Hydrogen production from cellulose in a two-stage process combining fermentation and electrohydrogenesis

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\textbf{Abstract}

A two-stage dark-fermentation and electrohydrogenesis process was used to convert the recalcitrant lignocellulosic materials into hydrogen gas at high yields and rates. Fermentation using \textit{Clostridium thermocellum} produced 1.67 mol H\textsubscript{2}/mol-glucose at a rate of 0.25 L H\textsubscript{2}/L-d with a corn stover lignocellulose feed, and 1.64 mol H\textsubscript{2}/mol-glucose and 1.65 L H\textsubscript{2}/L-d with a cellobiose feed. The lignocellulose and cellobiose fermentation effluent consisted primarily of: acetic, lactic, succinic, and formic acids and ethanol. An additional 800 \pm 290 mL H\textsubscript{2}/g-COD was produced from a synthetic effluent with a wastewater inoculum (fermentation effluent inoculum; FEI) by electrohydrogensis using microbial electrolysis cells (MECs). Hydrogen yields were increased to 980 \pm 110 mL H\textsubscript{2}/g-COD with the synthetic effluent by combining in the inoculum samples from multiple microbial fuel cells (MFCs) each pre-acclimated to a single substrate (single substrate inocula; SSI). Hydrogen yields and production rates with SSI and the actual fermentation effluents were 980 \pm 110 mL/g-COD and 1.11 \pm 0.13 L/L-d (synthetic); 900 \pm 140 mL/g-COD and 0.96 \pm 0.16 L/L-d (cellobiose); and 750 \pm 180 mL/g-COD and 1.00 \pm 0.19 L/L-d (lignocellulose). A maximum hydrogen production rate of 1.11 \pm 0.13 L H\textsubscript{2}/L reactor/d was produced with synthetic effluent. Energy efficiencies based on electricity needed for the MEC using SSI were 270 \pm 20\% for the synthetic effluent, 230 \pm 50\% for lignocellulose effluent and 220 \pm 30\% for the cellobiose effluent. COD removals were \sim 90\% for the synthetic effluents, and \sim 70–85\% based on VFA removal (65\% COD removal) with the cellobiose and lignocellulose effluent. The overall hydrogen yield was 9.95 mol-H\textsubscript{2}/mol-glucose for the cellobiose. These results show that pre-acclimation of MFCs to single substrates improves performance with a complex mixture of substrates, and that high hydrogen yields and gas production rates can be achieved using a two-stage fermentation and MEC process.

\section*{Keywords:}
Biohydrogen
Microbial
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1. Introduction

Biohydrogen production from cellulose has received considerable attention as a carbon neutral method for producing hydrogen from renewable resources and wastes. Lignocellulose is the most abundant biopolymer on earth and the main component of plant biomass. It is available in many human-created wastes such as straw, wood-chips, grass residue, and paper waste. Hydrogen has a high energy content (120 MJ/kg compared with 44 MJ/kg of gasoline) and is a useful energy carrier for transportation when produced using sustainable and renewable energy sources such as biomass. There are multiple ways of producing hydrogen from diverse feedstock using microorganisms with no external energy input necessary, such as biophotolysis, photo-fermentation, and dark-fermentation [1–5]. Dark-fermentation has a maximum hydrogen yield of 2.4–3 mol H₂/mol glucose in practice [6,7]. However, this is only 20–25% of the 12 mol of hydrogen possible based on stoichiometric conversion of glucose to hydrogen [6,7], resulting in residual organic matter containing end-products (such as acetic and butyric acids, and ethanol) that cannot be further converted by fermentation to hydrogen [3–5].

