Performance Evaluation of a Continuous-flow Bioanode Microbial Electrolysis Cell Fed with Furanic and Phenolic Compounds

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Abstract

Furanic and phenolic compounds, formed during the pretreatment of lignocellulosic biomass, are problematic byproducts in down-stream biofuel processes. Microbial electrolysis cell (MEC) is an alternative technology to handle furanic and phenolic compounds and produce renewable hydrogen (H_2). The present study evaluated the performance of a continuous-flow bioanode MEC fed with furanic and phenolic compounds at different operating conditions. All hydraulic retention times (HRTs) tested (6 - 24 h) resulted in complete transformation of the parent compounds at an organic loading rate (OLR) of 0.2 g/L-d and applied voltage of 0.6 V. Increasing the OLR to 0.8 g/L-d at HRT of 6 h resulted in an increased H_2 production rate from 0.07 to 0.14 L/L_{anode}-d, but an OLR of 3.2 g/L-d did not lead to a higher H_2 production rate. Significant methane production was observed at an OLR of 3.2 g/L-d. The lack of increased H_2 production at the highest OLR tested was due to a limited rate of exoelectrogenesis but not fermentation, evidenced by the accumulation of high acetate levels and higher growth of fermenters and methanogens over exoelectrogens. Increasing applied voltage from 0.6 to 1.0 V at an OLR of 3.2 g/L-d and HRT of 6 h enhanced exoelectrogenesis and resulted in a 1.7-fold increase of H_2 production. Under all operating conditions, more than 90% of the biomass was biofilm-associated. The present study provides new insights into the performance of continuous-flow bioelectrochemical systems fed with complex waste streams resulting from the pretreatment of lignocellulosic biomass.

1. Introduction

Furanic and phenolic compounds, formed during the pretreatment of lignocellulosic biomass, are significant components of hydrolysates, pyrolysates and wastewaters, posing serious challenges in down-stream biofuel processes. Furanic and phenolic compounds are known inhibitors of ethanol and H_2 producing microorganisms used in dark fermentation, negatively impacting the efficiency of biofuel production from lignocellulosic hydrolysates.[1] Another problem with furanic and phenolic compounds is that their polar and in some cases acidic nature makes pyrolysates corrosive and unstable.[2] As lignocellulosic biomass has been recognized as a promising feedstock for biofuel production, alternative technologies are needed to handle furanic and phenolic compounds bearing waste streams generated during biomass pretreatment.[2,3]

Bioelectrochemical systems have been assessed for the bioconversion of furanic and phenolic compounds. Microbial fuel cells (MFCs) are reported to produce 8 A/m^2 current density from these compounds,[4] and to treat hydrolysates and fermentation waste streams generated during the conversion of corn-stover biomass to ethanol.[5] Microbial electrolysis cell (MEC), another bioelectrochemical system, was recently investigated for the conversion of furanic and phenolic compounds to H_2.[6-7] The MEC technology not only offers an alternative in dealing with waste streams bearing furanic and phenolic compounds, but also produces renewable H_2, large quantities of which are needed for the hydrogenation process in bio-oil production via pyrolysis. Currently, H_2 is produced by reforming natural gas (i.e., methane), a non-renewable H_2 source.[2] The above-mentioned advantage of MEC cannot be achieved by other technologies, such as solvent extraction or use of genetically engineered, tolerant microorganisms. Zeng *et al.*[6] reported that a mixture of two furanic and three phenolic compounds was completely transformed as the sole carbon and energy source in a batch-fed MEC bioanode, at a Coulombic

efficiency of 44 – 69%, demonstrating great potential for H_2 production even from these problematic compounds. In addition, Lewis et al.[7] used a complex pyrolysate stream, which contained furanic and phenolic compounds, in a continuously-fed, batch MEC, and achieved a H_2 production rate as high as 4.3 L/L-d. These proof-of-concept studies based on batch systems demonstrate that MEC technology can be an attractive integrative solution for improving the sustainability of biofuel production. However, assessment of continuous-flow MEC operating conditions, a crucial step to promote the implementation of MEC technology in the overall biofuel production process, is currently lacking.

Several studies have investigated continuous-flow MECs with domestic and synthetic wastewater, as well as digestate.[8-10] However, our fundamental understanding of the effect of continuous-flow operating conditions on MEC performance is still limited, in part due to the complexity of the waste streams used. As stated above, furanic and phenolic compounds are challenging and problematic components of lignocellulose-derived waste streams. In-depth evaluation of the effect of continuous-flow bioanode MEC operating conditions on the conversion of these compounds will enhance our understanding of the performance of bioelectrochemical systems fed with complex waste streams, resulting from the pretreatment of lignocellulosic biomass. The objective of the present study was to evaluate the response of a continuous-flow MEC bioanode, fed with a mixture of two furanic and three phenolic compounds, to various hydraulic retention times (HRT), organic loading rates (OLR), and applied voltage.

2. Materials and Methods

2.1 Chemicals

Furfural, 5-hydroxymethylfurfural, syringic acid, vanillic acid, and 4-hydroxybenzoic acid, purchased from Sigma-Aldrich (St. Louis, MO) and Alfa Aesar (Ward Hill, MA), were used as

the anode substrates. The properties of these chemicals were previously summarized in Zeng *et al.*.[6]

2.2 MEC

An H-type MEC system was developed with two identical glass chambers (250 mL empty bed volume each) separated by a cation exchange membrane (Nafion 117, 5.7 cm^2; Dupont, Wilmington, DE). Each chamber was a modified square glass bottle with three side-ports for gas sampling, influent addition, and effluent removal. A glass buret was connected to the headspace of each chamber for gas collection and volume measurement by the displacement of an acid brine solution (10% NaCl w/v, 2% H_2SO_4 v/v) (Fig. 1). The anode electrode was carbon-felt (5 stripes, 0.5 inch × 0.5 inch × 3 inch each) and the cathode was a platinum-coated carbon cloth (5 cm × 6 cm, 0.5 mg Pt/cm^2; Fuel Cell Etc). The MEC anode was inoculated with electrode-attached biofilm from a MFC as previously described,[6] enriched with a mixture of the above-described five compounds at an applied voltage of 0.6 V (anode relative to cathode), and maintained batch-fed once a week for over a year. Both the anode and cathode contents were continuously mixed magnetically.

