Original Research

Enhancement of hydrogen production and energy recovery through electro-fermentation from the dark fermentation effluent of food waste

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A B S T R A C T

To enhance hydrogen production efficiency and energy recovery, a sequential dark fermentation and microbial electrochemical cell (MEC) process was evaluated for hydrogen production from food waste. The hydrogen production, electrochemical performance and microbial community dynamics were investigated during startup of the MEC that was inoculated with different sludges. Results suggest that biogas production rates and hydrogen proportions were 0.83 L/L d and 92.58%, respectively, using anaerobic digested sludge, which is higher than that of the anaerobic granular sludge (0.55 L/L d and 86.21%). The microbial community were predominated by bacterial genus Acetobacterium, Geobacter, Desulfuvibrio, and archaeal genus Methanobrevibacter in electrode biofilms and the community structure was relatively stable both in anode and cathode. The sequential system obtained a 53.8% energy recovery rate and enhanced soluble chemical oxygen demand (sCOD) removal rate of 44.3%. This research demonstrated an important approach to utilize dark fermentation effluent to maximize the conversion of fermentation byproducts into hydrogen.

1. Introduction

The energy crisis and climate change has created a special spotlight on identifying renewable and sustainable energy sources [1,2]. Hydrogen (H₂) is considered an attractive future fuel since it is a clean energy carrier and has a higher energy density relative to fossil fuels [3]. Traditional hydrogen production methods, such as water electrolysis and fossil fuel gasification, are cost-intensive because of the high-energy requirements. These methods are also unsustainable [4-6]. Dark fermentative hydrogen production from organic wastes has drawn great attention due to its lower energy requirements and the ability to use less expensive and abundant raw materials, which reduce production costs and makes it feasible in practical applications [7-9]. However, the maximum theoretical hydrogen yield using dark fermentation is only 4 mol. H₂/mol glucose. These conditions will also result in a fermentation end product (acetate; 2 mol/mol glucose) that bacteria are unable to further convert to hydrogen. Another drawback is the energy yield is less than 3 mol. H₂/mol glucose in practical operation [10,11]. Thus, large surpluses of effluent from dark fermentation end product is difficult to utilize and most of the remaining organic matter is essentially wasted as a mixture of primarily acetic and butyric acids even though the stoichiometric potential of hydrogen production is 12 mol. H₂/mol glucose [12]. The biochemical barrier is due to the insufficient terminal electron acceptor and a fermentative thermodynamic obstacle, which can be overcome by microbial bioelectrochemical technologies [10,13,14]. By deriving protons and electrons from the dark fermentation effluent, hydrogen can be obtained directly under low voltage conditions using microbial bioelectrochemical systems that do not require oxygen [12,15].

Recently, a MEC have been used as a typical microbial bioelectrochemical system to explore the conversion of organics into hydrogen using different biomass and inoculants [16-19]. Several literatures have reported improvement of hydrogen yield in a combined treatment process of dark fermentation and MEC using fermentation effluent, since the effluent from a dark fermentation process is rich in organics that cannot be further fermented to produce hydrogen. Lalaurette et al. [20] showed a nearly 10 mol. H₂/mol glucose from a cellobiolose feedstock, compared to 1.65 mol. H₂/mol glucose by dark fermentation alone. Dhar et al. [21] also reported a highest hydrogen yield (6molH₂/mol hexose) from sugar beet juice, which corresponds to 25%
of initial COD in a sequential hydrogen process. Recently, some reports
focus on the electro-fermentation (EF) of food waste, high volatile fatty
acids and biomethane production were obtained [22,23]. However, there
were few reports about the sequential process with dark fermentation
and MEC of food waste.

Although the advantages of electro-fermentation are efficient biomass
conversion and high Coulombic efficiencies using simple substrates,
hydrogen recovery from fermentation effluent, is unsatisfactory in MECs
[24–27]. Functional regulation and adaptive evolution approaches were
also used on electroactive microorganisms to confer the ability to
enhance commercial efficiencies and hydrogen recovery by
electro-fermentation [28,29]. To enhance hydrogen production effi-
ciency and substrate utilization, it is important to clarify the dynamic
mechanisms of electro-fermentation hydrogen production from dark
fermentation effluent, especially the incredible diversity of electroactive
microorganisms and the basics of substrate conversion and energy re-
cover [30].

In this study, the potential of hydrogen production from food waste
was evaluated using a hydrogen production process of dark fermentation
and MEC. The analysis of the MECs startup inoculated with different
sludges from were carried out to determine the electrochemical perfor-
mances, microbial morphologies and community dynamics. Based on this
analysis, the performance of electro-fermentation hydrogen production
from food waste dark fermentation effluent and energy recovery using
MECs were investigated on the macro- and micro-scales. The metabolic
pathways of the sequential system combining dark fermentation with
electro-fermentation hydrogen production were investigated according
to the change of metabolites and based on the ecological niche of the
electroactive microorganisms.