A new method was recently developed, called electrohydrogenesis, that can be used to convert biomass to hydrogen gas in a device called a microbial electrolysis cell (MEC) [8]. In an MEC, bacteria on the anode oxidize organic matter, releasing electrons through the circuit to the cathode where hydrogen can be formed from protons in the water. This reaction is endothermic, and therefore additional electrical input is needed that is provided by a power source. The MEC efficiency relative to the electrical input has reached over 30% [9], proving that the electrical energy needed (typically >1.6–1.8 V applied) is much less than that used for water electrolysis (>2 V applied) [9–11]. Hydrogen has been produced in MECs using many different substrates, including acetic acid, butyric acid, lactic acid, glucose, cellulose, and wastewater [10,12–15], but few tests have been conducted using complex mixtures of substrates such as wastewaters [16,17]. No test has been conducted using effluent of a bioreactor fermenting carbohydrates.

In this study we examined the use of a two-stage process for converting lignocellulose into hydrogen. This process consists of a dark-fermentation process to optimize the conversion of pre-treated lignocellulosic biomass into hydrogen, carbon dioxide, acetic, formic, succinic, and lactic acids, and ethanol, followed by electrohydrogenesis to convert the residual volatile fatty acids (VFAs) and alcohols into hydrogen gas. MECs have previously been used to produce hydrogen directly from cellulose, but the process efficiency and hydrogen production rates were low compared to those achieved with single VFAs [12]. Thus we reasoned that a more efficient process could be developed by optimizing the fermentation and electrohydrogenesis processes in separate reactors. For the fermentation process, we used a pure culture of Clostridium thermocellum, a gram-positive, acetogenic, and thermophilic microbe. It produces an active extracellular cellulase system [18] called the cellulosome [19], and it has one of the highest known growth rates on crystalline cellulose [20,21]. The influent to the fermentor was either the dilute-acid pre-treated lignocellulose feed from corn stover [22] or a cellobiose solution. Inocula for the MEC were developed from wastewater and acclimated either to a synthetic feed containing the primary constituents in the fermentation effluent (acetic acid, ethanol, succinic acid, lactic acid, and formic acid) or the individual substrates.

2. Materials and methods

2.1. Biomass pre-treatment

Corn stover biomass was pretreated at 20% (w/w) solid concentration by dilute-acid hydrolysis (H₂SO₄; 1.08%, w/w) at 190 °C for 90 s in a pilot scale reactor at NREL’s Alternative Fuel User Facility [22]. The solid lignocellulose fraction (containing mostly cellulose and lignin) was separated from the aqueous hemicellulose by centrifugation, followed by washing in water at 3800 × g for 25 min. After pressing to remove excess water, the final material (45% moisture content) was analyzed according to NREL’s Laboratory Analytical Procedure [27] and contained on a dry weight basis: 59.1% cellulose, 25.3% lignin, 5.1% xylan, 0.7% arabinan, 0.4% galactan, 0.2% mannan, 0.1% acetic acid, 1.9% protein, and 3.7% ash.

2.2. Fermentation

Clostridium thermocellum 27405 (from David Levin, Univ. of Manitoba) was maintained at 55 °C by routinely transferring 10% (v/v) inocula into fresh ATCC 1191 medium [23], supplemented with avicel cellulose (0.5%, w/v) as the sole carbon substrate. Fermentation experiments were carried out in a fermentor (Electrolab 2400, Gloucestershire, UK) containing 600 ml of 1191 medium continuously sparged with N₂ gas (10 ml/min) at 50 °C (Electrolab 240 Temperature Control). Approximately 0.25% (dry w/v) of either pre-treated corn stover lignocellulose or cellobiose was added to the fermentor, followed by inoculation with 100 ml of C. thermocellum. The stirring rate was maintained at 120 rpm, and pH at 6.8 (Electrolab 260 pH Control Module) using an anaerobic NaOH solution (1 N). Hydrogen, CO₂, and N₂ gas measurements were recorded every hour in triplicate according to Datar et al. [6] using an online GC (Varian, Palo Alto, CA) connected to the fermenter. Concentrations of H₂ and CO₂ were calculated based on the continuous flow rate of N₂ gas (10 ml/min) and its content in the sample gas, after correcting for the altitude (0.82 atmosphere at 5280 ft) and the laboratory and reactor temperatures. At the end of fermentation, the supernatant was collected by centrifugation at 3800 × g for 10 min to remove the cells along with any residual solid biomass.

