

Article

Electric Field-Driven Direct Interspecies Electron Transfer for Bioelectrochemical Methane Production from Fermentable and Non-Fermentable Substrates

Gyung-Geun Oh ¹, Young-Chae Song ^{1,*}, Byung-Uk Bae ² and Chae-Young Lee ³

¹ Department of Environmental Engineering, Korea Maritime and Ocean University, 727 Taejong-ro, Yeongdo-Gu, Busan 49112, Korea; sambo8866@kmou.ac.kr

² Department of Environmental Engineering, Daejeon University, 62 Daehak-ro, Dong-Gu, Daejeon 34520, Korea; baebu@dju.kr

³ Department of Civil and Environmental Engineering, The University of Suwon, 17 Wauan-gil, Bongdam-eup, Hwaseong-si 18323, Korea; chaeyoung@suwon.ac.kr

* Correspondence: soyg@kmou.ac.kr; Tel.: +82-51-410-4417

Received: 24 August 2020; Accepted: 13 October 2020; Published: 15 October 2020

Abstract: The bioelectrochemical methane production from acetate as a non-fermentable substrate, glucose as a fermentable substrate, and their mixture were investigated in an anaerobic sequential batch reactor exposed to an electric field. The electric field enriched the bulk solution with exoelectrogenic bacteria (EEB) and electrotrophic methanogenic archaea, and promoted direct interspecies electron transfer (DIET) for methane production. However, bioelectrochemical methane production was dependent on the substrate characteristics. For acetate as the substrate, the main electron transfer pathway for methane production was DIET, which significantly improved methane yield up to 305.1 mL/g chemical oxygen demand removed (COD_r), 77.3% higher than that in control without the electric field. For glucose, substrate competition between EEB and fermenting bacteria reduced the contribution of DIET to methane production, resulting in the methane yield of 288.0 mL/g COD_r, slightly lower than that of acetate. In the mixture of acetate and glucose, the contribution of DIET to methane production was less than that of the single substrate, acetate or glucose, due to the increase in the electron equivalent for microbial growth. The findings provide a better understanding of electron transfer pathways, biomass growth, and electron transfer losses depending on the properties of substrates in bioelectrochemical methane production.

Keywords: direct interspecies electron transfer; fermentable substrate; non-fermentable substrate; bioelectrochemical methane production

1. Introduction

Anaerobic digestion is a sustainable bioprocess that stabilizes organic waste, while recovering methane as a useful by-product. In anaerobic digestion, extracellular hydrolytic enzymes first break down complex organic matter into monomers. Acidogenic bacteria ferment the monomers to intermediates, including acetate, hydrogen, and formic acid, and methanogenic archaea convert the intermediates to methane [1–3]. Thus, anaerobic digestion is a kind of indirect interspecies electron transfer (IIET) process, in which intermediates shuttle electrons between acidogenic bacteria and methanogenic archaea [2–4]. However, the IIET involved in methane production is a series of multi-step enzymatic reactions with significant electron losses [1,4–6]. The enzymatic reactions cannot fully transfer electrons thermodynamically from the substrate to methane [4,6]. Therefore, the methane yield that can be obtained from organic matter does not reach its theoretical value of 350 mL/g COD_r.

In addition, the physiological properties of acidogenic bacteria, such as their growth rate and susceptibility to environmental conditions, are different from those of the methanogenic archaea [4,6,7]. Therefore, the anaerobic digestion process can easily be destabilized by the imbalance between the IIET steps, even with a small external shock [7,8].

However, direct interspecies electron transfer (DIET), without any electron shuttle from a microbial species to another species, can be more thermodynamically and kinetically advantageous over the IIET [4,9]. DIET can be a breakthrough to address the limitations in the anaerobic digestion based on IIET. The microbial species involved in DIET from organic matter to methane are electroactive microorganisms, including exoelectrogenic bacteria (EEB) and electrotrophic methanogenic archaea (EMA) [2,4,9]. The electroactive microorganisms are the microbial species with conductive proteins including cytochrome C over-expressed to the outer membrane of the cell and conductive pili as an appendage connecting microbial species [5,10,11]. EEB releases electrons derived from the oxidation of low molecular organics, while EMA directly reduces carbon dioxide using those electrons to produce methane [9,11]. In anaerobic digestion, electroactive microorganisms are generally abundant in anaerobic microbial aggregates, conductive material surfaces, and polarized electrode surfaces [7,9,12,13]. These microbial species can directly transfer electrons for their syntrophic metabolism through the electrical connection by physical contact with each other (biological DIET), or through the mediation by conductive materials (cDIET) or polarized electrodes (eDIET) [6,9,12,14]. In bioelectrochemical anaerobic digesters with polarized electrodes such as microbial electrolysis cells, the potential difference between the electrode surface and the bulk solution causes the faradaic current for methane production through eDIET. However, electrical energy is required in proportion to the amount of methane produced through eDIET [2,4,7]. Interestingly, electroactive microorganisms can also be abundant in the bulk solution of bioelectrochemical anaerobic digesters with polarized electrodes, and improve methane production [4,15,16]. It is worth noting that the bulk solution around the polarized electrode is exposed to an electric field. This indicates that the electric field formed by polarized electrode enriches the bulk solution with electroactive microorganisms, and significantly promotes biological DIET [16–18]. Meanwhile, the faradaic current for eDIET can be blocked by insulating the electrode surface with a dielectric material. This indicates that polarizing the insulated electrodes creates the electric field in the bulk solution, which improves methane production through biological DIET without the consumption of electric energy.

In general, both DIET and IIET can simultaneously contribute to methane production in bioelectrochemical anaerobic digesters with polarized electrodes [4]. As the relative contribution of DIET increases, the anaerobic digestion process becomes more robust, and methane production from organic matter further increases, improving the performance of anaerobic digestion [2,4,9]. In the electric power supply sector, the share of renewable energy such as wind and solar power is increasing significantly. However, wind and solar power are fluctuating and intermittent energies have to be balanced through long-term storage and reserve production to stabilize the power grid [19,20]. Power to gas (PtG) technology that converts excess renewable energy to hydrogen or methane might contribute to mitigating the fluctuation and intermittence of renewable energy [19,20]. Bioelectrochemical anaerobic digestion that improves methane production with small electric power has great potential as a PtG technology for intermittent renewable energy in the near future.

However, DIET and IIET can compete for electrons to produce methane in bioelectrochemical anaerobic digesters with polarized electrodes [16,18,21]. In thermodynamics, the equilibrium constant (K) for the interspecies electron transfers, including IIET and DIET, depends on the free energy change ($\Delta G = -RT \ln K$) [22,23]. The free energy (G) is a function of the enthalpy (H), entropy (S), and temperature ($G = H - TS$) [23]. This indicates that the substrate type can affect the relative contribution of DIET and IIET to methane production in anaerobic digesters with polarized electrodes. Among organic substrates, acetate is a simple and non-fermentable substrate. Acetate can be easily converted to methane by mainly acetoclastic or syntrophic acetate oxidation pathway in anaerobic digestion [1,18,24]. Acetate is also a suitable substrate for EEB [21,25]. In anaerobic digesters with polarized electrodes, this implies that acetate can be converted to methane in the bulk solution

by biological DIET between EEB and EMA. However, as of yet, there is little information reporting the transfer rate and conservation of electrons associated with DIET for methane production from acetate. Unlike acetate, glucose is a fermentable substrate [21]. In anaerobic digestion, one of the main pathways for electron transfer that produces methane from glucose is IIET between acidogenic bacteria and methanogenic archaea through the intermediates, such as acetate and hydrogen. However, EEB can metabolize glucose, as well as acetate, and release electrons outside the cell [25–27]. In anaerobic reactors with polarized electrodes, it seems that DIET and IIET can compete or cooperate to produce methane from glucose. In the case of a substrate mixture of acetate and glucose, the routes for the electron transfer for methane production would be similar to those of glucose. However, it is believed that the contribution of DIET and IIET to methane production depends on the relative fraction of acetate to glucose in the mixture. The characteristics of methane production from the mixture of acetate and glucose would be slightly different from those of the acetate or the glucose alone.