2.3 MEC bioanode continuous-flow operation

Two influent pumps and one effluent pump were connected to the anode inlet and outlet ports, respectively (Fig. 1). Two positive displacement pumps (Fluid Metering Inc., Syosset, NY) were used for anolyte influent and effluent. A syringe pump (Cole Parmer, Hills, IL) was used for substrate addition. All three pumps ran for 10 min every 3 h controlled by an electronic timer (ChronTrol Corporation; San Diego, CA); the influent anolyte and substrate pumps were turned ON after the effluent pump was turned OFF. The anolyte was microbial growth medium and the catholyte was phosphate buffer, both at pH 7.0, with composition as previously described.[6] The

substrate stock solution was a mixture of the five compounds at equal electron equivalents (each at 3.12 g COD/L) and a total concentration of 10 g/L (15.6 g COD/L) dissolved in the anolyte solution. The effect of HRT, OLR and applied voltage on MEC performance was evaluated in three consecutive operational phases. During each phase, one of the parameters was varied while the values of the other two parameters were fixed. First, the HRT was reduced step-wise from 24 to 12 and then to 6 h at a constant OLR of 0.2 g/L-d (Phase I), followed by the increase of OLR from 0.2 to 0.8 and then to 3.2 g/L-d at a constant HRT of 6 h (Phase II), by changing the influent substrate concentration accordingly. The applied voltage was then increased from 0.6 to 1.0 V (anode relative to cathode) at an HRT of 6 h and OLR of 3.2 g/L-d (Phase III). Each change of MEC condition was made after stable operation was reached and maintained for at least 4 days. While the cathode was not operated as a continuous-flow, the catholyte was replaced every 2-4 days when the pH increased to 7.5.

In the present study the OLR values are expressed based on the total mass of the five compounds mixture used as the bioanode substrate; a factor of 1.56 can be used to convert g/L-d to g COD/L-d based on the theoretical oxygen demand of the compounds.[6] Thus, the applied OLR values are equivalent to 0.3, 1.2 and 5.0 g COD/L-d. Lewis *et al.* tested a switchgrass pyrolysate in a MEC anode at an OLR up to 10 g COD/L-d, in which approximately 27% was contributed by furanic and phenolic compounds.[7] Thus, the maximum OLR used in the present study (5 g COD/L-d) is well above the equivalent OLR of furanic and phenolic compounds in the pyrolysate used by Lewis *et al.*.[7]

2.4 Experimental controls

In order to assess potential H_2 production and compound transformation through abiotic reactions, an abiotic batch assay was conducted with an uninoculated anode at 0.6 and then 1.0

V. Assessment at each condition lasted for 7 days with close monitoring of the current, H_2 production and the concentration of the five compounds. Additionally, an open circuit control batch assay was conducted with an active MEC bioanode for 7 days, in order to assess the fermentative transformation of the five compounds mixture in the bioanode. The initial concentration of the five compounds mixture used in the control assays was 0.8 g/L, which was the highest influent concentration of the five compounds mixture used in the continuous-flow bioanode MEC.

2.5 Microbial community analysis

Analysis of the MEC anode microbial community was performed after stable operation was achieved with OLR of 0.2 (day 35) and 3.2 g/L-d (day 58), both at an HRT of 6 h, representing the microbial communities at low and high OLR conditions. The genomic DNA extraction from the biofilm, followed by DNA purity check and quantification, was conducted as previously described.[6] Each extracted DNA sample was sequenced in duplicate for 16S rRNA gene using Illumina MiSeq (Research and Testing Laboratory; Lubbock, TX). Primer sets 28F/388R (5'-GAGTTTGATCNTGGCTCAG -3'/5'-TGCTGCCTCCCGTAGGAGT-3') and 519wF/909R (5'-CAGCMGCCGCGGTAA -3'/5'-TTTCAGYCTTGCGRCCGTAC-3') were used for partial 16S rRNA gene of Bacteria and Archaea, respectively. The obtained sequences were clustered into Operational Taxonomic Units (OTUs) at 4% divergence using the UPARSE. The centroid sequence of each I was used for taxonomic classification using the USEARCH global alignment program. The sequences of the abundant species (>1 %) have been deposited to GenBank, National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/) with sequence accession numbers from KT799852 to KT799875.

2.6 Analytical methods

The furanic and phenolic compounds and their metabolites were quantified after sample filtration through 0.2 μm polycarbonate membrane, using a high performance liquid chromatography (HPLC) unit equipped with a HPX-87H column, as previously described.[6] Soluble chemical oxygen demand (sCOD) and pH were measured following procedures outlined in Standard Methods.[11] Total gas production was measured by acid brine solution displacement in glass burets, after equilibration to 1 atm. All gas data reported here are at 20°C and 1 atm. Headspace gas composition (H_2, CO_2, and CH_4) was determined with a gas chromatography unit equipped with two columns and two thermal conductivity detectors.[12]

The protein concentration of the MEC anode biofilm and planktonic biomass was measured and used for biomass quantification. For each biofilm sample, two pieces of 0.5 × 0.5 × 0.5 inch anode electrode were removed from the MEC. Protein was extracted by bead beating followed by 30 min heating at 100 °C in 0.1 N NaOH. To quantify the planktonic protein, 60 mL of bioanode liquid was centrifuged at 10,000 rpm for 15 min. The pellet was then washed three times with clean anolyte followed by centrifugation. The pellet was re-suspended in 0.1 N NaOH, followed by 30 min heating at 100 °C. The extracted protein was quantified using the Pierce™ BCA protein assay kit (Thermo Scientific, Waltham, MA), according to the manufacturer's instructions.