2.3. MECs

Anodes used in MEC tests were initially enriched by operating reactors as microbial fuel cells (MFCs) as previously described [24]. Both reactors consisted of a 4-cm long cylindrical chamber formed in a solid block of Lexan, with a liquid volume of 28 ml. The anodes were graphite fiber brush electrodes pretreated using an ammonia gas process. Cathodes
were flat carbon cloth containing a Pt catalyst (10% Pt/C) on the anode-facing side of the electrode [9]. Cathodes used in MFCs also contained 4 diffusion layers applied to the air-facing side [25].

Anodes were enriched in MFCs using a 1:1 (v/v) ratio of wastewater (primary clarifier effluent from the Penn State University’s Wastewater Treatment Plant) and a nutrient buffer solution (NBS; pH = 7) containing a substrate as previously described [9]. When the reactor’s voltage output exceeded 0.100 V with a 1 kΩ resistor, the inoculum was omitted from subsequent cycles. Two types of substrates were fed into MFCs: a complex substrate containing a mixture of chemicals modeled after the lignocellulase effluent (synthetic effluent); or a single substrate. The synthetic effluent contained (per liter): 1.56 g acetic acid, 0.64 g ethanol, 0.66 g succinic acid, 0.16 g lactic acid, and 0.03 g formic acid (conductivity = 8 mS/cm; pH = 7). The same constituents were used as single substrates, and were added to individual MFCs at a concentration of 1 g/L [26]. The anodes enriched in MFCs were fed with these different substrates for ~2 months (data in reference [26]). After an MFC exhibited a reproducible maximum voltage for at least 3 cycles, anodes were transferred to MECs in the laboratory under ambient atmospheric conditions.

MECs experiments were conducted using an applied voltage of ±0.5 V (duplicate reactors). The first set of MEC tests were conducted using brushes acclimated to the synthetic effluent (fermentation effluent inoculum; FEI) or individual substrates and the same solutions used in MFC tests. A separate set of MEC tests were also conducted using brushes acclimated to the synthetic effluent and a single substrate inoculum (SSI, a combination of equal volumes of solutions from the MFCs acclimated to individual substrates) instead of a wastewater inoculum. Experiments were then conducted using the MECs with the FEI and SSI inocula using two effluents from celllobiose fermentation (conductivity = 8 mS/cm; pH = 7.3) or corn stover lignocellulose fermentation (conductivity = 7 mS/cm; pH = 7.2). The reactors were re-filled with solution when gas production stopped and there was a sharp decrease in current production. New cathodes were used for each new type of substrate to avoid differences in performance due to the cathodes.

2.4. Chemical analysis

Organic acids and ethanol in the fermentor supernatant were measured using high performance liquid chromatography (HPLC; Agilent 1050) equipped with UV–VIS and Refractive Index (RI) detectors [6]. Cellulose content within the lignocellulose fraction, before and after fermentation, was measured by an acid hydrolysis method [27] with resulting glucose determined via HPLC (Agilent 1050), equipped with a RI detector and a Shodex column (SP0810) using water as the mobile phase. Total chemical oxygen demand (COD) was determined using standard methods (TNT plus COD Reagent; HACH Company). Liquid concentrations of acetic, lactic, formic and succinic acids before and after MEC experiments were analyzed by HPLC (Waters 2695) with sulfuric acid as the mobile phase, and ethanol was measured by gas chromatography (GC Agilent 6890 N). Gas chromatography was used to analyze gas composition as previously described by Call and Logan [9].