The purpose of this study was to investigate the bioelectrochemical methane production depending on the substrate type, including non-fermentable, fermentable, and mixed substrates, in the anaerobic batch reactor in which the bulk solution was exposed to an electric field. For this, the yield and production rate of methane from acetate were compared with those of glucose and their mixture and also discussed based on the electron balance and the contribution of DIET to methane production. In addition, the microbial community and electrochemical activity of the bulk solution depending on the substrate type were analyzed.

2. Materials and Methods

2.1. Anaerobic Medium, Seed Sludge and Electrode

Anaerobic medium containing $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 15.7 mM (2.45 g/L) (Daejung chemical and metals Co., Ltd., Gyeonggi, Korea), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 12.8 mM (4.58 g/L) (Daejung chemical and metals Co., Ltd., Gyeonggi, Korea), NH_4Cl 2.4 mM (0.13 g/L) (Junsei chemical Co., Ltd., Tokyo, Japan), KCl 4.16 mM (0.31 g/L) (Junsei chemical Co., Ltd., Tokyo, Japan), NaHCO_3 50 mM (4.2 g/L) (Junsei chemical Co., Ltd., Tokyo, Japan), and small amounts of vitamins and trace metals were prepared according to previous studies [4,28]. Analytical grade sodium acetate ($\text{CH}_3\text{COONa} \cdot 12\text{H}_2\text{O}$) and glucose ($\text{C}_6\text{H}_{12}\text{O}_6$) were used to prepare three types of substrates of acetate, glucose, and their mixture. Anaerobic sludge was collected from an anaerobic digester at a sewage treatment plant (S WWTP, Busan, Korea). The anaerobic sludge was sieved with a screen (1 mm opening) to remove the impurities, and placed in a refrigerator (4 °C) for 24 h to settle down. The settled anaerobic sludge was used as the inoculum. The initial pH of the inoculum was 7.17–7.32, and the alkalinity and volatile suspended solids (VSS) were 3450–3849 mg/L as CaCO_3 and 12.5–14.2 g/L, respectively. For the electrodes, a thin copper plate (0.3 T, copper 99.9 %, KDI Co., Korea) was cut into two different sizes (small size, 5.5 cm × 7 cm; large size, 26 cm × 9 cm). The copper plates were coated with a dielectric polymer (alkyd enamel, VOC 470 g/L, Noroo Paint Co., Korea) to insulate their surface.

2.2. Set-up for Bioelectrochemical Anaerobic Batch Reactor

A cylindrical anaerobic batch reactor (effective volume 0.5 L, diameter 8.5 cm, height 10 cm) was prepared using acrylic resin (Figure 1). The upper part of the reactor was covered with an acrylic plate to seal, and a mixing blade was installed inside the reactor. The blade was connected to a DC motor installed on the cover plate using a vertical steel shaft. Sampling ports to collect liquid and biogas and a valve to vent biogas were installed in the cover plate. The sampling ports were covered with n-butyl rubber stoppers. In the cover plate, the sampling port for liquid and the hole for the steel shaft were sealed by attaching their bottoms with sealing tubes that extended into the liquid inside. The biogas venting valve was connected with a rubber tube to a floating type gas collector. The gas collector was filled with an acidic solution saturated with NaCl to avoid biogas dissolution. The small and large electrodes were rolled into annular form, and installed on the outer wall of the sealing tube for the steel shaft, and the inner wall of the main body of the reactor, respectively. The electrodes

were connected to the terminals of a DC voltage source (OPM series, ODA Technologies Co., Korea) with titanium wire. For the experiment, the anaerobic medium of 0.25 L and inoculum of 0.25 L were added into three anaerobic batch reactors, respectively, and one of acetate, glucose, or their mixture was fed into each reactor to be 3.0 g/L, based on chemical oxygen demand (COD). In order to expose the bulk solution to the electric field, the electrodes in the anaerobic reactor were polarized by applying a DC voltage. The electric field exposed to the bulk solution was 0.33 V/cm, which was selected based on previous studies [4,15–18]. The anaerobic batch reactors exposed to the electric field were referred to as AcEF, GluEF, and MixEF, depending on the substrate type. An anaerobic reactor without electrodes with acetate as the substrate was prepared separately, to use as the control. Another anaerobic blank reactor without any substrate was used to correct methane production from the inoculum. The prepared reactors were flushed with nitrogen gas to remove oxygen inside and placed in a closed room. The temperature inside the reactor was maintained to 35 ± 2 °C by controlling the room temperature with a PID controller. The anaerobic batch reactors began to operate by mixing the anaerobic medium using the blade. When the biogas production in each batch cycle was not observed because of substrate depletion, the anaerobic batch experiment was repeated in sequential batch mode by stopping the mixing for 30 min to precipitate the suspended sludge, and replacing the supernatant (ca. 0.25 L) with fresh medium.

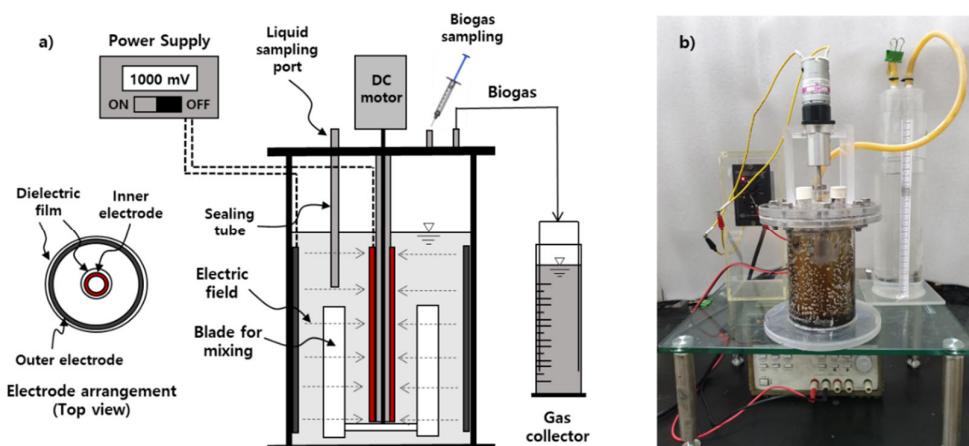


Figure 1. Bioelectrochemical anaerobic batch reactor used in the experiment: (a) schematic diagram, (b) photograph.