2.7 Calculations

Coulombic efficiency was calculated based on the number of electrons recovered as electrical current per electron equivalent of COD removed. Electrical or overall energy efficiency was calculated as the ratio of energy recovered as H_2 to the electrical energy input, or to the sum of electrical and substrate energy input, respectively. Biomass yield coefficient was quantified by

177 considering both planktonic and biofilm biomass accumulation during operation at OLR of 0.2
178 and 3.2 g/L-d. Details on the calculations are described in Text S1.

179 **3. Results and Discussion**

180 MEC operation consisted of three consecutive phases (total duration 66 days) for the evaluation
181 of the effect of HRT, OLR and applied voltage: Phase I (40 days), varying HRT values from 24
182 to 6 h at constant OLR of 0.2 g/L-d and voltage of 0.6 V; Phase II (18 days), varying OLR values
183 from 0.2 to 3.2 g/L-d at constant HRT of 6 h and voltage of 0.6 V; Phase III (8 days), varying
184 applied voltage at constant HRT of 6 h and OLR of 3.2 g/L-d (Fig. 2A, B). The MEC
185 performance in response to each operating condition is discussed below.

186 *3.1 Phase I – Effect of HRT on MEC performance*

187 HRT is an important design parameter for a continuous-flow process. For a MEC, a short HRT
188 allows a high substrate throughput and H_2 production rate for a given influent substrate
189 concentration, but may result in a low extent of substrate biotransformation and COD removal.
190 In the present study, the effect of HRT was assessed at a constant OLR of 0.2 g/L-d and an
191 applied voltage of 0.6 V.

192 Upon each change of HRT from 24 to 12, and then to 6 h, current reached a relatively
193 stable value within 2 days, followed by a stable effluent sCOD in 7 days (Fig. 2). The operation
194 continued for another 4-8 days after a stable effluent sCOD was reached, during which the MEC
195 performance was evaluated (Fig. 3). At a constant OLR of 0.2 g/L-d, the current increased from
196 2.0 to 2.4 mA when the HRT was reduced from 24 to 12 h, and then decreased to 1.9 mA when
197 the HRT was further reduced to 6 h (Fig. 3A; Phase I). Consistent with the measured current, the
198 H_2 production rate at an HRT of 12 h was slightly higher than at an HRT of 24 h or 6 h (Fig. 3B;
199 Phase I), but the difference was not statistically significant ($P = 0.208$). The bioanode and

effluent acetate concentration remained below 35 mg/L at all HRT values (Fig. 3C; Phase I). In the open circuit control assay, where only fermentation took place, acetate was produced reaching 502 mg/L at an initial substrate concentration of 800 mg/L of the mixture of the five compounds (Fig. S1). Therefore, acetate was a major fermentation product, which has also been shown in other bioelectrochemical studies which used various fermentable substrates.[6,13] Acetate, on the other hand, is a key substrate for exoelectrogenesis.[6,13] Therefore, the observed low acetate concentration during the continuous-flow MEC operation in the present study implies comparable rates of acetate production (i.e., fermentation) and consumption (i.e., exoelectrogenesis) at an HRT of 6 - 24 h and an OLR of 0.2 g/L-d. The five furanic and phenolic compounds were completely transformed at all HRT values tested during Phase I (Table 1). The sCOD removal ranged from 28 to 41%, and was the highest at an HRT of 12 h (Table 1), which is consistent with the higher current measured at an HRT of 12 h than at HRTs of 24 and 6 h. The Coulombic efficiency, which ranged from 60 to 76%, was not statistically significantly different at the three HRT values tested at an OLR of 0.2 g/L-d ($P = 0.088$) (Table 1).

Overall, complete transformation of the five parent compounds at an OLR of 0.2 g/L-d was observed at all HRT values tested. The MEC performance at the three HRTs and an OLR of 0.2 g/L-d varied by no more than 25% in terms of H_2 production rate, sCOD removal, and Coulombic efficiency (0.11 ± 0.01 L/L-d, 34 ± 7%, and 73 ± 6%, respectively; mean ± standard deviation); a HRT of 12 h resulted in a slightly higher current and sCOD removal. Due to the fact that the influent concentration (C_{in}), HRT, and OLR are inter-related (i.e., OLR = C_{in}/HRT), a decrease of HRT at a constant OLR was achieved by decreasing the influent concentration. Thus, the influent concentration, in addition to the HRT, can have an effect on the overall MEC performance. On the other hand, the relatively small variation of the MEC performance in

response to the change of HRT is attributed to the fact that more than 90% of the anode biomass was in the biofilm (Fig. 4). Thus, the impact of HRT on the overall anode biomass retention time was expected to be less significant than that in a fully suspended-growth biomass bioreactor. The shortest HRT tested (6 h) is considered as an appropriate operating condition because of the following: (1) a short HRT allows a relatively high substrate throughput and a small anode volume; (2) a nearly complete transformation of the furanic and phenolic compounds was achieved (even at higher OLR values; Table 1, Phase II); and (3) the overall efficiency of H_2 production was not considerably lower than that at longer HRT values. Compared to previously reported HRT values used for bioelectrochemical systems, which ranged from hours to days,[8-10] even the relatively short HRT of 6 h used in the present study was sufficient for the transformation of the furanic and phenolic compounds.

3.2 Phase II – Effect of OLR on MEC performance

When the OLR was increased from 0.2 to 0.8 g/L-d, both the current and H_2 production rate increased significantly from 1.9 to 2.7 mA and from 0.10 to 0.14 L/L-d ($P < 0.05$), respectively. However, the current and H_2 production rate did not increase any further when the OLR was increased from 0.8 to 3.2 g/L-d (Fig. 3; Phase II). To understand which sub-process (i.e., fermentation or exoelectrogenesis) limited the H_2 production rate at an OLR of 3.2 g/L-d, the acetate concentration was examined. As discussed in Section 3.1 above, the measured acetate concentration was the result of the relative rate of production by fermentation and consumption by exoelectrogenesis. At an OLR of 3.2 g/L-d, the effluent acetate level was 332 mg/L, more than 6-fold higher than that at lower OLR values ($P < 0.05$) (Fig. 3C; Phase II). The high acetate concentration in the effluent shows that the rate of acetate consumption by exoelectrogenesis was not able to keep up with the rate of acetate production by fermentation. Considering the liquid

phase of the MEC anode as a continuous-flow, stirred tank reactor (CSTR) at steady-state, the following equation applies relative to acetate:

$$0 = r_f - r_e - \frac{C}{HRT} \quad (1)$$

where r_f is the acetate production rate by fermentation, r_e is the acetate consumption rate by exoelectrogenesis, and C is the measured acetate concentration in the bioanode and the effluent during stable operation. The acetate consumption rate (r_e) at an OLR of 3.2 g/L-d was back calculated as equal to 1.4 mM/d based on the measured H_2 production rate and the following stoichiometric equation:

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (2)$$

Based on eq 1, the acetate production rate (r_f) at an OLR of 3.2 g/L-d was back calculated as 23.5 mM/d. The fact that r_f (23.5 mM/d) is 17-fold greater than r_e (1.4 mM/d) demonstrates that the rate of exoelectrogenesis was considerably slower than that of fermentation. Moreover, compared with the r_f at OLR of 0.8 g/L-d (4.7 mM/d), the acetate production rate was 5-fold higher at an OLR increased by 4-fold, while the acetate consumption rate (r_e) remained the same. Thus, increasing the OLR improved the rate of acetate production by fermentation, but not the rate of acetate consumption by exoelectrogenesis. Therefore, it is concluded that the H_2 production at an OLR of 3.2 g/L-d was limited by the rate of acetate-supported exoelectrogenesis, as opposed to the rate of fermentation resulting in acetate production.

Possible reasons for the limited rate of exoelectrogenesis at an OLR of 3.2 g/L-d are discussed below. The measured acetate concentration at an OLR of 3.2 g/L-d (332 mg/L) was substantially higher than the previously reported value (111 mg/L) of the half saturation constant (K_s) for acetate utilization in mixed bioanode communities.[14] Thus, substrate concentration could

not be considered as a rate-limiting factor of exoelectrogenesis at the highest OLR of 3.2 g/L-d used in the present study. Although furanic and phenolic compounds can be inhibitory to microbial activity, the influent concentration of 0.8 g/L (at an OLR of 3.2 g/L-d and HRT of 6 h) was not inhibitory as shown in our previous study.[6] Thus, inhibition of exoelectrogenesis was not the cause of the lack of increased H_2 production at an OLR of 3.2 g/L-d. However, a combination of electrochemical constraints can be a plausible cause of the observed limited exoelectrogenesis at high OLR. The H-type reactor used in the present study is a proof-of-concept design, has a relatively large internal resistance due to the distance between the anode and cathode electrodes, as well as the use and/or the type of the ion exchange membrane.[15] In addition, Harrington *et al.*[16] reported that a carbon-felt anode, which is the type of electrode used in the present study, can suffer from ion transport limitation, although its porous structure provides a large surface area for biofilm attachment and development. For electrons to be transferred from the exoelectrogens to the anode and then to the cathode, an equal number of charges (i.e., ions) must be transported out of the anode biofilm to the anolyte and then to the catholyte to achieve electroneutrality. Thus, ineffective ion transport can limit the current and thus cathodic H_2 production. Another electrochemical constraint can be the relatively small specific surface area of the cathode used in the present study (12 m^2/m^3) compared with the typical range of 10-100 m^2/m^3 for a 250 mL-reactor according to a recent review.[17] Other reasons for the lack of increased H_2 production, from the perspective of microbial interactions, are discussed in Section 3.4, below.

Similar to the limited increase of H_2 production observed in the present study at an OLR of 5.0 g COD/L-d, Escapa *et al.*[8] reported a Monod-type saturation of H_2 production above an OLR of 2.0 g COD/L-d in a membrane-less MEC fed with domestic wastewater. However, the

cause of the observed plateaued H_2 production may be different from that in the present study. Escapa *et al.*[8] observed a significant loss of cathodic H_2 to methanogenesis and anodic re-oxidation. In contrast, in the present study, the cathode efficiency was as high as 99%, and thus cathodic H_2 loss was minimal. Besides, as discussed above, the H_2 production at high OLR used in the present study was limited by exoelectrogenesis as opposed to fermentation; the limiting sub-process in the study conducted by *Escapa et al.* was not clear.

The sCOD removal and Coulombic efficiency decreased with the increase of OLR (Table 1). At an OLR of 3.2 g/L-d, aromatic metabolites were detected in the effluent, such as catechol (0.12 g/L) and phenol (0.11 g/L), which indicates a relatively low extent of biodegradation of the phenolic compounds. Nevertheless, more than 98% of the parent compounds were transformed at the two OLR values tested in Phase II (Table 1). Therefore, increasing the OLR enhanced the H_2 production rate to a certain extent, but with the trade-off of lower effluent quality, i.e., higher effluent sCOD concentration.

Compared to our previously reported, batch-fed MEC bioanode study, which used the same substrate (i.e., mixture of the five compounds) at an initial/influent substrate concentration range (0.2 – 0.8 g/L), the H_2 production rate during the continuous-flow MEC bioanode operation was 1.4 to 2-fold higher than the maximum rate obtained during batch operation.[6] The H_2 loss in the batch MEC, possibly caused by H_2 diffusion from the cathode to the anode,[6] was not observed during the continuous-flow operation in the present study (i.e., cathode efficiency > 99%). However, the extent of sCOD removal was lower in the continuous-flow MEC (13 - 28% vs. 49 - 61%) due to the shorter residence time (6 h) compared to the batch incubation duration (7 d).

3.3 Phase II – Effect of OLR on COD balance

A COD balance was performed based on the rate of sCOD removal, H_2, biomass and methane production at the various OLRs. The sCOD removal rate (g/L-d) increased linearly with increasing applied OLR (Fig. 5A). However, the fraction of the COD removed as H_2 decreased (Fig. 5B). Thus, the lack of increased H_2 production at high OLR was not due to the MEC's capacity to utilize COD, but rather to a limited contribution of the COD removed towards exoelectrogenesis.

