 90%, 95%, and 100% ethanol/distilled water solutions successively. Afterwards, samples were freeze-dried for overnight to get the powder specimens. The specimens were coated with a gold thin layer (Quorum sputter coater, UK) and observed by SEM-EDS at 3.0 kV.

Microbial community analysis

To characterize changes in microbial community before and after operation, the raw granules, and the enriched granules and anodic biofilm after strategy 3 were all collected by using sterilized scalpel or spoon as previously described²². Granules were sampled at either in the direct vicinity, or further away from the anode. All the samples were collected in triplicate except the biofilm which was sampled in duplicate. Total DNA extraction was performed using PowerSoil DNA Isolation Kit (MoBio PowerSoil, Carlsbad, CA, USA). Total genomic DNA amplification using universal primers 515F/806R was conducted on

V4 hypervariable region of 16S rRNA gene, and amplicons were sequenced by Illumina MiSeq desktop sequencer (Ramaciotti Centre for Genomics, Kensington, Australia).

Raw data was deposited in the Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/sra>) under the accession number PRJNA485399. OTU clustering and taxonomy identification were performed using microbial genomics module plug of CLC Workbench software (V.8.0.2, QIAGEN) as previously described²³. OTU was chosen to represent the Alpha diversity, while Principal Component Analysis (PCA) performed by STAMP software²⁴ was selected to represent Beta diversity. The taxonomical assignments of the selected interesting OTUs (relative abundance over 0.5%) was performed including a manual comparison of CLC results with 16S ribosomal RNA sequences (Bacteria and Archaea) database at the National Center for Biotechnology Information (NCBI) by using BLAST²³. Microbial relative abundance and folds change were visualized in heat maps using Multi experiment viewer software (MeV 4.9.0). Statistics regarding to the significant differences in microbial communities were identified by t-test in STAMP software.

Results and discussion

Different strategies to enhance the electroactivity of AGS for bioelectricity generation and wastewater treatment

Impact of external resistance

Figure 1 is here.

212 The strategy of varying external resistance was first applied to the MFC reactor inoculated
213 with AGS. The current density, as representative of electricity generation, showed a
214 different behavior with different resistances (Figure 1A). When external resistance was
215 changed from 1000 to 10 Ω , 14 fold increase of maximum current density (from 0.41 to
216 5.84 A/m²) was observed at the same acetate level (1500 mg/L). The trend of current
217 generation at different external resistances was consistent with previous studies^{25, 26}.
218 During the same period (Figure 1B), The COD removal was greatly improved (from 67%
219 to 87%) when MFC was switched from 1000 to 10 Ω . The higher COD removal rate at 10
220 Ω indicated that the substrate oxidation rate was enhanced when subjected to lower
221 resistance. Regarding to the biogas production rate and methane yield, it was noticeable
222 that the methane production was significantly increased at 10 Ω (Figure 1C). The result
223 showed that the increase of COD removal after changing resistance to 10 Ω was partly due
224 to the anaerobic methanation. The results were different from the previous studies that
225 methanogens activity was inhibited at lower resistance²⁷⁻²⁹. In this study, the AGS was
226 originally cultivated for biomethanation which was different from previous MFC studies.
227 In addition, decreasing external resistance could be an effective way to enrich
228 exoelectrogens, but it may also facilitate interspecies electron transfer between
229 exoelectrogens and methanogens as reported previously³⁰. There was no significant
230 difference in anodic potential (around -500 mV) and pH (approx. pH 7) during the
231 operation with two different external resistances (data not shown). The influence of pH in
232 the methanogenic activity could be neglected. Thus, strategy 1 referred to changing
233 resistance was not an effective way to inhibit methanogenic activity. Considering the
234 enhanced electricity production, R-10 Ω was selected for the following experiments.