2.3. Analysis and Calculations

The liquid sample in the anaerobic batch reactor was collected from the bulk solution at the start and end of each batch cycle, and the physicochemical properties, such as the total COD (TCOD), soluble COD (SCOD), VSS, and alkalinity, were analyzed according to Standard Methods [29]. The pH was measured with a pH meter (YSI pH1200 laboratory pH meter 115–230 V (T1)). Biogas production of the anaerobic batch reactors was monitored daily from the gas collector, and the composition of the biogas was also analyzed by gas chromatography (Gow-Mac series 580, Bethlehem, PA, USA; TCD detector; Porapak-Q, 6 ft × 1/8 in SS). Methane production was estimated by biogas production and its methane content, and then converted to the value at STP after correcting the methane production from the inoculum, as described in previous studies [4,16,28]. The cumulative methane production during the third and fourth batch cycles where biogas production was stabilized was fitted into the Modified Gompertz equation, as shown in Equation (1), to obtain the initial lag phase (λ , day), the maximum production rate (μ_m , mL/d), and the ultimate methane production (P_u , mL), using the nlstools package in R [30].

$$P = P_u \times \exp\left[-\exp\left(\frac{\mu_m \times \exp(1)}{P_u}(\lambda - t) + 1\right)\right] \quad (1)$$

The methane yield for the substrate was estimated by dividing the ultimate methane production (mL) by the amount of COD removal (g COD_i) during the batch cycle. The electron balance between the substrates and products was estimated from the electron equivalents for COD removed, methane production, and grown biomass. However, hydrogen was excluded in the electron balance because it was not observed during the third and fourth batch cycles. The electron equivalents were obtained by multiplying the moles of removed COD, produced methane, and grown biomass by their stoichiometric electron equivalent factors. The stoichiometric electron equivalent factors were 4 e⁻ eq/mole for COD, 8 e⁻ eq/mole for methane, and 20 e⁻ eq/mole of biomass (C₅H₇O₂N). The difference in electron equivalents between the removed COD and the products was considered as electron loss during the conversion process from the substrate to methane. The methane fraction dissolved in the liquid was considered as a part of losses. The cyclic voltammetry (CV) was performed for the bulk solution within the potential range of −1.0 to 1.0 V using a potentiostat (ZIVE SP1, WonA Tech, Korea). The scan rate was 10 mV/sec, which was a value in the range used in previous studies [15,30–32]. For the CV, small pieces of stainless steel mesh (1 cm × 1 cm) were used as the working electrode and the counter electrode, respectively, and Ag/AgCl electrode (ALS Co., Ltd., Japan) was used as the reference electrode. Smart manager software (Zive Lab, WonATech, Korea) was used to estimate the redox peak and the peak height from the voltammograms to estimate the electrochemical activity of electroactive microorganisms, including the EEB and the EMA.

2.4. Microbial Community Analysis

The suspended sludge was sampled from the bulk solution for the anaerobic batch reactors at the end of the experiment and then used for the microbial community analysis, based on 16S rRNA. The DNA was extracted from the suspended sludge sample using Power soil DNA isolation kit, according to the kit protocol (MO BIO laboratories, Inc., San Diego, CA, USA). The variable region (V3V4 for bacteria, V1V9 for archaea) of the 16S rRNA in the metagenomic DNA was amplified with fusion primers (bacteria: 27F/1492R, archaea: A25F/U1492R). The 16S rRNA was pooled, and sequenced on the MiSeq Personal Sequencer (Illumina, San Diego, CA, USA). The amplification, construction of the sequencing library, and bioinformatic analysis were performed as described in previous study [33]. Chimera was checked, and taxonomic assignments of the readings were the obtained from the EzBioCloud (<http://ezbiocloud.net/>). Microbial community and the statistical taxonomical assignments were obtained based on the operational taxonomic units (OTUs). The bioinformatic analysis for species-level classification of microbes was conducted by the EzBiocloud (Chunlab, Inc., Seoul, Korea).

3. Results and Discussion

3.1. Bioelectrochemical Methane Production

In the anaerobic batch reactors, cumulative methane production increased in the form of sigmoid type curves; and then as the batch cycle repeated, the production features stabilized. The methane production in the anaerobic reactors exposed to the electric field was dependent on the substrate type, but significantly higher than the control without the electric field. However, hydrogen production from all of the reactors was very small as less than 0.2 mL, observed only at the start of the first batch cycle. The initial and final pH values for each batch cycle were around 7.2–7.4 and 7.2–7.6, respectively. In the control, the ultimate methane production from acetate was only 156.7 mL, and the maximum methane production rate was 44.9 mL/d (Figure 2). However, in AcEF, the ultimate methane production from acetate was 407.1 mL, which was 2.6 times more than that of the control. The SCOD residual in AcEF at the end of batch cycles was 922 mg/L, which was less than the 1574 mg/L of the control (Table 1). This implies that the electron transfer for methane production from acetate in AcEF is thermodynamically advantageous over that in the control. However, the greater methane production in AcEF was not explained only by more COD removal than the control. It is well known that DIET between electroactive microorganisms, including EEB and EMA, better conserves electrons [4–6]. In AcEF, the main electron transfer route for methane production is likely to be biological DIET, considering that its ultimate methane production was significantly higher than

that of the control. This indicates that the electric field enriches the bulk solution with electroactive microorganisms, and promotes biological DIET for methane production [15–18]. On the other hand, in anaerobic digestion, acetate can be converted to methane by acetoclastic methanogenesis, or hydrogenotrophic methanogenesis through syntrophic acetate oxidation pathway [1,24]. The acetoclastic methanogenesis is a biochemical reaction in which acetate is first converted to acetyl-CoA with ATP and coenzyme A (CoA) [1,34]. Subsequently, the methyl group is transferred to tetrahydromethanopterin ($\text{CH}_3\text{-H}_4\text{M(S)TP}$), and then to coenzyme M (HS-CoM). The methyl-CoM is finally reduced to methane with coenzyme B (HS-CoB). However, the hydrogenotrophic methanogenesis through syntrophic acetate oxidation pathway is a niche mechanism that produces methane from acetate occurring at high temperature or inhibitory conditions [24]. This indicates that the acetoclastic- and hydrogenotrophic methanogenesis from acetate are biochemical reactions with high energy loss, based on the low methane production in the control [5,6]. Interestingly, the maximum methane production rate in AcEF was only 1.7 times higher than that of the control (Table 1). The ultimate amount and rate of methane production are related to the thermodynamic and kinetic properties of the electron transfer pathway, respectively, in methane production. This indicates that, compared to acetoclastic methanogenesis, biological DIET further improves the ultimate methane production from acetate, rather than the methane production rate. It is revealed that the electric field alters the free energy change for redox reaction by changing the molecular polarity of the reactant, bridging the charged particles, or oscillating the permanent dipole [23,35]. It seems that, compared to acetoclastic methanogenesis, biological DIET improves methane production from acetate kinetically, and further, thermodynamically.