The anode biomass, quantified by protein measurement, increased significantly as the OLR increased from 0.2 to 3.2 g/L-d, but the biofilm-associated biomass fraction of the total anode biomass did not change (93 and 91 % at OLR of 0.2 and 3.2 g/L-d, respectively; Fig. 4). The biomass observed yield coefficient (Y_{obs}) was estimated as 0.23 g biomass-COD/g COD removed (or 0.16 g VSS/g COD removed) at both OLR of 0.2 and 3.2 g/L-d (Text S1). Thus, the fraction of electron equivalents used for biomass synthesis (23%) was constant at the increased OLR. The Y_{obs} value estimated in the present study is consistent with the theoretical yield coefficient of exoelectrogens calculated based on thermodynamics (0.1 – 0.3 g VSS/g COD) by Wilson and Kim,[18] as well as the biomass yield estimated in a glucose-fed bioanode (up to 0.54 g biomass-C/g substrate-C, equivalent to 0.38 g VSS/g COD) by Freguia *et al.*.[19]

It is noteworthy that methane was detected in the bioanode only at an OLR of 3.2 g/L-d with a production rate of 0.09 L/L-d, accounting for 19% of the measured sCOD removed (Fig. 5B). The electron flow diverted to methane is equivalent to 0.36 L/L-d of H_2 production, which is a significant loss compared to the observed H_2 production rate of 0.13 L/L-d. If methane production had not occurred, the H_2 production rate at OLR of 3.2 g/L-d would have been 0.49 L/L-d, representing a 3.5-fold increase from that observed at an OLR of 0.8 g/L-d. The forgoing

discussion assumes that the observed methane production was due to hydrogenotrophic methanogenesis. As discussed in Section 3.4 below, acetoclastic methanogens were not detected.

After accounting for H_2, CH_4, and biomass production, 5 – 45% of the COD removed remained unaccounted for (Fig. 5B). Bacterial carbon storage as polymers represented 34-65% of the total bioanode biomass has previously been reported.[19] Because in the present study the biomass was quantified by protein measurement, carbon storage in polymeric substances was not accounted for. Therefore, increasing the OLR to 3.2 g/L-d affected the distribution of electron equivalents, resulting in a higher fraction used for methanogenesis and a lower fraction used for exoelectrogenesis, but the fraction used for biomass production remained constant.

3.4 Phase II – Effect of OLR on anode microbial community

The anode microbial community consisted of three major bacterial phyla: *Proteobacteria, Firmicutes* and *Bacteroidetes*. However, the relative abundance of these phyla was considerably different at OLR of 0.2 and 3.2 g/L-d (Fig. 6). At the low OLR (0.2 g/L-d), the microbial community was dominated by *Proteobacteria*, in which more than 80% were *Geobacter spp.*, closely related to *G. sulfurreducens*, a well-known exoelectrogen, and other *Geobacter spp.* reported in bioelectrochemical systems (> 97% identity).[20,21] In contrast, at the high OLR (3.2 g/L-d), *Firmicutes* was the major phylum, distributed in the genera of *Anaerovorax* (16-19%; duplicate analysis), *Acetobacterium* (12-14%), *Eubacterium* (7-9%), *Phascolarctobacterium* (3-4%), *Clostridium* (3%), and unclassified *Clostridia spp.* (42-50%). The characteristics of the detected *Firmicutes* phylotypes are discussed below. The only described species in the *Anaerovorax* genus is *A. ordorimutans*, which ferments putrescine to acetate, butyrate and hydrogen.[22] A number of strains of *Acetobacterium* and *Eubacterium* are acetogenic and able to metabolize methoxylated aromatic compounds, such as syringic and vanillic acid.[23-25] In

particular, the *Eubacterium sp.* detected in the present study is phylogenetically close to *Eubacterium limosum* (99% identity), which is known to perform homoacetogenesis and can also grow on methoxylated aromatic compounds.[24,25] *Phascolarctobacterium sp.* detected in the present study was closely related to *P. faecium* (99% identity), which can convert succinate to propionate.[26] *Clostridium spp.* are mostly obligate anaerobes and usually produce mixtures of organic acids and alcohols from carbohydrates.[27] To date, only a few exoelectrogens belonging to *Firmicutes* have been isolated, such as *Clostridium butyricum* EG3,[28] *Desulfitobacterium hafniense* strain DCB2,[29] and two *Thermincola* strains.[30,31] However, none of these species was detected or was closely related to the phylotypes identified in the present study. Therefore, the *Firmicutes* species in the present study are considered to be fermenters and acetogens, which metabolically differ from the *Proteobacteria* dominated by exoelectrogenic *Geobacter spp.*.

Although the relative abundance of exoelectrogens decreased at the high OLR used in the present study, it does not necessarily mean that the size of the exoelectrogenic population decreased, because the total biomass concentration increased at the high OLR by a factor of 3 (Fig. 4). However, it is clear that the relative size of the non-exoelectrogenic population increased considerably and disproportionally to that of the exoelectrogens with the increase of OLR from 0.2 to 3.2 g/L-d (Fig. 6). Excessive growth of fermenters and acetogens was expected to result in a higher acetate production rate and acetate accumulation at higher levels, which is consistent with the calculated production rate and measured acetate concentration as discussed in Section 3.2, above. Therefore, the higher OLR favored the growth of the non-exoelectrogens to a higher extent than that of the exoelectrogens. The furanic and phenolic compounds used in the present study are fermentable compounds, which are not directly used by exoelectrogens.[6] Thus, increasing the loading rate of these substrates enriched fermenters more directly than

exoelectrogens. The change of biofilm composition was observed in 24 days (6 days at an OLR of 0.2 g/L, 8 days at 0.8 g/L-d, and then 10 days at 3.2 g/L-d). For the observed dramatic change in the bioanode microbial community composition in a relatively short time, the biofilm coverage was more likely low, which was confirmed based on visual observation and the measured, anode surface area-normalized biomass (0.02 and 0.07 g VSS/m^2 at OLR of 0.2 and 3.2 g/L-d, respectively). Such low biofilm coverage is not uncommon. Harrington *et al.*[16] observed sporadic monolayer biofilm on a MEC graphite felt electrode using scanning electron microscopy, and suggested that low biofilm coverage may be characteristic of high surface area electrodes, in contrast to high biofilm coverage on small and flat electrodes.