235 ***Impact of substrate concentration***

236 According to the previous study³¹, the methanogens activity could be manipulated by
237 organic loading. Therefore, as strategy 2, the acetate concentrations ranging over 1000,
238 1500 and 3000 mg/L was applied consecutively. As shown in Figure 1A, the current
239 response significantly decreased when the acetate concentration increased from 1000 to
240 1500 or 3000 mg/L. It suggested the exoelectrogens weren't activated by elevated organic
241 loading. The electricity production was inhibited by increasing organic loading, as the
242 current density at 1000 mg/L was the highest among all conditions. From the COD removal
243 performance, it was clearly observed that COD removal rate was greatly enhanced with
244 elevated acetate concentrations. The average COD removal rate for 1000, 1500 and 3000
245 mg/L was 70.99, 110.59 and 360.60 mg/L/d, respectively. The COD removal was probably
246 contributed by (1) acetate oxidation by exoelectrogens; (2) acetate oxidation by
247 methanogens; (3) acetate oxidation by aerobic microbes. On one hand, from the
248 aforementioned current response (Figure 1A), it was clearly observed that current density
249 didn't increase dramatically with increasing of organic loading, which indicated that the
250 contribution of exoelectrogens to acetate oxidation wasn't enhanced with improved carbon
251 loading. On the other hand, due to the anolyte was flashed with nitrogen gas before starting
252 each test, the contribution by aerobic oxidation could be limited. Thus, the only possible
253 reason would be due to the activity of methanogens. To confirm our speculation, the biogas
254 production rate and methane yield were further analysed. As depicted in Figure 1C, the
255 higher acetate concentration, the faster methane production rate was observed, suggesting
256 the active methanogenesis process at elevated acetate concentration. Also, the methane
257 yield increased accordingly, which indicated the unsuccessful inhibition of methanogens

activity by improving organic loading. It was noticeable that in all cases, methane contents always kept almost ten times higher than carbon dioxide. This could be due to its high solubility of CO₂. Recirculation of liquid was applied in the anode, which may promote the dissolution of CO₂. Overall, the above results demonstrated that the acetate concentration of 1000 mg/L was good to obtain a superior electricity generation, and the substrate concentration was not the contributing for turning methanogenic AGS into electrogenic.

Impact of anodic potential

Figure 2 is here.

In addition to external resistance and organic loading, the anode potential has been reported to impact microbial community structure and electrochemical performance^{29, 32}. Therefore, controlling anode potential at +20 mV (VS Ag/AgCl) as the third strategy was employed. Clearly, during the period of anode potential control, the acetate was degraded rapidly in 5 days (Figure 2B). In fact, after 3 days, the acetate concentration already decreased from 800 to 33 mg/L, resulting in 96% removal. Correspondingly, the peak current density increased significantly to 10.32 A/m² (Figure 2A). The high current response with fast acetate degradation indicated that anode potential motivated the exoelectrogenic reactions other than methanogenic. On the one hand, positive anode potential meant more energy to support electroactive bacterial growth. It was reported that at relatively higher anode potential, exoelectrogens can theoretically gain more energy for their growth and maintenance^{33, 34}, according to:

$$\Delta G^{0'} = -nF(E_{\text{anode}} - E_{\text{donor}}^{0'})$$

where $\Delta G^{0'}$ is the Gibbs free energy change at standard conditions (pH 7 and 25°C), n is the number of electrons transferred, F is Faraday's constant ($96485 \text{ C mol}^{-1} \text{ e}^{-}$), E_{anode} is the anode potential, $E_{donor}^{0'}$ is the standard biological redox potential of electron donor. On the other hand, positive anode potential may affect the electron transfer kinetics and attract bacteria to move towards the electrode to form a thick biofilm³⁵. Therefore, when the anode potential was increased from -550 mV (measured anode potential at closed-circuit) to +20 mV, both of the carbon removal and current generation were greatly enhanced. To confirm the inhibited methanogenic activity at high anode potential, biogas production rate and methane yield were analysed. As shown in Figure 2C, methane yield almost decreased to 0, which indicated that the methanogenic activity was fully suppressed at high anode potential. Furthermore, it was reported that the amounts of proteins (i.e. OmcA), which are responsible for extracellular electron transfer, increased with elevating anodic potential³⁵. More direct electron transfer-related protein at positive potential helped to stimulate an electroactive-biofilm formation³⁵.