2. Materials and methods

2.1. MEC system configuration and startup

Experiments were conducted using single chamber MEC reactors
made of polycarbonate. The total volume was 125.6 mL and consisted of
a 56.5 mL chamber (3 cm inner diameter × 8 cm long) and a gas
collection tube attached to the top of the reactor (1.6 cm inner diameter
× 7.5 cm length; 14.5 mL capacity). The anode brush was made of carbon
fiber with a diameter of 25 mm (wos1009, CeTech Co., Ltd, Taiwan), and
all anode brushes were soaked in acetone for 24 h and subsequently
heated in a muffle furnace at 450 °C for 30 min before use. The cathode
was a carbon cloth coated with Pt/C, which was placed on the side
opposite the anode brush. All reactors were operated in batch mode. The
electrical voltage was 0.8 V and was supplied from a switching power
supply (CHI 1000B, CH Instrument, Shanghai, China). The voltages
across a resistor (10.0 Ω for MECs) were measured using a multimeter
(model 2700; Keithley Instruments, U.S.A.) every 10 min.

The MEC startup was divided into two periods and consisted of an
electroactive microorganism enrichment period and a microorganism
growth period. The electroactive microorganism enrichment period
was inoculated with anaerobic digested sludge (ADS) from the anaerobic
treatment of pig manure and anaerobic granular sludge (AGS) from a
citric acid wastewater anaerobic treatment plan. During the electroactive
microorganism enrichment period, all the reactors were fed 40 mL of
food waste. The reactors were run for 6 months, and the
organic load rate (OLR) increased from 3.0 g VS/L d to 6.0 g VS/L d. The
pH value was controlled at 6.0 through the addition of aqueous solutions
of 1 M HCl and 1 M NaOH.

2.2. Electro-fermentation hydrogen production

2.2.1. Dark fermentation hydrogen production

Dark fermentation hydrogen production systems were operated in 2 L
continuous stirred tank reactors at 35 ± 1 °C. Motorized automatic
stirring was used (120 rpm). A seed sludge of 50 g was loaded into each
bioreactor during the initiation stage, and the working volume was
increased to 1.5 L using distilled water. The reactors were operated in a
semi-continuous mode at a total hydraulic retention time of 8 days;
manual sampling was conducted at the sampling opening before the daily
addition of food wastes. The reactors were run for 6 months, and the
organic load rate (OLR) increased from 3.0 g VS/L d to 6.0 g VS/L d. The
pH value was controlled at 6.0 through the addition of aqueous solutions
of 1 M HCl and 1 M NaOH.

2.2.2. Electro-fermentation hydrogen production

After the startup of the MEC reactors, electro-fermentation hydrogen
production from dark fermentation effluent was conducted. All MEC
reactors were fed 40 mL of dark fermentation effluent (48 h batch cycles)
from continuous systems of dark fermentation, instead of acetate me-
dium. The initial pH value was adjusted to 7.0 through the addition of
aqueous solutions of 1 M HCl and 1 M NaOH. The control group was fed
sodium acetate as a single substrate and the culture medium was the
same as the electroactive microorganism enrichment period.

2.3. Microbial morphology and community analysis

At the end of the startup period, the MEC reactors were aseptically
disassembled and the biofilms were scraped from the anode brushes and
cathode cloths to characterize the microbial morphology using a scan-
nning electron microscopy (SEM; JSM-IT300LV, JEOL Ltd., Tokyo, Japan).
A microbial community analysis was also performed using the electrode
biofilm and electrolyte samples. The samples were stored at –80 °C prior
to deoxyribonucleic acid (DNA) extraction. The total DNA was extracted
from each sample with the Powersoil DNA isolation kit (MoBio Labora-
tories, Inc., USA) following the manufacturer’s method. The bacterial
DNA was amplified using the 16S rRNA gene primers 338F (5’-ACTCCT-
TACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-
3’) [31]. Archaeal DNA was amplified using 16S rRNA gene primers
524F-10-extF (5’-TGYAGCCGCGCGGTATAA-3’) and Arch958r-modR
(5’-YCYYCGGTGTTGVCATTTAAT-3’) [32].

The polymerase chain reaction (PCR) products were sequenced on
an Illumina Hiseq 2500 PE250 pyrosequencing platform (Illumina, San
Diego, USA). The raw sequences were trimmed, qualified and then
clustered into operational taxonomic units (OTUs). The initial removal
of the sequences was aimed at those shorter than 50 bp and with quality
scores smaller than 20. OTUs were clustered with 97% similarity cutoffs
using UPARSE (version 7.1 http://drive5.com/uparse/), and the
chimeric sequences were identified and removed using UCHIME.
The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP
Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva
(SSU123) 16S rRNA database with a confidence threshold of 70%. Richness
estimators of the Sobs, Shannon, Simpson, Abundance-based
coverage estimator (ACE) and Chao1 index were calculated by
MOTHUR (version v.1.30.1 http://www.mothur.org/wiki/Schloss-
SOP#Alpha_diversity). A Principal Component Analysis (PCA) was con-
ducted using a web-based platform (http://www.s-sanger.com/).