2.5. Calculations

The performance of the MEC reactors was measured and compared on the basis of hydrogen recovery, hydrogen production rate, and the electrical energy recovery as described by Logan et al. [28]. Synthetic effluent calculations were based on the composition of the medium (54% acetic acid, 29% ethanol, 12% succinic acid, 4% lactic acid and 1% formic acid; Table 1), and fermentation effluent calculations were based on their measured composition.

Hydrogen recoveries (mol H2/mol substrate) were based on actual recovery (measured) and compared to the maximum possible recovery (theoretical). Actual hydrogen recoveries were calculated on the basis of COD removal. Maximum hydrogen recoveries were calculated assuming stoichiometric conversion of the substrate to hydrogen, based on the following equations:

\[ \text{Acetic acid: } \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} \rightarrow 4\text{H}_2 + 2\text{CO}_2 \]  
(1)

\[ \text{Ethanol: } \text{CH}_3\text{CH}_2\text{OH} + 3\text{H}_2\text{O} \rightarrow 6\text{H}_2 + 2\text{CO}_2 \]  
(2)

\[ \text{Succinic acid: } \text{COOHCH}_2\text{CH}_2\text{COOH} + 4\text{H}_2\text{O} \rightarrow 7\text{H}_2 + 4\text{CO}_2 \]  
(3)

\[ \text{Lactic acid: } \text{CH}_3\text{CHOHCOOH} + 3\text{H}_2\text{O} \rightarrow 6\text{H}_2 + 3\text{CO}_2 \]  
(4)

\[ \text{Formic acid: } \text{HCOOH} \rightarrow \text{H}_2 + \text{CO}_2 \]  
(5)

Thus, maximum hydrogen recoveries were 4 mol H2/mol acetic acid, 6 mol H2/mol ethanol, 7 mol H2/mol succinic acid, 6 mol H2/mol lactic acid, and 1 mol H2/mol formic acid.

The predicted results for the synthetic effluent (SSI-Pr) were calculated on the basis of the solution composition and the performance in the MECs with individual substrates as:

\[ \text{SSI - Pr} = 0.54P_A + 0.29P_E + 0.12P_S + 0.04P_L + 0.01P_F \]  
(6)

<table>
<thead>
<tr>
<th>Table 1 – Substrate COD conversion characteristics and individual substrates concentrations in fermentation effluents.</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD</td>
</tr>
<tr>
<td>g-cod/ g</td>
</tr>
<tr>
<td>Acetic acid</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Succinic acid</td>
</tr>
<tr>
<td>Lactic acid</td>
</tr>
<tr>
<td>Formic acid</td>
</tr>
</tbody>
</table>
where P indicates the performance (for example, the hydrogen yield or the COD removal) and the subscripts indicate chemical species of acetic acid (A), ethanol (E), succinic acid (S), lactic acid (L), and formic acid (F). Maximum hydrogen recoveries for actual fermentation effluents in MECs were also calculated with this expression with solution compositions as defined in Table 1.

Hydrogen production from the MEC stage ($M_{H_2,\text{MEC}}$) was calculated on the basis of the COD content of 0.7 L effluent of cellobiose, and MEC performance in terms of COD removal and the corresponding volume of hydrogen produced. The equation used for this calculation was:

$$M_{H_2,\text{MEC}} = \frac{Y_{H_2} V_{H_2} \Delta \text{COD} V_R}{C_{g0}}$$

(7)

where $Y_{H_2}$ (mL H$_2$/g-COD$_{cons}$) is the hydrogen yield of the MEC, $V_{H_2}$ (0.0402 mM H$_2$/mL H$_2$ at 30°C and 1 atm) the conversion of hydrogen from volume to moles, $\Delta$COD (g-COD/L) the concentration of COD consumed by the MEC, $V_R$ (L) the volume of the reactor, and $C_{g0}$ (mol glucose/L) the initial concentration of substrate entering the MFC (the fermentation effluent) expressed in terms of moles of glucose added as cellulose. The overall system performance for hydrogen production was calculated based on the combined systems.