To examine the persistence of this strategy for electroactive bacteria enrichment, the reactor was subsequently switched to MFC mode (without potential control) again. It was shown that the peak current density increased from 3.30 (before potential control) to 6.41 A/m² (after potential control) when it was fed with 1000 mg/L acetate (Figure 2A). The acetate removal efficiency increased from 50% (9 days) to 100% (8 days), indicating effectiveness of anode potential control on enrichment of exoelectrogens. The pH was quite stable (around pH 7 during each batch run, data not shown) before and after potential control. The contribution of capacitive effect to the high current generation after potential control could be neglected, since the maximum stable current generation lasted for a few

hours while discharging behaviour is normally around few seconds to minutes. To further explore the contribution of AGS in electricity generation and carbon removal, the performance of both MFC after removing AGS from anode chamber (Control 1) and new MFC anode with removed AGS (Control 2) were investigated, respectively. In control 1, the peak current density immediately decreased from 6.59 to 0.52 A/m², suggesting that the AGS was partially involved in electron transfer. Accordingly, the acetate concentration decreased from 731 to 166 mg/L after 7 days, resulting in 77% acetate removal. Since no methane was produced in control 1, the contribution of methanogens to acetate removal could be excluded. The current response and carbon removal observed in control 1 could be due to small amount of residual AGS on anode since it is impossible to remove all AGS from the anode. As shown in Figure S1, a completely new anode with cultivated AGS produced a maximum current density of 1.11 A/m² after 1 day, which was higher than control 1. But it didn't recover to the level observed before moving. The results of two controls indicated that both AGS and formed biofilm on electrode played a vital/cooperative role for the current generation. Even the AGS was exoelectrogenic, the last step of electron transfer from bulk solution to solid electrode may still require an electroactive biofilm as electron conduit.

To short conclude, the above results indicated that anode potential controlled at +20 mV is effective to facilitate electroactive species growth and electron transfer in AGS. Methanogens are well known strict anaerobes i.e. they require very low potential to grow (<-300 mV). Therefore, exoelectrogens could dominate and got exclusively the chance to use acetate as substrate.

324 Acid resistance

325 Figure 3 is here.

326 In the previous studies, the most common inoculum for MFC electroactive biofilm
327 enrichment was domestic wastewater, which was either attached on anode or suspended in
328 liquid^{36, 37}. Comparatively, the AGS with diverse microbes and intrinsic granular structure
329 was used as inoculum in our work. It was previously reported that wastewater pH would
330 significantly affect MFC performance²⁹. Thus, in this section, the effect of pH shock on the
331 electrogenic capacity of the enriched AGS anode was investigated. Figure S2 depicts
332 acetate removal rates which showed good agreement with the current output at different
333 pH ranging over 5 to 7 (Figure S2). The maximum current density at each condition was
334 shown in Figure 3. The highest maximum current density (5.21 A/m^2) was observed at pH
335 7 in AGS-MFC. When pH was decreased from 7 to 5, the ability of electron production
336 was significantly deteriorated for both reactors. It was reported that exoelectrogens
337 couldn't survive in the acidic environment when pH was lower than 5.5³⁸. Although both
338 of reactors were negatively affected by the acid pH, AGS-MFC showed a relatively
339 stronger resistance to low pH compared to typical MFC inoculated with wastewater.
340 Assuming first-order kinetics, the rate coefficient was calculated according to Eq. (1), as
341 displayed in Figure 3. In both reactors, the rate coefficient showed a similar trend to pH
342 variations. The highest rate of 0.35 d^{-1} was obtained at pH 7 in AGS-MFC. In AGS-MFC,
343 when pH was decreased from 7 to 5, the rate coefficient decreased correspondingly from
344 0.35 to 0.20 d^{-1} , indicating diminished substrate degradation at acidic environment.
345 Similarly, for control MFC, the rate coefficient decreased from 0.20 to 0.12 d^{-1} . The rate
346 coefficient at pH 5 in AGS-MFC was even close to the value of control reactor at pH 7,