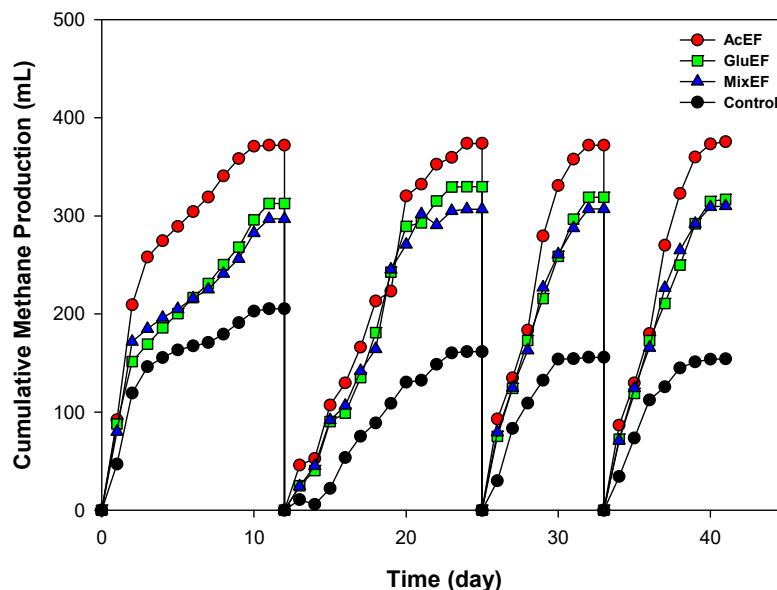


Figure 2. Cumulative methane production depending on the substrate type of the anaerobic reactor exposed to an electric field.

In the cases of glucose and the mixture of acetate and glucose, the methane productions in the anaerobic reactors exposed to the electric field were slightly different from those of the acetate. The ultimate methane production in GluEF and MixEF was 345.6 and 325.7 mL, respectively, less than the AcEF. This indicates that when the substrate is glucose, the electrons captured to methane are smaller than the acetate; and when it is a mixture of acetate and glucose, are smaller again. However, the maximum methane production rate in GluEF was 58.0 mL/d, which was slightly lower than that in MixEF. This suggests that compared to only glucose, the mixture of acetate and glucose as the substrate has better kinetics.

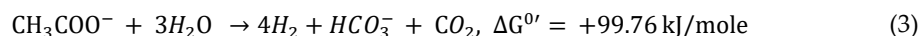
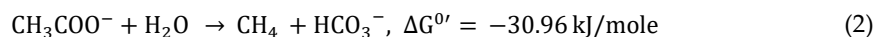
Table 1. Summary of methane production, physicochemical properties, and electron balance in the repeated sequential batch cycle.

Contents		AcEF	GluEF	MixEF	Control
P _u (mL CH ₄)		407.1 ± 3.7	345.6 ± 1.1	325.7 ± 1.0	156.7 ± 1.2
μ _m (mL CH ₄ /d)		74.9 ± 0.8	58.0 ± 1.3	64.4 ± 1.2	44.9 ± 1.9
λ (d)		0.23 ± 0.03	0.02 ± 0.01	0.05 ± 0.05	0.32 ± 0.04
CH ₄ yield (mL/g COD _r)		305.1 ± 6.2	288.0 ± 4.9	276.1 ± 4.2	172.1 ± 5.1
SCOD (mg/L)	Initial	3392 ± 24	3787 ± 33	3565 ± 50	3643 ± 11
	Final	922 ± 51	1467 ± 17	1345 ± 29	1574 ± 35
TCOD (mg/L)	Initial	9473 ± 48	9398 ± 38	9184 ± 67	9426 ± 53
	Final	6276 ± 37	6452 ± 43	6175 ± 56	6675 ± 105
VSS (mg/L)	Initial	8578 ± 176	8764 ± 69	8755 ± 191	8560 ± 121
	Final	9235 ± 29	9157 ± 97	9328 ± 143	8732 ± 46
CH ₄ (%)		87.2 ± 1.8	82.3 ± 1.4	78.9 ± 1.2	49.2 ± 1.5
Electron Balance	Biomass (%)	9.8 ± 4.0	7.5 ± 0.8	11.0 ± 1.4	4.7 ± 2.1
	Losses (%)	3.1 ± 2.3	10.2 ± 2.2	10.1 ± 2.6	46.1 ± 0.6

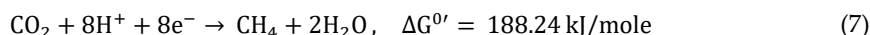
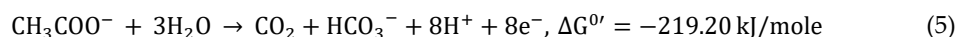
3.2. Methane Yield and Electron Balance

In anaerobic digestion, the final sinks of electrons derived from the substrate are mainly methane, hydrogen, and biomass. However, some of the electrons are lost in the process of transfer from the substrate to methane. Thus, the electron balance provides details of the electron transfer pathway for methane production that varies with the substrate type. In control, the methane yield from acetate was 172.1 mL/g COD_r (Table 1). The electrons transferred from acetate to methane and biomass were 49.2% and 4.7%, respectively, while the electron loss was high at 46.1%. In previous studies, the methane yield for acetate in anaerobic digestion ranged from 185.5 to 220.5 mL/g COD_r, and the biomass yield was 0.040 to 0.108 g VSS/g COD_r [36–38]. The yields of methane and biomass in control are slightly lower than those of previous studies. It seems that the percentages of electrons captured to methane and biomass during the anaerobic conversion of acetate into methane are dependent on the relative contribution of the acetoclastic pathway to the syntrophic acetate oxidation pathway. The significant electron loss in control implies that in terms of electron conservation, the processes of enzymatic conversion from acetate to methane in anaerobic digestion are inefficient.

In AcEF, there are possibly substrate competitions between electron transfer pathways for methane production. The stoichiometric Reactions (2)–(4) show acetoclastic and syntrophic acetate oxidation pathways for methane production [38–40]:

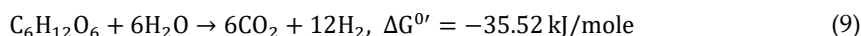
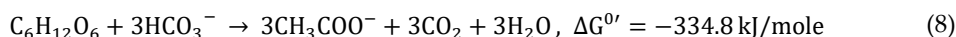


The value $\Delta G^{0'}$ represents the free energy change released under standard conditions and at pH 7. However, EEB oxidizes acetate directly into electrons, protons, and carbon dioxide Reaction (5), and hydrogen to electrons and protons Reaction (6) [25,40–42]. EMA reduces the carbon dioxide with those electrons to methane Reaction (7):

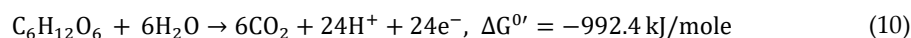


Interestingly, the free energy change in DIET pathway for methane production is the same as for the acetoclastic or syntrophic acetate oxidation pathways. However, the loss of electrons derived from acetate was only 3.1% in AcEF, much lower than the control (Table 1). In previous studies, electrons captured in methane through DIET ranged 70 to 96% in microbial electrolysis cells [43,44]. The methane yield for acetate in AcEF was as high as 305.1 mL/g COD_r, which was 87.2% of the theoretical value of 350 mL/g COD_r (Table 1). Considering that the percentage of electrons captured in control was 49.2%, the DIET contribution to methane production in AcEF can be estimated as up to 81.2% from the electron balance ($96 \times x + 49.2 \times (100 - x) = 100 \times 87.2, x = 81.2$). This suggests that the electric field in AcEF improved methane production from acetate by promoting DIET [15,16,30].