3. Results

3.1. Hydrogen production via fermentation

Using cellobiose (0.25% or 14.6 mM glucose for 2500 mg/L of cellobiose) as the substrate for fermentation, hydrogen and CO$_2$ production was observed almost immediately after reactor inoculation (Fig. 1). The rate of hydrogen production during the linear phase was 1.65 L H$_2$/L-day, with all the cellobiose consumed at the end of 40 h. Using lignocellulose (0.25% or 9.1 mM glucose) as a substrate, however, there was a longer lag phase of 10 h (Fig. 1). The subsequent hydrogen production rate during the linear phase was 0.25 L H$_2$/L-day, which was much slower than that of cellobiose. Analysis of the residual solids at the end of 90 h indicated that the cellulose component of the added lignocellulose was totally consumed. Based on the consumption of either cellulose or cellobiose, the hydrogen molar yield was 1.67 mol/mol-glucose for the corn stover lignocellulose, and 1.64 mol/mol-glucose for the cellobiose. The ratio of H$_2$ to CO$_2$ was slightly larger for cellobiose (0.93) than for lignocellulose (0.85), but both results are within the range reported for fermentation of α-cellulose and delignified wood [23].

Both fermentation reactions yielded acetic acid and ethanol as the major by products along with minor amounts of formic, lactic, and succinic acids (Table 1). However, the original lignocellulose feed contained sugars and acetic acid derived from hemicellulose (see Materials and Methods), although C. thermocellum was unable to metabolize them [21]. As a result, 17% (2.4 mM) of the 14 mM acetic acid in the fermentation effluent was present in the fermentation influent for the lignocellulose substrate.

3.2. Volumetric hydrogen production in MECs

Hydrogen production using an inoculum acclimated to the synthetic fermentation effluent and the synthetic fermentation effluent (FEI-Syn) declined over successive cycles (Fig. 2A). However, when the combined inoculum from the MFCs acclimated to individual substrates was used in MEC tests (SSI-Syn), there was more consistent gas production over
multiple cycles (Fig. 2B). The total gas production over the three cycles with SSI-Syn was 110 ± 10 mL per batch based on duplicate reactors, with an average of 79 ± 2% of hydrogen and 10 ± 2% methane (Table 2), and a maximum current density of 1.15 ± 0.05 A/m². In contrast, gas production with FEI-Syn varied between 80 and 159 mL (average 97 ± 31 mL) and contained 76 ± 6% of hydrogen. The proportion of methane in the gas produced for FEI-Syn varied between 1 to 45%, with an average of 21 ± 16% over the first five cycles with the duplicate reactors.

The use of the actual fermentation effluents produced comparable or slightly lower amounts of gas per batch cycle than when the reactor was fed with synthetic effluent (Table 2). There was 105 ± 17 mL of gas produced using the cellobiose fermentation effluent, and 97 ± 16 mL with corn stover lignocellulose fermentation effluent. The percent of hydrogen in the gas, however, declined compared to the synthetic effluent. There was 69 ± 4% H₂ in the gas from the cellobiose effluent, and 69 ± 6% H₂ with the lignocellulose.

### 3.3. Hydrogen yields in MECs

Hydrogen yields were calculated on the basis of COD removal and molar conversion for defined substrates (pure chemicals, cellobiose and synthetic effluents). For the individual substrates, acetic acid produced the most hydrogen with 1400 ± 170 mL-H₂/g-COD, with a range of 810 ± 260 mL-H₂/g-COD (formic acid) to 1100 ± 130 mL-H₂/g-COD (succinic acid) for the other single substrates (Fig. 3A). The molar yield was highest for acetic acid based on the maximum possible percent yield (3.6 mol/mol, or 90% conversion) (Fig. 4A), but there were apparent larger molar yields produced with succinic (4.8 mol/mol or 68.5%) and lactic acids (4 mol-H₂/mol or 68%) but a lower overall conversion efficiency relative to the possible percent yields.