meaning a superior performance of acetate oxidative reaction in AGS-MFC even at unfavourable pH conditions. Clearly, neutral pH conditions proved to be the optimal environment for the exoelectrogenic bacteria. AGS inoculated MFC would have better resistance considering that the biofilm from AGS might become even thicker during long-term operation.

Morphological characteristics and elemental composition of AGS

Figure 4 is here.

The morphological image of single AGS taken after strategy 3 was depicted in Figure 4. As shown in Figure 4A, an AGS has a spherical rough surface and macroporous carbon architecture. A zoomed in image of the surface is shown in Figure 4B, in which the entire surface of AGS was covered with compact rod-shaped bacterial cells. The porous structure and rough surface would be beneficial for microbial growth and biofilm formation³⁹. In addition to the excellent porous structure, granules exhibit good mechanical strength for microbes to resist the changes of surrounded environments (such as extreme pH or organic loading shock) compared to the conventional flocs or biofilm^{12, 40}. High-resolution of SEM images of AGS channels (Figure 4C) demonstrated deep channels of ca. 1 μm diameter, with rod-shaped bacteria aligned on the channel sides. It revealed that the bacterial cells were densely adhered not only to AGS surface, but also interior sections of AGS, indicating the porous structure of AGS permitted sufficient substrate exchange from outside to inside to support internal biofilm growth⁴¹. All of these attractive properties (the porous structure, rough surface and dense microbes) enabled AGS as an ideal inoculum candidate for MFC exoelectrogens enrichment.

According to the EDS results (Figure 4D), the raw AGS contained high levels of carbon (53%) and oxygen (32%), and small amounts of minerals such as silicon, calcium and other traditional metals including iron. These minerals were reported as the main skeleton of granular structure, and may be involved in the electrical double layer formation of AGS as reported before^{42, 43}. After strategy 3, the cultivated AGS contained higher amounts of carbon, which could be assigned to the increasing biomass contents. The low deviation suggested a homogeneous mineralized granular structure. To get further information of the granule size, Mastersizer 2000 was used to measure the particle size distribution (Figure 4E). It was found the mean diameter based on the volume weighted was significantly increased from 122 (raw AGS) to 760 μm (cultivated AGS). It means over 50% granules had the diameter of 760 μm after anode potential control. The bigger size demonstrated that granulation of AGS was enhanced after being shifted from methanogenic to electrogenic condition⁴⁴. This is also consistent with the higher energy gain of bacteria at higher anodic potential which would inevitably result in higher cell biomass.

The influence of anode potential manipulation in microbial community dynamics

After the selection and comparison of three strategies, strategy 3 (anode potential) was demonstrated to be the most effective to inhibit methane production and improve current generation. To gain an insight into microbial communities residing in granules and in biofilm of carbon brush, 16S rRNA gene analysis was employed.

According to alpha diversity results shown in Figure S3 (Supporting Information), an increase in microbial diversity (represented as OTU) regardless of sampling position was observed after potential control. The results indicate that a more diverse microbiome was

enriched after a positive anode potential. Beta diversity shown in Figure S4A demonstrated a distinct microbial dynamic change before and after anode potential control. A dramatic change was found between raw granules and enriched granules/biofilms after strategy 3 based on the principal percentage (PC1 and PC2) (Figure S4A). When taking further analysis of PC3, AGS taken from different positions (close and far from anode) were also different from each other in microbial community compositions. Same distinct difference was observed between granules and anodic biofilm. The above results were in agreement with previous findings that the anode potential significantly affected the microbiome clustering in anode³⁴.