The electron transfer pathways for methane production from glucose can be more complex than the non-fermentable acetate. In GluEF, the methane yield was 288.0 mL CH₄/g COD_r, which was less than the AcEF. Glucose in GluEF can be fermented by acidogenic fermentation bacteria (AFB) to produce the intermediates, such as acetate and hydrogen Reactions (8) and (9). The intermediates can be converted to methane by acetoclastic or syntrophic acetate oxidation pathways and hydrogenotrophic methanogens Reactions (2)–(4):



However, EEB can also directly oxidize glucose, as well as acetate and hydrogen, to produce electrons, protons, and carbon dioxide Reactions (5), (6), and (10), and then EMA can reduce carbon dioxide with the electrons to produce methane Reaction (7) [25,42].



In the electron balance for GluEF, the percentage of electrons converted from glucose to methane decreased slightly to 82.3%, compared to the AcEF, but the electron loss increased to 10.2% (Table 1). In GluEF, the DIET contribution to methane production can be estimated as up to 70.7% from the electron balance ($96 \times x + 49.2 \times (100 - x) = 100 \times 82.3, x = 70.7$). The enzymatic IIET with higher transfer losses of electrons might be more contributed to methane production from fermentable substrate than from the non-fermentable acetate [4–6]. Likely, the substrate competition between exoelectrogenic- and acidogenic fermentation bacteria for the fermentable substrate decreased the contribution of biological DIET to methane production.

In MixEF, both IIET and DIET can be involved in methane production from the mixture of glucose and acetate, as in GluEF. However, the methane yield for MixEF was 276.1 mL CH₄/g COD_r, which was slightly lower than that in GluEF (Table 1). In the electron balance, the electrons captured to methane for MixEF was 78.9%, less than for the GluEF, while the electrons for the microbial growth increased to 11.0%. In MixEF, the DIET contribution to methane production can be estimated from the electron balance ($96 \times x + 49.2 \times (100 - x) = 100 \times 78.9, x = 63.5$) as up to 63.5%. This suggests that complex mixed substrates stimulate the growth of various microbial species and significantly change the electron transfer pathways for methane production [25,42].

3.3. Bioelectrochemical Activity of the Suspended Microorganisms

The cyclic voltammogram obtained from the bulk solution visualizes the electrochemical activity of suspended anaerobic microorganisms related to the ability of biological DIET for methane production. In the voltammogram, the peak and formal potentials generally depend on the types of redox substances [45–47]. In the AcEF, the peak potentials for oxidation and reduction were 0.17 V vs. Ag/AgCl, and −0.23 V vs. Ag/AgCl, respectively (Figure 3). In previous studies, the peak potential in bioelectrochemical reactors was observed in the range −0.20 to −0.01 V vs. Ag/AgCl for EEB, and −0.23 to −0.41 V vs. Ag/AgCl for EMA, slightly different from those of the AcEF [4,46,47]. However, the formal potential of the AcEF was −0.03 V vs. Ag/AgCl, which was close to the cytochrome C-550 of -0.02 ± 0.01 V vs. Ag/AgCl in the membrane of *Bacillus subtilis* or the new exoelectrogens of SCS5 [47]. It seems that the redox peaks in the AcEF were primarily expressed by electroactive

microorganisms, including EEB and EMA. This means that the peak heights obtained from the voltammogram are mainly related to the electrochemical activities of EEB and EMA. The peak heights in AcEF were as high as 0.41 mA for both oxidation and reduction, higher than the others (Table 2). This suggests that the bulk solution in AcEF was enriched with electroactive microorganisms by the electric field; thus, DIET contributed more to methane production from acetate.

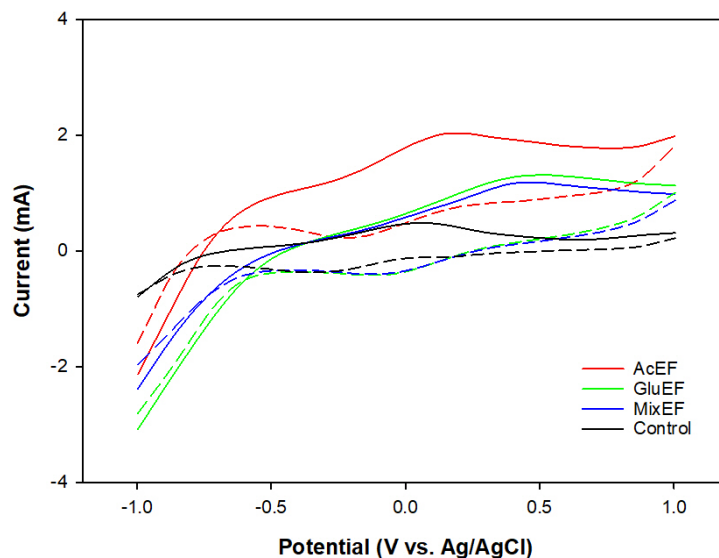


Figure 3. Cyclic voltammogram for the bulk solution depending on the substrate type in the anaerobic reactor exposed to an electric field (solid line: oxidation, dashed line: reduction).

Table 2. Redox peaks for the bulk solution depending on the substrate type in the anaerobic reactor exposed to an electric field.

	AcEF	GluEF	MixEF	Control
$E_{p,o}$ (V) vs. Ag/AgCl	0.17	0.41	0.40	0.07
$E_{p,r}$ (V) vs. Ag/AgCl	−0.23	−0.08	−0.07	−0.35
E_f (V) vs. Ag/AgCl	−0.03	0.17	0.17	−0.14
$I_{p,o}$ (mA)	0.41	0.29	0.21	0.19
$I_{p,r}$ (mA)	0.41	0.29	0.23	0.20

($E_{p,o/r}$: peak potentials for oxidation and reduction, E_f : formal potential, $I_{p,o/r}$: peak heights for oxidation and reduction).

In GluEF, the redox peak potentials were observed at 0.41 V vs. Ag/AgCl and −0.08 V vs. Ag/AgCl, which were shifted to the positive direction, compared to those in the AcEF (Figure 3). It seems that EEB and EMA species enriched from glucose were different from the AcEF. In addition, the peak heights were 0.29 mA for both oxidation and reduction, which were smaller than those in the AcEF. It is likely that EEB competes with AFB for glucose as a substrate, and some portion of methane is produced from the intermediates, such as acetate and hydrogen, via IIET. Thus, the contribution of DIET to methane production in the GluEF was possibly less than in the AcEF. This is the reason why in the GluEF, the electron transfer loss was higher than in the AcEF, and the methane yield was smaller (Table 1).

In the MixEF, the oxidation and reduction peaks in the voltammogram were observed at 0.40 V vs. Ag/AgCl and −0.07 V vs. Ag/AgCl (Table 2), similar to those in the GluEF. However, the peak heights were less than those in the GluEF. In the MixEF, the electron equivalent for the biomass growth was higher than in the GluEF (Table 1), and the contribution of DIET to methane production was less. This suggests that the contribution of DIET to methane production in the MixEF might be further decreased compared to the GluEF by the growth of non-electroactive microorganisms.