The hydrogen yield with the synthetic effluent was higher (980 ± 110 mL-H₂/g-COD) using the combined inoculum from the individual substrates (SSI), than that obtained without the special acclimation procedure (FEI) (800 ± 290 mL-H₂/g-COD) (Fig. 3B). Based on the composition of the synthetic effluent, and the results achieved with the individual substrates in the MECs, we expected a hydrogen yield of 1200 mL-H₂/g-COD. Thus, the FEI-Syn produced 33% less hydrogen than expected. On a molar basis, we obtained an average of 3.2 mol-H₂/mol-substrate using the SSI inoculum, and 2.6 mol-H₂/mol-substrate using the FEI inoculum with the synthetic effluent (Fig. 4B).

### Table 2 – Gas production and composition for different fermentation effluent in either non-acclimated or acclimated reactors (Standard deviations are calculated for duplicate reactors over 3 cycles: n = 6).

<table>
<thead>
<tr>
<th>Fermentation effluent</th>
<th>Total gas production (mL)</th>
<th>H₂</th>
<th>CH₄</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic (SSI-Syn)</td>
<td>110 ± 10</td>
<td>79 ± 2 10 ± 2 11 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lignocellulose</td>
<td>97 ± 16</td>
<td>69 ± 6 12 ± 3 19 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose</td>
<td>105 ± 17</td>
<td>69 ± 4 16 ± 4 14 ± 1</td>
<td></td>
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</tbody>
</table>

The hydrogen yield with the cellobiose fermentation effluent of 900 ± 140 mL-H₂/g-COD (Fig. 3B) was close to that achieved with the synthetic effluent (980 ± 110 mL-H₂/g-COD). The hydrogen recovery efficiency was also higher (73%) than that achieved using the synthetic effluent or the lignocellulose effluent. The lignocellulose fermentation effluent produced less hydrogen in MEC tests, with an average of 750 ± 180 mL-H₂/g-COD over 3 batch cycles (duplicate reactors). This is equivalent to molar yields of 2.9 mol H₂/mol substrate for the cellobiose effluent. We obtained an overall yield of 8.31 mol H₂/mol-glucose based on 0.7 L of cellobiose fermentor effluent calculated on the basis of the initial concentration of 8.8 mmol of glucose fed to the fermentation reactor. A molar yield for the lignocellulose effluent cannot be calculated on this initial sugar basis, however, due to the presence of other substrates in the lignocellulose feed such as the hemicellulose-derived acetic acid and sugars.

### 3.4. Methane production in MECs

The production of methane varied in the tests for the different single substrates and fermentation effluents (Fig. 3). There was generally less methane production using single substrates than effluents, with very little or no methane observed using acetic, succinic, or lactic acids (Fig. 3A). There was 49 ± 24 mL-CH₄/g-COD with ethanol and 54 ± 32 mL-CH₄/g-COD with formic acid. The lignocellulose effluents produced
120 ± 14 mL-CH₄/g-COD, while much more methane was obtained (210 ± 30 mL-CH₄/g-COD) using the cellobiose effluent. These values are both more than the expected 15 mL-CH₄/g-COD based on results with the individual substrates in the MEC tests (SSI-Pr), and 120 mL-CH₄/g-COD obtained with the synthetic effluent (SSI-Syn).

3.5. **Maximum hydrogen production rates in MEC tests**

The maximum hydrogen production rates ranged from 1.17 ± 0.07 L-H₂/L-d with acetic acid to 0.62 ± 0.20 L-H₂/L-d with formic acid (Fig. 5). Using the synthetic effluent, the rate was 1.11 ± 0.13 L-H₂/L-d with the SSI inoculum, and 0.59 ± 0.21 L-H₂/L-d with FEI inoculum. MEC tests with the actual fermentation effluents produced rates similar to those obtained with the synthetic effluent, with 1.0 ± 0.19 L-H₂/L-d for the corn stover effluent, and 0.96 ± 0.16 L-H₂/L-d for the cellobiose effluent (Fig. 5B).