Archaeal species were detected at an OLR of 3.2 g/L-d, but not at 0.2 g/L-d. The detected *Archaea* were two methanogens: *Methanobacterium palustre* (99%) and *Methanobrevibacter arboriphilus* (1%) (Fig. 6). The detection of methanogens is consistent with the methane production at an OLR of 3.2 g/L-d discussed in Section 3.3, above. In addition, both *Methanobacterium palustre* and *Methanobrevibacter arboriphilus* are hydrogenotrophic methanogens using H_2 and CO_2, but not acetate,[32] which is consistent with the observed acetate accumulation at an OLR of 3.2 g/L-d (Fig. 3C, Phase II). Absence of acetoclastic methanogens in bioanode has previously been reported and explained by outcompetition by exoelectrogens, given that the half-maximum rate concentration (K_s = 177– 427 mg COD/L) of acetoclastic methanogens is orders of magnitude higher than that of exoelectrogens (K_s = 0.64 mg COD/L).[33] The development of methanogens is considered a consequence of the excessive growth of fermenters at high OLR, which could have produced higher levels of H_2 and CO_2. In contrast, at low OLR, the H_2 produced by fermentation could have been rapidly utilized by the exoelectrogens which dominated the microbial community over the fermenters (Fig. 6). The

406 negative impact of methanogenesis is primarily the diversion of electrons away from
407 exoelectrogenesis and current production, resulting in lower Coulombic efficiency. A secondary
408 impact is competition of hydrogenotrophic methanogens with exoelectrogens for substrate (i.e.,
409 H_2) and nutrients in the biofilm.

410 The microbial community analysis confirmed that a high OLR caused an imbalanced
411 growth of fermentative and exoelectrogenic bacteria. This is another explanation of the limited
412 rate of exoelectrogenesis relative to fermentation, besides the electrochemical constraints
413 discussed in Section 3.2 above. Pinto *et al.*[34] developed a multi-population MEC mathematical
414 model which included microbial physiological groups such as fermenters, exoelectrogens, and
415 methanogens, which predicted that the increase of H_2 production rate was less significant at
416 higher organic loadings. However, it is not clear how the effect of organic loading on microbial
417 interactions was accounted for and related to the H_2 production. Consistent with the model
418 prediction,[34] the present study showed a similar H_2 production plateau, but has also provided
419 microbial community evidence on the shift relative to the abundance of fermenters and
420 exoelectrogens and the development of methanogens as a result of increased OLR. These
421 findings have important implications on MEC applications with complex, fermentable waste
422 streams derived from the pretreatment of lignocellulosic biomass.

423 *3.5 Phase III – Effect of applied voltage on MEC performance*

424 The effect of increased applied voltage was investigated at an OLR of 3.2 g/L-d and HRT of 6 h,
425 a condition where exoelectrogenesis was limited as discussed in Section 3.2, above. Upon the
426 increase of voltage from 0.6 to 1.0 V, the current gradually increased from 2.5 to 4.2 mA in 2
427 days, and then reached a plateau at 4.2 ± 0.1 mA (Fig. 2C; 60-68 d). The abiotic controls
428 conducted at 0.6 V and 1.0 V showed negligible current production (< 0.05 mA). The fate of the

furanic and phenolic compounds in the abiotic controls at 0.6 and 1.0 V was very similar (Fig. S2). It is noteworthy that electrochemical reactions and diffusion of the compounds through the ion exchange membrane to the cathode took place during the abiotic control assays, as described in detail in Text S2. However, the extent of diffusion and electrochemical reactions was the same at both 0.6 and 1.0 V. Therefore, the observed improvement in current production at 1.0 V was not associated with any abiotic electrochemical reactions triggered by the voltage increase.

The H_2 production rate at 1.0 V was 1.7-fold higher than at 0.6 V (Fig. 3B, Phase III). The acetate level at 1.0 V (264 mg/L) was lower than that at 0.6 V (332 mg/L), indicating that the rate of acetate consumption by exoelectrogenesis was enhanced at the higher voltage (Fig. 3C; Phase III). A higher applied voltage potentially increased the free energy gain of the exoelectrogens, and facilitated electron and charge transport, thus compensating for the electrochemical constraints discussed in Section 3.2, above. The sCOD removal efficiency increased by 2.6-fold, but the Coulombic efficiency decreased (Table 1), as the extent of current increase was less than that of sCOD removal. The differences in current, H_2 production, sCOD removal, Coulombic efficiency, and acetate concentration at 0.6 and 1.0 V were all statistically significant ($P < 0.05$). The concentrations of catechol and phenol, two detected metabolites (both at 0.12 ± 0.01 g/L), were not significantly different from those at the lower voltage (0.12 ± 0.02 g/L, $P = 0.705$ and 0.11 ± 0.004 g/L, $P = 0.169$, respectively). The open circuit control assay confirmed that catechol and phenol were fermentation products (Fig. S1). Our previous study showed that catechol and phenol were not used by exoelectrogens.[6] Thus, the change in applied voltage was not expected to affect the effluent concentration of catechol and phenol. The electrical and overall energy efficiencies at 1.0 V decreased from 242 to 150 %, and from 14 to 9%, respectively, compared with those at 0.6 V. A recent review by Lu and Ren summarized

MEC performance at various applied voltage values in studies using a large variety of substrates from acetate to lignocellulosic biorefinery byproducts.[35] They showed a general trend of increased MEC performance but decreased energy efficiency with an increased voltage in the range of 0.2 - 1.2 V, attributed to a higher energy input which in turn facilitates electron transfer in exoelectrogenesis.