3.4. Microbial Communities

The contribution of biological DIET to methane production depending on the substrate type, which characterizes bioelectrochemical methane production, could be well supported by not only in the electrochemical activity of the bulk solution but also in the microbial community. The dominant bacterial groups at the phylum level were commonly Bacteroidetes, Firmicutes, Chloroflexi, and Proteobacteria in all of the anaerobic reactors (Figure 4). However, Actinobacteria was a bacterial group that is abundant in the control and AcEF containing acetate as the substrate, while Verrucomicrobia was abundant in the GluEF and MixEF with glucose as the substrate. In the archaeal group, Euryarchaeota was the most dominant phylum, at over 97% in all of the anaerobic reactors.

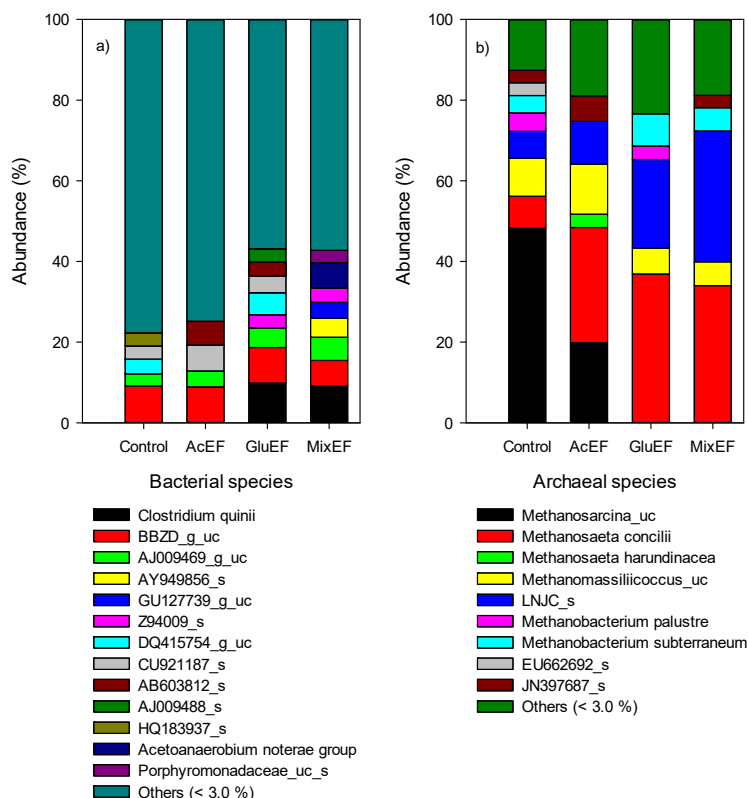


Figure 4. Microbial communities depending on the substrate type in the anaerobic reactor exposed to the electric field: (a) bacterial species, and (b) archaeal species.

In microbial communities, the number of bacterial species that were abundant over 3% was greater in GluEF and MixEF than those of in the control and AcEF. As a fermentable substrate, glucose is believed to increase bacterial species diversity by the growth of fermentation bacteria, compared to the acetate. The commonly abundant species in all of the samples were *BBZD_g_uc* and *AJ009469_g_uc*. Syntrophic acetate oxidation is the only intersection of bacterial metabolic pathways for methane production from acetate and glucose in all of the anaerobic reactors. It seems that *BBZD_g_uc* and *AJ009469_g_uc* are the bacterial species that are involved in the acetate oxidation to hydrogen and carbon dioxide. The species *CU921187_s* was abundant in AcEF, GluEF, and control, and *AB603812_s* was abundant in AcEF and GluEF. However, *CU921187_s* and *AB603812_s* were minorities in MixEF. *CU921187_s* and *AB603812_s* are uncultured species belong to Bacteroidetes phylum, which were isolated from the anaerobic digester for sewage sludge and observed also in the bioelectrochemical conversion of coal to methane [15,48]. It seems that while *CU921187_s* and *AB603812_s* are involved in the syntrophic acetate oxidation or hydrogen-oxidizing EEB. Interestingly, *Clostridium quinii* and *Z94009_s* were abundant in GluEF and MixEF. *C. quinii* is a species isolated from anaerobic granular sludge, which produces hydrogen, carbon dioxide, formate,

acetate, ethanol, and butyrate from glucose fermentation [49]. *C. quinii* is also known as a species that is involved in biological DIET for methane production from glucose in the bioelectrochemical anaerobic reactor [4]. *Z94009_s* is an uncultured species isolated from activated sludge [50]. *C. quinii* and *Z94009_s* are likely to be glucose-oxidizing EEB. *DQ415754_g_uc* and *HQ183937_s* were abundant in both GluEF and control. It seems that these species are involved in syntrophic acetate oxidation, but are not electroactive bacterial species. *AY949856_s*, *GU127739_g_uc*, *Acetoanaerobium noterae* group, and *Porphyromnadaeae_uc_s* were abundant in MixEF only. In MixEF, the acetate fraction of the substrate was higher than that in the GluEF. These bacterial species are likely to be the bacterial species that are involved in syntrophic acetate oxidation, or are acetate-oxidizing EEB.

In the control, the predominant archaeal species was *Methanosarcina_uc*, followed by *Methanosaeta concilii*, *Methanomassiliicoccus_uc*, *LNJC_g*, *LNJC_s*, *Methanobacterium palustre*, and *Methanobacterium subterraneum*. The routes for methane production in the control can be acetoclastic and hydrogenotrophic methanogenesis through syntrophic acetate oxidation. It is well known that acetoclastic methanogens include *Methanosarcina_uc* and *M. concilii*, and hydrogenotrophic methanogens are the other archaeal species. In AcEF, the abundances of archaeal species *M. concilii*, *L. LNJC_s*, *Methanobacterium harundinacea*, and *Methanosaeta JN397687_s* increased, compared to the control, while *Methanosarcina_uc* decreased. In the anaerobic reactor exposed to an electric field, methane can be produced through biological DIET. The archaeal species that increased in AcEF compared to the control are likely EMA. In previous studies, *M. concilii* was observed in the bioelectrochemical anaerobic reactor that fed with glucose as the substrate [4]. The archaeal species *L. LNJC_s*, *M. harundinacea*, and *M. JN397687_s* were observed in the bioelectrochemical reactor for coal conversion to methane [15]. Surprisingly, *Methanosarcina_uc* was not observed in GluEF or MixEF, indicating that acetoclastic methanogenesis is not an important route in GluEF or MixEF. However, the abundances of *M. concilii*, *L. LNJC_s*, and *M. subterraneum* were significantly high in both GluEF and MixEF. It seems that *L. LNJC_s* and *M. subterraneum* are EMA species that are involved in methane production from fermentable substrate, like glucose.

4. Conclusions

The contribution of DIET to methane production in the anaerobic reactor exposed to an electric field depends on the substrate characteristics and affects the yield and production rate of methane. In anaerobic digestion without an electric field, the main pathways for the production of methane from acetate as a non-fermentable substrate are the acetoclastic process and syntrophic acetate oxidation that have significant electron transfer losses. However, the electric field in the anaerobic digestion promotes biological DIET pathway that is a primary electron transfer route for methane production from acetate. The DIET significantly improves the yield and production rate of methane from acetate. In the case of glucose, a fermentable substrate, the substrate competition between exoelectrogenic bacteria and acidogenic fermentation bacteria under the electric field decreases the contribution of biological DIET to methane production, which decreases the yield and production rate of methane compared to the acetate by increasing the electron transfer loss. The mixture of acetate and glucose further decreases the methane yield by stimulating microbial growth. These findings provide a better understanding of the biological DIET for methane production under an electric field, which is of great importance in designing the high-rate bioelectrochemical anaerobic process.