2.4. Analysis methods

The soluble chemical oxygen demand (sCOD) and pH were measured
according to the APHA standard methods [33]. The composition of the
volatile fatty acids (VFAs) was analyzed by gas chromatography
(GC7980, Fu Li, Zhejiang) equipped with a flame ionization detector and
bags (0.1 L capacity; Cali-5-Bond, Calibrated Instruments) for analysis.

Environmental Science and Ecotechnology 1 (2020) 100006

X. Jia et al.
2.5. Calculations

The reactor performance was evaluated in terms of Coulombic efficiency (%), current density (mA), hydrogen recovery (%) and hydrogen production rate (L/L d), which were all calculated as previously described [35].

The cathodic hydrogen recovery efficiency, defined as the fraction of electrons reaching the cathode that are recovered as hydrogen gas, calculated as:

\[ n_{H_{2, cat}} = \frac{n_{H_2}}{n_{H_2, cat}} \times 100\% \] (1)

where \( n_{H_2} \) is the number of moles of hydrogen recovered, and \( n_{H_2, cat} \) is the number of moles of hydrogen that can theoretically be produced from the current, calculated as:

\[ n_{H_2, cat} = \frac{\sum I \Delta t}{2F} \times 100\% \] (2)

where 2 is used to convert moles of electrons to moles of hydrogen gas, \( F \) is Faraday’s constant (96,485 C/mol e⁻), and \( I = E/R_{ex} \) is the current (A) calculated from the measured voltage (E, V) across the resistor (R_{ex} = 100Ω).

The overall hydrogen recovery, \( r_{H_2, COD} \), which is defined as the ratio of the hydrogen recovered to the maximum possible hydrogen recovery based on the organic matter oxidized on the basis of COD,

\[ r_{H_2, COD} = \frac{n_{H_2}}{n_{H_2, COD}} \times 100\% \] (3)

where \( n_{H_2, COD} \) is the COD (mg/L) consumed during the batch cycle, \( V_L \) is the volume of the liquid, and \( bH_2/S \) is a conversion factor based on stoichiometric conversion of electrons in COD to hydrogen gas equaling 1 mol. H₂ per 16 g O₂.

The Coulombic efficiency was calculated as follow:

\[ CE = \frac{n_{H_2, cat}}{n_{H_2, COD}} = \frac{r_{H_2, cat}}{r_{H_2, COD}} \times 100\% \] (5)

The energy calculation includes values for both consumption and generation. Energy consumption includes the electric current demand of energy for heating and mixing and compensates for the heat losses during fermentation. Energy generation is produced from hydrogen production. The fermentation tanks used included a single continuously stirred reactor made of steel plate splicing technology with a working volume of 0.15 m³ with 12 cm in diameter and 13.26 cm in height. The reactor wall surface area (A) was calculated from the useable reactor volume, while the reactor bottom was not included. The interlayer is made of rock wool for insulation, and the inner wall included a heating coil.

The calculation formula of energy consumption for the feed heating during the dark fermentation is as follows:

\[ E_0 = C_p Q (T_a - T_i) \] (6)

where \( E_0 \) is the energy consumption for feed heating (kJ/d); \( C \) is the Specific heat of the material (4.18 kJ/kg °C); \( p \) is the density of fermentation liquor (1000 kg/m³); \( Q \) is the flow rate (1.87 × 10⁻⁴ m³/d); \( T_a \) is the pretreatment temperature (55 °C); and \( T_i \) is the ambient temperature (20 °C).

Fermentation tank using single CSTR (continuous stirred reactor) made of steel plate, the interlayer is made of rock wool for insulation, and the inner wall is added with heating coil. The tank insulation calories computation is mainly from rock wool due to the higher heat conduction coefficient (\( \lambda = 16 \text{ W/m K} \)) of the steel plate compared to the rock wool heat conduction coefficient (\( \lambda = 0.04 \text{ W/m K} \)). The tank insulation calories computation formula is as follows:

\[ E_i = \frac{A \bar{h} (T_i - T_a) \times \Delta t}{h} \] (7)

where \( E_i \) is the energy consumption for heat preservation (kJ/d); \( A \) is the surface area of the process tank (3.63 × 10⁻⁴ m²); \( h \) is the thickness of the rock wool (5 × 10⁻⁳ m); \( T_i \) is the observed dark fermentation temperature (°C); \( T_a \) is the ambient temperature (20 °C); and \( \Delta t \) is the time of the dark fermentation process (1 d).

The energy required for biomass pumping and reactor mixing is as follows:

\[ E_m = V_0 \rho \] (8)

where \( E_m \) is the input electricity for reactor mixing (kJ/d); \( V \) is the working volume (1.87 × 10⁻⁴ m³); and \( \omega \) is the electricity consumption for mixing (300 kJ/m³ reactor d).