Figure 5 is here.

High throughput 16S RNA amplicon sequencing was used to analyze the microbial dynamics in AGS and attached anodic biofilm, and the relative abundance of taxa over 0.5% is illustrated in Figure 5A. The vast majority of raw AGS microbial community was composed of 90% bacteria (based on the average relative abundance). *Bacteroidetes* (23%), *Firmicutes* (23%), *Proteobacteria* (12%), followed by *Synergistetes* (9%), and others (23%), were the most dominant phyla (Figure S5). The microbial composition in raw AGS was in agreement of common mesophilic AGS as reported before⁴⁵. *Bacteroidetes*, *Firmicutes* and *Proteobacteria* has always been detected in MFC, which were supposed to be responsible for electricity generation^{32, 46}. Thus, the raw AGS probably already contained electroactive bacteria in the innate microbial community, which could enable its utilization as biocatalyst in an MFC.

To get an insight into how the microbial community composition in MFC, the changes between the raw and enriched AGS after manipulating anode potential were compared. The

414 results are shown in Figure 5B. As illustrated, in a cluster of taxa (Figure 5B, Group 1),
415 increasing significantly in relative abundance after manipulating anode potential was
416 mainly composed by exoelectrogenic bacteria. For example, *Synergistaceae* spp. (5 and 6)
417 increased from 0.2% to over 10% of relative abundance in both the granules and biofilm,
418 indicating that the proliferation of species belonging to *Synergistaceae* was due to the
419 improved anode potential. The family *Synergistaceae* was often found in MFC anode⁴⁷. It
420 was noticeable that the strain of *Arcobacter butzleri* spp. (16 and 8), known as
421 exoelectrogens⁴⁸, appeared after the potential control (accounting for 6.5% of relative
422 abundance in the biofilm sample), strongly supported the enrichment of exoelectrogens.
423 Also, *Desulfurmonadales* spp. appeared after improving anode potential, suggesting a
424 strong correlation to the potential change. The *Desulfurmonadales* spp. (22 and 65) showed
425 a 97% similarity to *Pelobacter propionicus* and *Geobacter chapellei*. *Pelobacter*
426 *propionicus* was known as propionate producer from acetate, while no propionate was
427 detected during the experiment. Therefore, the high similarity of the strain was very likely
428 affiliated to *Geobacter chapellei*, which was reported as Fe(III) reducer⁴⁹. Since iron-
429 reducing bacteria are known to use electrode as electron acceptor, we deduce that
430 *Desulfurmonadales* spp. represented by *Geobacter chapellei* may also have been involved
431 in direct electron transfer to anode³⁵. Furthermore, the strain affiliated to the family
432 *Marinilabiliaceae* also increased in abundance, which has been previously found in MFC
433 bioanode⁵⁰. Interestingly, another known species *Methanobacterium beijingense* was
434 dominant after improving anode potential. *M. beijingense* was known as hydrogenotrophic
435 methanogens using H₂/CO₂⁵¹. However, since no methane was detected, the species might
436 contribute mainly to maintain the granular structure by acting as the nucleation center, as

described elsewhere⁵². It has to be mentioned that although the enrichment of exoelectrogens was demonstrated at positive anodic potential, the microbial community was different from the previous findings. The predominance of *Geobacter* species was typically reported for the acetate-fed MFCs^{53, 54}, while in this study, the microbial community was more diverse with relatively fewer numbers of *Geobacter*. It was mainly due to the different inoculum sources^{53, 55}. In this study, methanogenic AGS was used as the inoculum, while the domestic wastewater was often reported as the inoculum when *Geobacter* was the most abundant in the acetate-fed exoelectrogenic biofilms.