Author Contributions: Y.-C.S., B.-U.B. and C.-Y.L. conceived the original idea; Y.-C.S. and G.-G.O. designed the study; G.-G.O. carried out the experiment and collected the data; Y.-C.S., B.-U.B. and C.-Y.L. interpreted the data and developed the theory. All authors discussed the data and contributed to the final manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government(MSIT) (NRF-2017R1E1A1A01075325) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2019R1A6A3A1309621).

Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of this article.

References

- Enzmann, F.; Mayer, F.; Rother, M.; Holtmann, D. Methanogens: Biochemical background and biotechnological applications. *AMB Express* **2018**, *8*, 1, doi:10.1186/s13568-017-0531-x.
- Feng, Q.; Song, Y.C.; Yoo, K.; Kuppanan, N.; Subudhi, S.; Lal, B. Bioelectrochemical enhancement of direct interspecies electron transfer in upflow anaerobic reactor with effluent recirculation for acidic distillery wastewater. *Bioresour. Technol.* **2017**, *241*, 171–180, doi:10.1016/j.biortech.2017.05.073.
- Shrestha, P.M.; Erotaru, A.E. Plugging in or going wireless: Strategies for interspecies electron transfer. *Front. Microbiol.* **2014**, *5*, 237, doi:10.3389/fmicb.2014.00237.
- Feng, Q.; Song, Y.C.; Ahn, Y. Electroactive microorganisms in bulk solution contribute significantly to methane production in bioelectrochemical anaerobic reactor. *Bioresour. Technol.* **2018**, *259*, 119–127, doi:10.1016/j.biortech.2018.03.039.
- Lovley, D.R. Live wires: Direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. *Energy Environ. Sci.* **2011**, *4*, 4896–4906, doi:10.1039/c1ee02229f.
- Shen, L.; Zhao, Q.; Wu, X.; Li, X.; Li, Q.; Wang, Y. Interspecies electron transfer in syntrophic methanogenic consortia: From cultures to bioreactors. *Renew. Sustain. Energy Rev.* **2016**, *54*, 1358–1367, doi:10.1016/j.rser.2015.10.102.
- Song, Y.C.; Feng, Q.; Ahn, Y. Performance of the bio-electrochemical anaerobic digestion of sewage sludge at different hydraulic retention times. *Energy Fuels* **2016**, *30*, 352–359, doi:10.1021/acs.energyfuels.5b02003.
- Padilla-Gasca, E.; López-López, A.; Gallardo-Valdez, J. Evaluation of stability factors in the anaerobic treatment of slaughterhouse wastewater. *J. Bioremed. Biodegrad.* **2011**, *2*, 1000114, doi:10.4172/2155-6199.1000114.
- Baek, G.; Kim, J.; Kim, J.; Lee, C. Role and potential of direct interspecies electron transfer in anaerobic digestion. *Energies* **2018**, *11*, 107, doi:10.3390/en11010107.
- Leang, C.; Qian, X.; Mester, T.; Lovley, D.R. Alignment of the c-Type Cytochrome OmcS along Pili of *Geobacter sulfurreducens*. *Appl. Environ. Microbiol.* **2010**, *76*, 4080–4084, doi:10.1128/aem.00023-10.
- Blasco-Gómez, R.; Battle-Vilanova, P.; Villano, M.; Balaguer, M.D.; Colprim, J.; Puig, S. On the edge of research and technological application: A critical review of electromethanogenesis. *Int. J. Mol. Sci.* **2017**, *18*, 874, doi:10.3390/ijms18040874.
- Dubé, C.D.; Guiot, S.R. Direct interspecies electron transfer in anaerobic digestion: A review. *Adv. Biochem. Eng. Biotechnol.* **2015**, *151*, 101–115, doi:10.1007/978-3-319-21993-6_4.
- Kato, S.; Hashimoto, K.; Watanabe, K. Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals. *Environ. Microbiol.* **2011**, *14*, 1646–1654, doi:10.1111/j.1462-2920.2011.02611.x.
- Rotaru, A.-E.; Shrestha, P.M.; Liu, F.; Markovait, B.; Chen, S.; Nevin, K.P.; Lovley, D.R. Direct Interspecies Electron Transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*. *Appl. Environ. Microbiol.* **2014**, *80*, 4599–4605, doi:10.1128/aem.00895-14.
- Piao, D.M.; Song, Y.C.; Oh, G.G.; Kim, D.H.; Bae, B.U. Contribution of yeast extract, activated carbon, and an electrostatic field to interspecies electron transfer for the bioelectrochemical conversion of coal to methane. *Energies* **2019**, *12*, 4051, doi:10.3390/en12214051.
- Feng, Q.; Song, Y.C.; Yoo, K.; Kuppanan, N.; Subudhi, S.; Lal, B. Polarized electrode enhances biological direct interspecies electron transfer for methane production in upflow anaerobic bioelectrochemical reactor. *Chemosphere* **2018**, *204*, 186–192, doi:10.1016/j.chemosphere.2018.03.163.
- Joicy, A.; Song, Y.C.; Li, J.; Oh, S.E.; Jang, S.H.; Ahn, Y. Effect of electrostatic field strength on bioelectrochemical nitrogen removal from nitrogen-rich wastewater. *Energies* **2020**, *13*, 3218, doi:10.3390/en13123218.
- Feng, Q.; Song, Y.C.; Li, J.; Wang, Z.; Wu, Q. Influence of electrostatic field and conductive material on the direct interspecies electron transfer for methane production. *Environ. Res.* **2020**, *188*, 109867, doi:10.1016/j.envres.2020.109867.
- Belderbos, A.; Valkaert, T.; Bruninx, K.; Delarue, E.; D'Haeseleer, W. Facilitating renewables and power-to-gas via integrated electrical power-gas system scheduling. *Appl. Energy* **2020**, *275*, 115082, doi:10.1016/j.apenergy.2020.115082.