The power input of MEC is as follows:

\[ E_1 = \int_0^t (I E - I^2 R_{ex}) dt \] (9)

where \( E_1 \) is the power input of MEC (kJ); \( I \) is the external circuit current (A); \( E \) is the applied voltage (0.8 V); \( R_{ex} \) is the external resistance (10 Ω); and \( t \) is the processing cycle (2 d).

Hydrogen energy from dark fermentation was calculated as follows:

\[ E_1 = \rho_{H_2} \times q_{H_2} \times V_1 \] (10)

where \( E_1 \) is the generated energy of hydrogen from dark fermentation (kJ/d); \( \rho_{H_2} \) is the density of hydrogen (0.0899 kg/m³); \( q_{H_2} \) is the calorific value of hydrogen (1.43 × 10⁻⁵ kJ/kg); and \( V_1 \) is the hydrogen yield from dark fermentation (m³/d).

Hydrogen energy from the MEC was calculated as follows:

\[ E_2 = \rho_{H_2} \times q_{H_2} \times V_2 \] (11)

where \( E_2 \) is the generated energy of hydrogen from MEC (kJ), and \( V_2 \) is the hydrogen yield from MEC (m³).

To evaluate the energy recovery from dark fermentation hydrogen production, the energy recovery rate (\( \eta_1 \)) was calculated according as follows:

\[ \eta_1 = \frac{E_1}{E_i + E_f + E_m} \times 100\% \] (12)

The energy recovery rate of the MEC (\( \eta_2 \)) was calculated according to Eqs. (9) and (11):

\[ \eta_2 = \frac{E_2}{E_1} \times 100\% \] (13)

The energy recovery rate of the DF-MEC (\( \eta_3 \)) was calculated according to Eqs. (6)–(11):

\[ \eta_3 = \frac{E_i + E_j}{E_0 + E_f + E_m + E_r} \times 100\% \] (14)

a 30 m × 0.32 mm × 0.50 mm fused-silica capillary column (KB-Wax) after pretreatment with a 0.45 μm membrane filter. Gases (hydrogen, carbon dioxide, and methane) were analyzed using a gas chromatography system (GC7900, Tian Mei, Shanghai) with a thermal conductivity detector and a 2 m high-porosity polymer bead-packed column [34].

Speciﬁc heat of the material (4.18 kJ/kg °C); \( p \) is the density of fermentation liquor (1000 kg/m³); \( Q \) is the flow rate (1.87 × 10⁻⁴ m³/d);
3. Results and discussion

3.1. Reactor performance of the MEC startup under different inoculated sludges

3.1.1. Electrochemical performance of the MEC startup

In the MEC startup period, two typical inoculated sludges (i.e., ADS and AGS) were used in different MECs at a room temperature of 20 ± 2 °C. During the electroactive microorganism enrichment period, a feed-batch addition of 40 mL of medium was used in repetitive 48 h batch cycles. The maximum stable current and hydrogen production was observed by day 30 (Fig. 1a and c) and the startup period was continued for another 336 h (7 batch cycles) to confirm stable performance during the electroactive microorganism growth period (Fig. 1b and d). The peak currents produced by the MECs were higher than the early stage at 0.8 V after changing the new electrodes and inoculated with the enrichment effluent. The maximum currents were 5.63 mA and 4.00 mA in the startup period when inoculated ADS and AGS, respectively. The Coulombic efficiency was 42.11 ± 12% and 39.77 ± 12% over the last 5 feeding cycles when inoculated ADS and AGS, respectively. The sodium acetate was utilized and a COD removal of 40 ± 10% during each feeding cycle was achieved.

3.1.2. Biogas production performance of the MEC startup

Biogas production performances with the inoculation of different sludges in the electroactive microorganism enrichment and microorganism growth periods are shown in Fig. 2. The accumulative biogas and hydrogen proportion inoculated with ADS (Fig. 2a and b) were generally higher than those only inoculated with AGS. In addition, less methane product and a higher hydrogen recovery (Fig. 2c and d) was obtained for reactors inoculated with ADS relative to that of the AGS at 0.8 V applied voltages. In the electroactive microorganism enrichment period, the hydrogen proportion of more than 90.86% and the methane production was negligible using the ADS. However, a higher methane proportion (17.50%) was observed under the AGS inoculation. Additionally, the reactors inoculated with the ADS had a faster startup time and similar accumulative gas production (49 mL) compared with AGS (50 mL). During the electroactive microorganism growth period, the hydrogen proportion increased to 84.90% and the methane proportion decreased significantly to 2.28% using the AGS at the end of reactor startup. Nevertheless, the hydrogen proportion was still higher than 92.58% and methane proportion stayed below 7.42% using the ADS. But even more importantly, the accumulative biogas production by ADS increased to 66 mL, which was higher than AGS (44 mL). The results suggest that the accumulative biogas production and hydrogen proportion increased under ADS and AGS after replacing the new anodes and cathodes in the MECs startup period, respectively.