Comparatively, a cluster of taxa decreased significantly in relative abundance, which demonstrated that the transition from low anode potential (-550 mV) to high anode potential (+20 mV) created a hostile habitat to these taxa. More specifically, *Mesotoga infera*, which was involved in the conversion of acetate to H₂/CO₂⁵⁶, decreased from 9% close to 0%, indicating that this pathway was negatively affected by increasing anode potential. This was further supported by the undetectable H₂ throughout the whole experiment. Similarly, *Methanosaeta concilii*, known as acetoclastic methanogens that has ability of interspecies electron transfer with *Geobacter* species for CO₂ reduction into CH₄, was diminished from 2.89% to 0.15%. This significant decrease indicated its inability to survive at high anode potential +20 mV. It has been reported that methanogens require a reductive environment where potential should be less than -527 mV (vs SHE) for its growth⁵⁷. That simply explained the inhibition of methanogens at +20 mV. In a more recent work⁵⁸, long-term open circuit was found preferable for the growth of methanogens in the cathode of acetogenic microbial electrosynthesis process, which implies the important role of circuit potential on the microbial communities on the electrode.

Furthermore, regarding the competition between exoelectrogens and methanogens, it has been proposed that a special structure of tightly packed aromatic amino acids enabled a long-range electron transport between *Geobacter* and *Methanosaeta*¹⁵. In the methanogenic aggregates, the known role of *Geobacter* species is converting acetate to CO₂ with electrons generating. Through the metallic-like conductive pili, electrons are released and flow to *Methanosaeta* for CO₂ reduction. The final electron sink is methane. The direct interspecies electron transfer way is broken down at positive anode potential since the *Methanosaeta* is not able to survive/active at high potentials⁵⁹. Thus, when inserting a conductive electrode in the aggregates, the realised electrons from *Geobacter* metabolism would flow to the electrode instead of being involved in the methane production. In the exoelectrogenic condition, the solid electrode substitutes the *Methanosaeta* as the electron acceptor.

Figure 6 is here.

In order to elucidate the difference between the microbial community composition in anodic biofilm (taken from carbon brush), enriched AGS closed to anode electrode and enriched AGS far from anode, significant analysis based on the overall taxa were performed (Figure 6A and B). Distinct consortia were formed in enriched AGS samples and anodic biofilm. Compared to the anodic biofilm, a significant increase in relative abundance of 9 bacterial taxa was observed in the enriched AGS close to carbon brush. Particularly, well-known electrogenic bacteria such as *Marinilabiliaceae* spp., *Anaerobineaceae* spp., and *Desulfovibrionales* spp. were found significantly increased in the AGS close to carbon brush. Besides, the significantly high abundance of *Synergistaceae* sp., which was previously demonstrated to be potentially electrogenic, was in accordance

with the electricity generation of Control 1 (MFC after removing granules). Comparatively, no significant difference was observed between AGS far from carbon brush and anodic biofilm, except one strain.

To get an additional insight into the difference between two AGS samples (one taken close to carbon brush and the other taken far from carbon brush), the statistical analysis was performed as well (Figure 6C). Clearly, 10 bacterial taxa were observed in significantly higher relative abundance in AGS close to carbon brush compared to the AGS far from carbon brush. The most significant increase was found in *Arcobacter butzleri*, which was characterized to be capable of transfer electrons from acetate to the electrode⁶⁰. Therefore, the above results strongly implied the AGS close to carbon brush might play more important role in the electricity generation than the AGS far from carbon brush.

Implications

This study demonstrated the proof concept of using intact AGS as biocatalyst in an MFC for simultaneous carbon removal and electricity generation. Compared to the conventional biocatalyst (e.g., domestic wastewater), the AGS has several merits. Firstly, the large surface area of AGS enabled a substantial electrogenic bacterial growth. Secondly, the MFC inoculated with AGS generated much higher current compared to the conventional MFC at same level of substrate. Lastly, the coulombic efficiency improved from 13.62% (before potential control, 1g/L, 10 Ω) to 33.82% (with potential control, 1 g/L) as indicated in Table S2. Although small improvement, the coulombic losses from methanogenic process were diminished. The relatively low value (34%) might be due to other process such as the cathodic oxygen diffusion or competition from other biological species^{61, 62}.