20. Götz, M.; Lefebvre, J.; Mörs, F.; Koch, A.M.; Graf, F.; Bajohr, S.; Reimert, R.; Kolb, T. Renewable Power-to-Gas: A technological and economic review. *Renew. Energy* **2016**, *85*, 1371–1390, doi:10.1016/j.renene.2015.07.066.
21. Lee, H.-S.; Parameswaran, P.; Kato-Marcus, A.; Torres, C.I.; Rittmann, B.E. Evaluation of energy-conversion efficiencies in microbial fuel cells (MFCs) utilizing fermentable and non-fermentable substrates. *Water Res.* **2008**, *42*, 1501–1510, doi:10.1016/j.watres.2007.10.036.
22. Rozendal, R.A.; Jeremiasse, A.W.; Hamelers, H.V.M.; Buisman, C.J.N. Hydrogen production with a microbial biocathode. *Environ. Sci. Technol.* **2008**, *42*, 629–634, doi:10.1021/es071720+.
23. Wisseroth, K.; Braune, H. Thermodynamic equilibrium in strong electric fields and field chemistry consequences. *J. Phys.* **1977**, *38*, 1249–1255, doi:10.1051/jphys:0197700380100124900.
24. Karakashev, D.B.; Batstone, D.J.; Trably, E.; Angelidaki, I. Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of methanosaetaceae. *Appl. Environ. Microbiol.* **2006**, *72*, 5138–5141, doi:10.1128/aem.00489-06.
25. Pant, D.; Van Bogaert, G.; Diels, L.; Vanbroekhoven, K. A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. *Bioresour. Technol.* **2010**, *101*, 1533–1543, doi:10.1016/j.biortech.2009.10.017.
26. Chaudhuri, S.K.; Lovley, D.R. Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. *Nat. Biotechnol.* **2003**, *21*, 1229–1232, doi:10.1038/nbt867.
27. Xu, S.; Liu, H. New exoelectrogen *Citrobacter* sp. SX-1 isolated from a microbial fuel cell. *J. Appl. Microbiol.* **2011**, *111*, 1108–1115, doi:10.1111/j.1365-2672.2011.05129.x.
28. Feng, Q.; Song, Y.C. Surface modification of a graphite fiber fabric anode for enhanced bioelectrochemical methane production. *Energy Fuels* **2016**, *30*, 6467–6474, doi:10.1021/acs.energyfuels.6b00959.
29. APHA; AWWA; WEF. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association/American Water Works Association/Water Environmental Federation: Washington, DC, USA, 2005.
30. Piao, D.M.; Song, Y.C.; Kim, D.H. Bioelectrochemical enhancement of biogenic methane conversion of coal. *Energies* **2018**, *11*, 2577, doi:10.3390/en1102577.
31. Khan, N.; Khan, M.D.; Nizami, A.; Rehan, M.; Shaida, A.; Ahmad, A.; Khan, M.Z. Energy generation through bioelectrochemical degradation of pentachlorophenol in microbial fuel cell. *RSC Adv.* **2018**, *8*, 20726–20736, doi:10.1039/c8ra01643g.
32. Villano, M.; Aulenta, F.; Ciucci, C.; Ferri, T.; Giuliano, A.; Majone, M. Bioelectrochemical reduction of CO₂ to CH₄ via direct and indirect extracellular electron transfer by a hydrogenophilic methanogenic culture. *Bioresour. Technol.* **2010**, *101*, 3085–3090, doi:10.1016/j.biortech.2009.12.077.
33. Yoon, S.H.; Ha, S.M.; Kwon, S.; Lim, J.; Kim, Y.; Seo, H.; Chun, J. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 1613–1617, doi:10.1099/ijsem.0.001755.
34. Ferry, J.G. Fundamentals of methanogenic pathways that are key to the biomethanation of complex biomass. *Curr. Opin. Biotechnol.* **2011**, *22*, 351–357, doi:10.1016/j.copbio.2011.04.011.
35. Pivrikas, A.; Ullah, M.; Sitter, H.; Sariciftci, N.S. Electric field dependent activation energy of electron transport in fullerene diodes and field effect transistors: Gill's law. *Appl. Phys. Lett.* **2011**, *98*, 92114, doi:10.1063/1.3557503.
36. Lin, C.Y.; Sato, K.; Noike, T.; Matsumoto, J. Methanogenic digestion using mixed substrate of acetic, propionic and butyric acids. *Water Res.* **1986**, *20*, 385–394, doi:10.1016/0043-1354(86)90088-6.
37. Jia, X.S.; Furumai, H.; Fang, H.H.P. Yields of biomass and extracellular polymers in four anaerobic sludges. *Environ. Technol.* **1996**, *17*, 283–291, doi:10.1080/09593331708616386.
38. Yang, Y.; Chen, Q.; Guo, J.; Hu, Z. Kinetics and methane gas yields of selected C1 to C5 organic acids in anaerobic digestion. *Water Res.* **2015**, *87*, 112–118, doi:10.1016/j.watres.2015.09.012.
39. Hattori, S. Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ.* **2008**, *23*, 118–127, doi:10.1264/jsme2.23.118.
40. Burton, F.L.; Tchobanoglous, G.; Tsuchihashi, R.; Stensel, H.D. *Wastewater Engineering: Treatment and Resource Recovery*, 5th ed.; McGraw-Hill Education: New York, NY, USA, 2014; pp. 579–587.
41. Kimura, Z.I.; Okabe, S. *Hydrogenophaga electricum* sp. nov., isolated from anodic biofilms of an acetate-fed microbial fuel cell. *J. Gen. Appl. Microbiol.* **2013**, *59*, 261–266, doi:10.2323/jgam.59.261.

42. Mateo, S.; Cañizares, P.; Rodrigo, M.A.; Fernandez-Morales, F.J. Driving force behind electrochemical performance of microbial fuel cells fed with different substrates. *Chemosphere* **2018**, *207*, 313–319, doi:10.1016/j.chemosphere.2018.05.100.
43. Feng, Q.; Song, Y.C.; Bae, B.U. Influence of applied voltage on the performance of bioelectrochemical anaerobic digestion of sewage sludge and planktonic microbial communities at ambient temperature. *Bioresour. Technol.* **2016**, *220*, 500–508, doi:10.1016/j.biortech.2016.08.085.
44. Xafenias, N.; Mapelli, V. Performance and bacterial enrichment of bioelectrochemical systems during methane and acetate production. *Int. J. Hydrog. Energy* **2014**, *39*, 21864–21875, doi:10.1016/j.ijhydene.2014.05.038.
45. Carmona-Martinez, A.A.; Harnisch, F.; Fitzgerald, L.A.; Biffinger, J.C.; Ringeisen, B.R.; Schröder, U. Cyclic voltammetric analysis of the electron transfer of *Shewanella oneidensis* MR-1 and nanofilament and cytochrome knock-out mutants. *Bioelectrochemistry* **2011**, *81*, 74–80, doi:10.1016/j.bioelechem.2011.02.006.
46. Feng, C.; Li, J.; Qin, D.; Chen, L.; Zhao, F.; Chen, S.; Hu, H.; Yu, C.P. Characterization of exoelectrogenic bacteria enterobacter strains isolated from a microbial fuel cell exposed to copper shock load. *PLoS ONE* **2014**, *9*, e113379, doi:10.1371/journal.pone.0113379.
47. Sharma, S.C.D.; Feng, C.; Li, J.; Hu, A.; Wang, H.; Qin, D.; Yu, C.P. Electrochemical characterization of a novel exoelectrogenic bacterium strain SCS5, isolated from a mediator-less microbial fuel cell and phylogenetically related to *aeromonas jandaei*. *Microbes Environ.* **2016**, *31*, 213–225, doi:10.1264/jsme2.me15185.
48. Rivière, D.; Desvignes, V.; Pelletier, E.; Chaussonnerie, S.; Guermazi, S.; Weissenbach, J.; Li, T.; Camacho, P.; Sghir, A. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J.* **2009**, *3*, 700–714, doi:10.1038/ismej.2009.2.
49. Svensson, B.G.; Nise, G.; Erfurth, E.M.; Olsson, H. Neuroendocrine effects in printing workers exposed to toluene. *Br. J. Ind.* **1992**, *49*, 402–408, doi:10.1136/oem.49.6.402.
50. Snaidr, J.; Amann, R.; Huber, I.; Ludwig, W.; Schleifer, K.H. Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Appl. Environ. Microbiol.* **1997**, *63*, 2884–2896, doi:10.1128/aem.63.7.2884-2896.1997.

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).