Therefore, the biogas production rate and hydrogen proportion were 0.83 L/L d and 92.58%, respectively, using ADS, which was higher than AGS (0.55 L/L d and 86.21%). The highest hydrogen recoveries were 50.24% and 49.84% during the electroactive microorganism growth period. These results indicate that the differences in the microbial community structure from the inoculation sludge affected the activities of electroactive microorganisms significantly. Additionally, the dramatically higher current density and Coulombic efficiency is attributed to an increase in the electron-transfer efficiency and the growth of the exoelectrogens in the pure biofilm due to the change of electrodes and the removal of sludge during the electroactive microorganism microorganism growth period. Thus, the ideal hydrogen production performance
and bioelectrochemical characteristics were attributed to the MECs startup in the presence of the inoculated ADS.

3.2. Microbial morphology and the microbial community dynamics

3.2.1. Difference of the microbial morphology on the electrodes

The microbial morphology was observed using SEM. As shown in Fig. 3, microorganisms were successfully attached to the electrode surface to form biofilms for different inoculated sludges. Meanwhile, the dominant microorganisms were affected by the inoculated sludge during the startup period, which resulted in a different shape of microorganisms and different community structures. The SEM images of the biofilms were composed of various forms of microorganisms such as globular, oval, filamentous and rod-like. SEM also confirmed the difference between the anode and cathode biofilms from different inoculated sludges at the end of the startup periods. The SEM images of the electroactive microorganisms occur on the carbon brush anodes made from graphite fibers and titanium wires, which can provide an open structure with a high surface area for microbial colonization. Only a few species of microorganisms in the biofilm dominated by small rod-like bacteria using the ADS were observed on the anodes, whereas short rod-like bacteria were prevalent in the MECs under the AGS. Additionally, the microbial morphology of the cathode biofilms was similar and the diversity was comparable to the different inoculated sludges but showed a richer species of microbes than the anode biofilms. The globular and rod-like bacteria were observed on the carbon cloth of the cathode. These results indicate the difference of the microbial communities under different inoculated sludges and electrodes.

3.2.2. Distinction of the microbial community under different inoculated sludges

To reveal the impacts of the inoculated sludge on microbial communities, the MiSeq Illumina sequencing at the 16S rRNA gene level was employed. The anode and cathode are exposed to the same electrolyte in single-chamber MECs, therefore the microorganisms on the cathode are exposed in the medium with inoculated sludge. Notably, the enrichment of hydrogenotrophic methanogens on cathodes. We examined the archaeal community from anode, cathode and electrolyte at the end of MECs startup. The bacterial community were also examined to determine whether there was any consistent relationship between the development of archaeal and bacterial community. Among these, the electrolyte bacterial DNA and cathode archaeal DNA under the AGS inoculation condition failed to amplify. The effective OTUs sequences were used to certify most of the community diversities. Moreover, the alpha diversity indexes, including the Sobs, Shannon, Simpson, ACE and Chao1 diversity index, are summarized in Table 1. Interestingly, distinct clusters of electrode and electrolyte microbial communities were different as observed using PCA of the bacterial and archaea communities inoculated with ADS and AGS during the MECs startup (Fig. 4). AGS-anode, AGS-cathode and ADS-cathode were closely clustered results, but the ADS-anode was further away regarding the bacterial community compositions, which indicated that there are some differences in bacterial species composition using different inoculated sludges. However, the distinct clusters of electrode and electrolyte were significantly different in the archaeal community compositions. The electrodes were closely clustered results and revealed a higher similarity in the archaeal community structure, whereas the ADS-electrolyte and AGS-electrolyte group were far away from the electrode groups. Compared with the electrode biofilm, the bacterial community diversity was lower in the electrolyte biofilm. Interestingly, the archaea community diversities of the electrolyte were 2.5 times higher than the electrode biofilm, except for the sampled ADS-anode biofilm. Most community diversities and PCA analyses revealed a better performance using the AGS than ADS during the MECs startup.

3.2.3. Difference of the microbial community dynamic

To further investigate the bacterial and archaea community constituent variations using different inoculated sludge, the level of the phylum and genus were analyzed in the anode and cathode biofilm samples (Fig. 5). The 16S rRNA gene-based sequencing showed that five bacterial phyla and one archaeal phylum were dominant in the electrode biofilms and the community structure remained relatively stable using the...
different inoculated sludge. (Fig. 5a and d).

Most sequences of bacterial phyla belonged to Firmicutes (46.25–52.50% of the total 16S rRNA gene sequences) and Proteobacteria (27.88–38.50% of the total 16S rRNA gene sequences). The highest relative abundance of the phyla Firmicutes and Proteobacteria was 86.33% in the ADS-anode followed by 83.07% in the ADS-cathode. Pure culture experiments have shown that many bacterial microorganisms are exoelectrogenic, mainly from three phyla microbial communities including the Firmicutes, Actinobacteria and Proteobacteria phyla. Numerous exoelectrogenic belong to Proteobacteria phyla, typically G. sulfurreducens and S. oneidensis [29,36]. Furthermore, compared with the cathode biofilms, the relative abundance of sequences of the phylum Actinobacteria was much higher using the ADS (6.21%) and AGS (9.51%) in the anode. In contrast, the relative abundance of the phylum Bacteroidetes was higher in the cathode biofilms than in the anode.