4. Conclusions

An HRT of 6 h resulted in complete transformation of furanic and phenolic substrates, which can be a feasible MEC operating condition for a lignocellulose-derived wastewater. OLR affects MEC performance and is critical in maintaining balanced growth between fermenters and exoelectrogens, especially for fermentable and slowly degraded substrates. Increasing the MEC voltage significantly improved the H_2 production rate when exoelectrogenesis was limited. The present study provides insights into how continuous-flow MEC operating conditions affect microbial activity and interactions in a mixed-population bioanode community, which apply to a broader range of complex, fermentable substrates used in bioelectrochemical systems.

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Figure Captions

Fig. 1. Continuous-flow bioanode MEC. 1, mixture of furanic and phenolic compounds; 2, anolyte; 3, effluent waste; 4, syringe pump; 5, positive displacement pump; 6, glass buret; 7, acid brine displacement reservoir; 8, anode chamber; 9, cation exchange membrane; 10, cathode chamber; 11, potentiostat.

Fig. 2. MEC response to various operating conditions. (A) HRT and OLR; (B) applied voltage; (C) current; (D) influent and effluent sCOD. Error bars represent mean values ± one standard deviation, $n = 3$.

Fig. 3. Current (A), H_2 production rate (B) and acetate concentration (C) during stable MEC operation at various HRT, OLR and applied voltage values. Error bars represent mean values ± one standard deviation, $n \geq 4$.

Fig. 4. MEC anode protein concentration normalized to anode empty bed volume at various OLR values and an HRT of 6 h. Error bars represent mean values ± one standard deviation, $n = 2$.

Fig. 5. Soluble COD removed (A) and its components (B) at various OLR values and an HRT of 6 h.

Fig. 6. Anode microbial community composition in duplicate at various OLR values and an HRT of 6 h.

Table 1. MEC Performance under Various HRT, OLR and Applied Voltage Conditions

Parameter	Operational Phase					
	I			II		III
Duration (d)	16	16	8	8	10	8
Applied voltage (V)	0.6	0.6	0.6	0.6	0.6	1.0
HRT (h)	24	12	6	6	6	6
OLR (g/L-d)	0.2	0.2	0.2	0.8	3.2	3.2
Influent parent compounds (mg/L)	200	100	50	200	800	800
Effluent parent compounds (mg/L)	ND [a]	ND	ND	4 ± 1 [b]	16 ± 2	5 ± 2
Parent compounds conversion (%)	> 99	> 99	> 99	98 ± 0.6	99 ± 0.2	99 ± 0.3
sCOD removal (%)	31 ± 1	47 ± 3	30 ± 10	18 ± 2	14 ± 1	37 ± 1
Coulombic efficiency (%)	74 ± 5	60 ± 4	76 ± 18	44 ± 3	13 ± 1	8.0 ± 0.3

[a] None detected.

[b] Mean ± standard deviation ($n \geq 4$).

Fig. 1

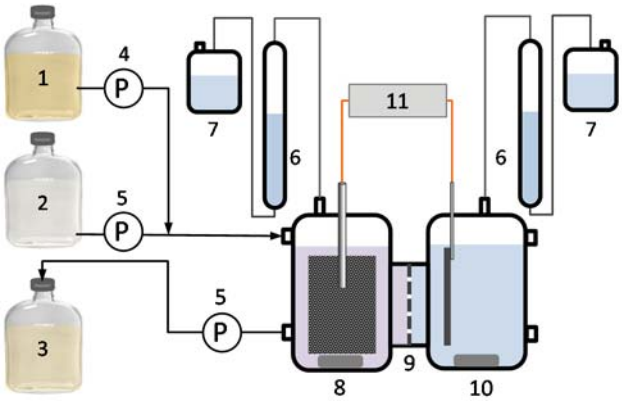


Fig. 2

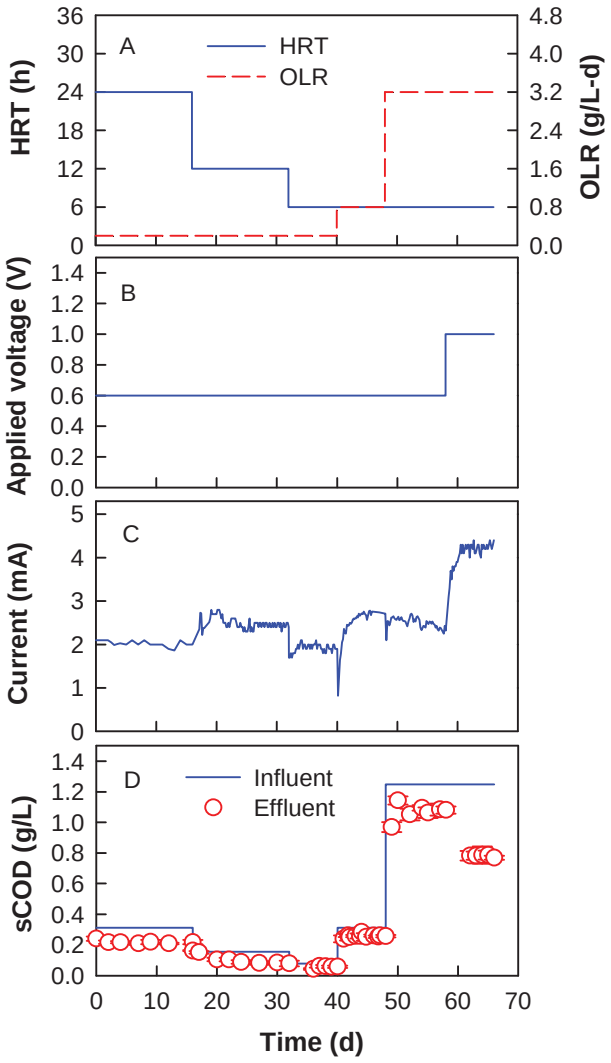

A
HRT
OLR
HRT (h)
OLR (g/L-d)
B
Applied voltage (V)
C
Current (mA)
D
Influent
Effluent
sCOD (g/L)
Time (d)