Meanwhile, it must be pointed out that though AGS contributed the major part of current generation, the biofilm derived from AGS on the surface of anode electrode is also crucial, as it might play a role of conduit for electron transfer between bulk AGS and electrode. The results indicate that it is possible to boost the current generation of MFC by employing AGS as biocatalyst, but a thin biofilm between AGS and electrode is still needed and may play an important role to efficiently harvest the energy generated by AGS. The special conductive property between AGS and the electrode may open up many other intriguing applications. For instance, the exoelectrogenic AGS could be used as the bed electrodes in METland (wetland plus MET) and other fluidized bed reactor systems⁶³.

Furthermore, more efforts should be made to further boost the application of AGS for energy recovery and simultaneous wastewater treatment. For instance, the mechanisms of electron transfer among granules should be explored to get better understanding of the system. In that case, how to accelerate the long distance of electron transfer in bacterial community could be identified and addressed well. Another interesting focus could be the studying of layer bacterial distribution in the granules and their involvements in the electron transfer. Further work should also focus on the continuous operation mode and reactor configuration, for example up flow MFC to optimize the settlement of granules for the future potential up scaling, or utilizing gas diffusion air cathode to bringing MFC closer to practical applications⁶⁴.

Supporting Information

Table S1, Figure S1, Figure S2, Figure S3, Figure S4, and Figure S5 as noted in the text.

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527 **Author Contributions**

528 YZ, IA and NZ designed the experiments, NZ and LT carried out the research. The
529 manuscript was written through contributions of all authors. YZ is responsible for
530 correspondence.

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725

726 **List of figure captions**

727 Figure 1 Current density (A), TCOD removal (B) and biogas production rate and methane
728 yield (C) over time at different external resistance and different acetate concentrations. Red
729 line and green line refer to the performance of R-10 ohm, 1500 mg/L in Strategy 1 and
730 Strategy 2, respectively.

731 Figure 2 Current density (A), acetate concentrations (B) and average biogas production rate
732 and methane yield (C) with time in different reactors. Control 1: MFC with only carbon
733 brush (after potential control and moving granules out).

734 Figure 3 The maximum current density and COD removal rate coefficient at varied pH
735 conditions. AGS-MFC: MFC after potential control; Control 3: MFC inoculated with
736 domestic wastewater.

737 Figure 4 SEM image of the surface structure of single GAS after anodic potential
738 control:(A) an intact granule; (B) high-resolution of SEM image of granular surface
739 showing the massive microbial colonization; (C) showing the rod-shape microbes aligned
740 on the side of deep channels. (D) Energy-dispersive X-ray (EDS) results of AGS before
741 and after strategy 3. (E) Particle size distribution of raw AGS and cultivated AGS after
742 strategy 3.

743 Figure 5 Microbial community compositions in raw AGS (G1), and enriched AGS after
744 anode potential control and close to carbon brush (G2), enriched AGS far from carbon
745 brush (G3), and biofilm on carbon brush (Biofilm). Relative abundance (%) and folds
746 change were reported in (A) and (B), respectively. Group 1: the taxa increased in relative

747 abundance after anode potential control. Group 2: the taxa decreased in relative abundance
748 after anode potential control.

749 Figure 6 OTUs that changed significantly ($p < 0.05$) in the comparison between G2
750 (enriched AGS taken from close to carbon brush after strategy 3) and Biofilm (A), between
751 G3 (enriched AGS taken far from carbon brush after strategy 3) and Biofilm (B), and
752 between G3 and G2 (C), respectively.

753

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

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