The genus level characterization further illustrates the difference and diversity of the functional communities (Fig. 5b). At the genus level, Acetobacterium from the phylum Firmicutes covered the highest relative
due to the favorable thermodynamics compare with most of the anaerobic microorganisms in the anode bacterial communities were a by-product of anaerobic metabolism. Complex inoculum enrichments for acetogens, which are predominantly responsible for making acetic acid as a major product, accounted for 32.63%, 45.51%, 42.97% and 29.54% in the ADS-anode, AGS-anode, ADS-cathode, AGS-cathode, respectively. These are acetogens, which are predominantly responsible for making acetic acid as a by-product of anaerobic metabolism. Complex inoculum enrichments for fermentative microorganisms in the anode bacterial communities were due to the favorable thermodynamics compare with most of the anaerobic respiratory metabolism [37]. Geobacter from phylum Proteobacteria accounted for 5.66% (AGS-anode) and 14.15% (AGS-cathode) using the AGS, which was higher than that of the ADS group (4.30% ADS-anode and 9.36% ADS-cathode) during the MECs startup. Logan et al. [29] reported that the current densities come from mixed cultures that are usually dominated by the genus Geobacter. The highest Geobacter spp. are well-studied exoelectrogens that use acetate and hydrogen as primary electron donors and directly transfer electrons from their membranes or through nanowire-like appendages to an electrode [38–40]. Establishing these exoelectrogens on the anode biofilms is an important determinant of the MEC performance [41]. In addition, the Geobacter with 5–9% relative abundant occurs in the cathode bacterial community. Several literatures of native and purified Geobacter c-type cytochromes have shown that the electron transfer reaction of the outer membrane cytochromes is reversible. The outer membrane cytochromes have to pass the electrons to more electro-positive electron acceptors within the periplasm and inner membrane [42,43]. It suggests Geobacter spp. can accept electrons from a cathode and act a role in the reduction of the cathode.

Interestingly, some special bacterial genera were found to be dramatically higher in different electrodes and with different inoculated sludge. Acinetobacter and Desulfovibrio from the phylum Proteobacteria accounted for 17.39% and 8.56% in the anode and cathode using the ADS, respectively, which were the highest among the other conditions. Desulfovibrio desulfuricans is a sulfate-reducing bacterium that can perform exoelectrogenesis through cytochrome c in anode biofilm [44, 45]. Moreover, Different types of cytochromes in the outer membrane of Desulfovibrio sp. as redox partners of hydrogenase was confirmed. Hydrogen detected by subatomic resolution protein crystallography in a [NiFe] hydrogenase from Desulfovibrio vulgaris [46]. Rosenbaum [43] suggested that majority of metabolic processes catalyzed by biocathodes typically involve microorganisms capable of metabolizing hydrogen. Similarly, these studies suggest a role of Desulfovibrio sp. in electronic interaction on electrodes. A totally different and special bacterial genera, Plesiomonadaceae from the phylum Proteobacteria and Fusibacter from phylum Firmicutes, accounted for 10.33% and 9.41% in the anode and cathode using the AGS, respectively, and were the highest among the other conditions.

The sequences of the archaeal phylum mainly consisted of Eurarchaeota (98.82–100%) and the relative abundance on the anode was greater than on the cathode. The archaeal genus Methanobrevibacter from the phylum Eurarchaeota was the most abundant at 96.02%, 94.95% and 77.84% in the ADS-anode, AGS-anode and ADS-cathode, respectively (Fig. 5e). As a typical hydrogenotrophic methanogen, the relative abundance of 97% in the cathodic archaea biofilm of the single-chamber MEC has reported (Rago et al., 2015). Methanobrevibacter spp. predominate on the platinum-catalyzed cathodes and produce methane when hydrogen gas readily arises by the catalysts of the cathodes [29]. Methanobacterium and Methanocorpusculum from the phylum Euryarchaeota mainly exist in the cathode biofilms using ADS and showed higher relative abundances (compared to the AGS group) of 12.92% and 8.78%.

Fig. 4. Principal Component Analysis of the bacterial community (a) and archaea community (b) from electrode biofilms and electrolyte inoculated ADS and AGS during the MECs startup.

Table 1
The results of the richness estimators (Sobs, Shannon, Simpson, ACE and Chao 1 index) and coverage of each sample.