Fig. 3

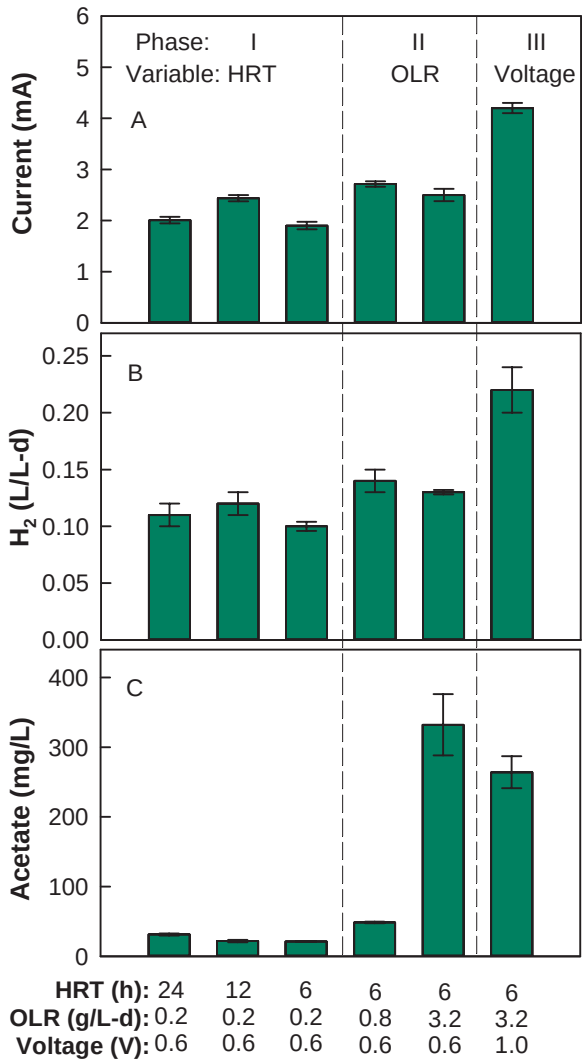

Phase: I II III
Variable: HRT OLR Voltage
A
B
C
Current (mA)
H2 (L/L-d)
Acetate (mg/L)
HRT (h): 24 12 6 6 6 6
OLR (g/L-d): 0.2 0.2 0.2 0.8 3.2 3.2
Voltage (V): 0.6 0.6 0.6 0.6 0.6 1.0

Fig. 4

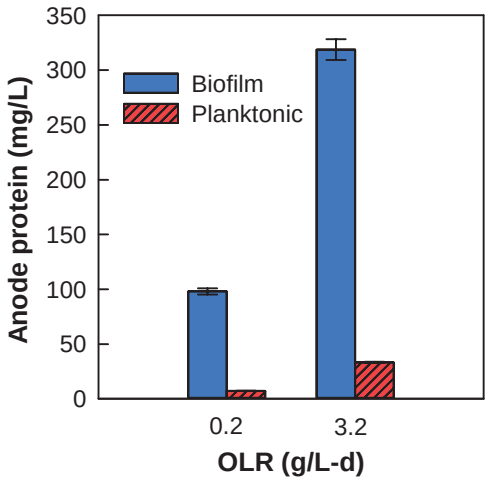

350
300
250
200
150
100
50
0
Anode protein (mg/L)
Biofilm
Planktonic
0.2
3.2
OLR (g/L-d)

Fig. 5

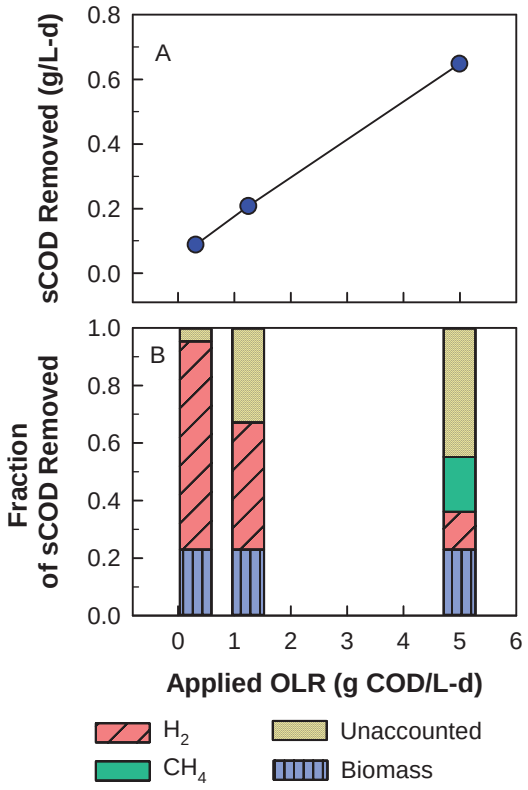
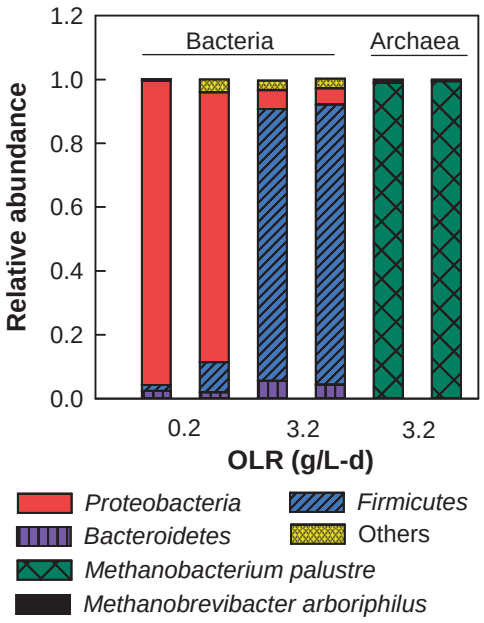

A
sCOD Removed (g/L-d)
0.0
0.2
0.4
0.6
0.8
B
Fraction of sCOD Removed
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
Applied OLR (g COD/L-d)
H2
CH4
Unaccounted
Biomass

Fig. 6


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative abundance
Bacteria
Archaea
0.2
3.2
3.2
OLR (g/L-d)
Proteobacteria
Firmicutes
Bacteroidetes
Others
Methanobacterium palustre
Methanobrevibacter arboriphilus