| Communities       | Sample      | Sobs index | Shannon index | Simpson index | ACE index | Chao 1 index | Coverage |
|-------------------|-------------|------------|---------------|---------------|-----------|--------------|----------|
| **Bacterial**     |             |            |               |               |           |              |          |
| AGS-anode         | 292         | 2.98       | 0.14          | 366.68        | 397.12    | 0.9981       |          |
| AGS-anode         | 242         | 2.56       | 0.23          | 306.89        | 317.64    | 0.9984       |          |
| ADS-cathode       | 302         | 2.96       | 0.18          | 363.52        | 355.05    | 0.9988       |          |
| AGS-cathode       | 264         | 3.20       | 0.11          | 321.53        | 308.68    | 0.9986       |          |
| ADS-electrolyte   | 203         | 2.26       | 0.23          | 266.03        | 303.65    | 0.9982       |          |
| **Archaear**      |             |            |               |               |           |              |          |
| ADS-anode         | 44          | 0.26       | 0.92          | 50.69         | 49.14     | 0.9998       |          |
| AGS-anode         | 14          | 0.22       | 0.90          | 67.88         | 19.00     | 0.9999       |          |
| ADS-cathode       | 15          | 0.70       | 0.63          | 45.48         | 18.75     | 0.9999       |          |
| ADS-electrolyte   | 40          | 0.54       | 0.77          | 52.01         | 49.43     | 0.9997       |          |
| AGS-electrolyte   | 40          | 0.82       | 0.49          | 43.43         | 41.43     | 0.9999       |          |
Siegert et al. [47] revealed that Methanobacterium spp. predominate on cathodes that poorly catalyze hydrogen production for a mixed culture and directly convert the current into methane. Additionally, Methanobacterium spp. achieved much higher methane production rates compared to those that were possible using hydrogen generated by abiotic cathodes. Though the behavior of anode biofilms has been well understood, the function of individual species in mixed microbial communities and microbial electron uptake is unclear on the cathode. Thus, further research is needed on electron uptake species and identification of these key roles [48].

The difference analysis of bacterial and archaeal genera in the anode biofilms is shown in Fig. 5c and f. The bacterial genera Acetobacterium, Acinetobacter and Pleomorphomonadaceae exhibit a significant change and Geobacter revealed relative stability using different inoculated sludges. Methanocorpusculum revealed a dramatic difference related to the archaeal genera in the anode biofilms using the ADS and AGS. Interestingly, the dominant genera Geobacter and Methanobrevibacter were similar with different inoculated sludges, which showed no relationship between microbial communities and the electrochemical performance on the anode biofilms during MECs startup. The ecology of electroactive microorganisms in the electrode biofilms shows an impressive diversity and is crucial for the MECs startup.

**3.3. Electro-fermentation hydrogen production from dark fermentation effluent**

**3.3.1. Reactor performance of electro-fermentation hydrogen production**

After the MECs startup, electro-fermentation hydrogen production and substrate cascade utilization were conducted using the fermentation effluent from the long-term dark fermentation hydrogen production (Fig. 6). The current was sharply increased to 1.6 mA after 40 mL of fermentation effluent was fed into the MEC, decreased to 0.7 mA at 20 h. Compared to sodium acetate as a single substrate, a lag time of 10 h appeared during electro-fermentation hydrogen production due to the complexity and refractory nature of the fermentation effluent. As the MEC adapted to the impact of effluent, the anode biofilm utilized the organic acids, and the falling current increased again to 1.2 mA at 40 h with the reactor operating. Colloidal particulates and complex organics in the real fermented wastewater resulted in high internal resistance and low output power (Nam et al., 2010). So, the current of MEC using fermentation effluent was much lower than control group. Besides, the Coulombic efficiency was 46.75% and decreased slightly compared to the reactor-fed sodium acetate (52.64%). The biogas production performance using dark fermentation effluent during the electro-fermentation hydrogen production is shown in Fig. 6c. The accumulative biogas production (28 mL) and hydrogen proportion (87.94%) reached a peak at 48
h and the methane proportion (0.24%) was negligible during the electro-fermentation system. The hydrogen yield and rate reached 280.0 mL/L d and 0.19 m³ H₂/m³ d, respectively. Particularly, the hydrogen recovery of 27.84% was obtained from dark fermentation effluent, which was lower than when the sodium acetate was seeded as the sole carbon source in electro-fermentation hydrogen production. The complex substrate from the dark fermentation effluent was presumably consumed by electrochemically inactive fermentative bacteria that also colonized on the anode surface and reduced the Coulombic efficiency in MECs, which resulted in slower kinetics for electron transfer and less potent hydrogen recovery than acetate as the substrate [37]. As Fig. 6(b) shown, the current rapidly decreased significantly after rising, and finally slowly increased and maintained around 1.1 mA after substrates were replaced.

3.3.2. Metabolic pathways of dark fermentation coupled with electro-fermentation hydrogen production

According to the metabolite and function prediction of the microbial community, the metabolic pathways of the sequential system combined dark fermentation with electro-fermentation hydrogen production is shown in Fig. 7 and Table 2. Dark fermentation is a biochemical process in which substrates, enzymes, and microorganisms interact with and inhibit each other. Furthermore, food wastes are complex and their biodegradation requires cooperation among different trophic groups (fermenters, homoacetogens and exoelectrogens), and also experiences competition with methanogens [25]. Based on the hydrolysis, carbohydrates in food waste were hydrolyzed into sugars, proteins into amino acids, and lipids into long chain fatty acids, which were then transformed to pyruvic acid through the glycolytic pathway. Pyruvic acid can be transferred through acetyl-CoA and it is an important hub in the metabolism of the three nutrient substances. The classic theory of hydrogen production using microorganisms is the hydrogen production process that uses pyruvic acid as either direct or indirect electron donors. This theory includes hydrogen production through decarboxylation of pyruvic acid and formic acid decomposition as well as the hydrogen production theory of NADH/NAD⁺ equilibrium regulation. Our previous study investigated the relationships among microbial community phylogeny and function in dark fermentation using food waste and analyzed by a metaproteomic method [49]. Additionally, many VFAs, such as acetic acid and butyric acid, were found in the dark fermentation effluent and were further decomposed into hydrogen and carbon dioxide by electro-active microorganisms during the electro-fermentation process (Fig. 7). Thus, the composition and concentration of VFAs were useful indicators for predicting the metabolic pathways of dark fermentation and electro-fermentation hydrogen production processes. In this study, the dominant VFAs are acetic acid and butyric acid from dark fermentation effluent. After electro-fermentation, the acetic acid concentration decreased from 1881.4 mg/L to 557.0 mg/L, and the utilization rate was 70.4% from dark fermentation effluent. Meanwhile, the butyric acid concentration decreased from 117.47 mg/L to 93.70 mg/L, and propionic acid appeared at a lower concentration of 42.18 mg/L. Using sodium acetate as a substrate permits much greater acetic acid (87.91%) utilization compared to dark fermentation effluent during electro-fermentation. These results indicate that acetic acid utilization dramatically increases as the main substrate for hydrogen production by electro-fermentation due to the dominant bacterial genus *Acetobacterium* from the phylum *Firmicutes*, which has the highest relative abundance and interaction with electroactive microorganisms on the electrode. Finally, methane production from archaeal microorganisms is able to reduce methyl in carbon dioxide and one-carbon compounds to methane under the common effects of many enzymes and coenzymes in methane metabolism. Methane metabolism may occur on the cathode biofilms through a pathway using the participation of the genera
Table 2: Energy evaluation of dark fermentation coupled with electro-fermentation from food waste and dark fermentation effluent for hydrogen production.

| Energy consumption (kJ) | Energy generation (kJ) | Energy recovery rate (%) |
|------------------------|------------------------|--------------------------|
| E₀                    | E₁                    | E₂          | E₃      | E₄      | E₅      | E₆      | η₁      | η₂      | η₃      |
| 11.72                 | 3.76                  | 0.45        | 2.758   | 8.34    | 1.48    | 53.83   | 53.67   | 53.81   |

E₀: energy consumption for feed heating. E₁: energy consumption for heat preservation. E₂: input electricity for biomass pumping and reactor mixing. E₃: energy input for MEC. E₄: energy generation from dark fermentation hydrogen production. E₅: energy generation from electro-fermentation hydrogen production. η₁: energy recovery rate from dark fermentation. η₂: energy recovery rate from electro-fermentation. η₃: energy recovery rate from the sequential system.

3.3.3. Energy evaluation of dark fermentation coupled with electro-fermentation hydrogen production

The energy evaluation of the sequential system involving dark fermentation coupled with electro-fermentation hydrogen production was based on the experimental results. Table 2 shows the energy consumption, generation, and recovery rate in the dark fermentation, electro-fermentation, and sequential systems, respectively. The energy consumption is mainly concentrated in the electricity demand for feed heating (E₀), heat preservation (E₁), biomass pumping and reactor mixing (E₂). Among these, the highest energy consumption of the dark fermentation was 11.72 kJ from feed heating. Energy generation is calculated according to the hydrogen production from dark fermentation (E₄) and electro-fermentation (E₅) systems. In the sequential system, food waste was converted to hydrogen and VFAs, and the effluent was then followed by a secondary electro-fermentation process for further hydrogen energy conversion efficiency. These results show that the energy recovery rate of the sequential system (η₃) was 53.81%, which is similar to the single process of dark fermentation (η₁, 53.83%) and electro-fermentation (η₂, 53.67%) for hydrogen production from food waste and dark fermentation effluent. Compared to the single dark fermentation hydrogen production system, electro-fermentation hydrogen production from the dark fermentation effluent can be utilized at 44.34% sCOD, which is a benefit for wastewater treatment.

4. Conclusions

In summary, electro-fermentation hydrogen production could significantly improve substrate conversion efficiency and energy benefits using effluent from a dark fermentation system. During the MECs startup, two typical inoculated sludges (ADS and AGS) were used. The best operating conditions for hydrogen production and bioelectrochemical characteristics were observed using ADS. The ecology of electroactive microorganisms in the electrode biofilms shows impressive diversity and has complex symbiotic interactions based on electron exchange. The metabolic pathways of electro-fermentation revealed a typically arctic acid degradation as electron donors occurred by exoelectrogens according to the change of metabolites and the ecological niche of electroactive microorganisms. This study provides an important technical foundation as an approach to the theoretical maximum hydrogen production capacity by sequential electro-fermentation hydrogen production from dark fermentation effluent.

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