3.6. **COD removal**

There as relatively good COD removal for individual substrates (91 ± 2% COD removal except for formic acid which only had 59% COD removal) as well as with the synthetic effluent with SSI (91%) and FEI (89%). However, a large portion of the total COD of the actual fermentation effluents was not removed in the MECs. HPLC analyses of the cellobiose and corn stover effluents showed that 27% (for cellobiose) and 14% (for lignocellulose) of the COD could be accounted for by VFAs (acetic, succinic, lactic and formic acids) and 20% (for cellobiose) and 14% (for lignocellulose) by ethanol. The removal of these VFAs and ethanol was 90 ± 4% (COD basis for both cellobiose and lignocellulose effluents). Overall, there was only 65% removal of the total COD (Fig. 6). There was little change in the concentration of the COD portion that was not associated with VFAs and ethanol, except for the cellobiose fermentation effluent (44% removal).

3.7. **Electrical energy efficiency**

The electrical energy efficiency only takes into account the electrical energy input into the process, and thus can have values above 100% [9,10,26,29]. Electrical energy efficiency was highest for acetic (280 ± 40%) and formic acids (270 ± 20%) (Fig. 7A). The synthetic effluent with the SSI inoculum had an electrical efficiency of 270 ± 20%, compared to 150 ± 50% with the FEI inoculum (FEI-Syn) (Fig. 7). The actual fermentation effluents had electrical energy efficiencies of 230 ± 50% for the lignocellulose, and 220 ± 30% for the cellobiose (Fig. 7B).

4. **Discussion**

Combining fermentation and electrohydrogenesis into a two-stage process resulted in an overall hydrogen yield of...
The first-stage lignocellulose fermentation step produced a similar yield of 1.67 mol-H$_2$/mol-glucose, but the overall molar yield could not be calculated due to the presence of other substrates in the feed (hemicellulose-derived acetic acid and sugars). The use of an MEC provides an additional advantage of converting certain components (hemicellulose-derived chemicals) into hydrogen that the first-stage fermentation cannot. The limiting step in increasing the rate of hydrogen production is clearly the conversion of solid substrate to soluble compounds that can be taken up by the cells. When cellobiose was the substrate, the rate of gas production in the fermentor was 1.65 L-H$_2$/L-d, but with insoluble lignocellulose this rate decreased to 0.25 L-H$_2$/L-d. Once fermented, the rates of hydrogen production in the MEC with the actual effluents were similar to that obtained with the synthetic fermentation effluent (1.11 ± 0.13 L-H$_2$/L-d), with the overall hydrogen production rates in the MEC of 1.00 ± 0.19 L-H$_2$/L-d using lignocellulose fermentation effluent and 0.96 ± 0.16 L-H$_2$/L-d with cellobiose fermentation effluent. This is the first time that a high rate of hydrogen production has been obtained directly using corn stover lignocellulose. Our finding that hydrogen production using lignocellulose is slower than that with cellobiose is consistent with our expectations based on previous studies with this microorganism and other substrates. For example, *C. thermocellum* has been shown to grow three times faster using cellobiose than z-cellulose [30]. Using pure z-cellulose, Magnusson et al. [31] observed in *C. thermocellum* a rate of hydrogen production at 0.13 L-H$_2$/L-day for a carbon-limited concentration of z-cellulose (0.11%, w/v or 6.8 mM glucose), which is 54% of the rate reported in this study using a slightly higher amount of lignocellulose substrate. The specific rate of hydrogen production is known to drop sharply with increasing hydrogen partial pressure (pH$_2$) [32,33]. In this study, the fermentor was continuously sparged with nitrogen gas to lower the pH$_2$, which likely accounts for the higher rate of hydrogen production here. Another cellulose-degrader Ruminococcus albus exhibited a rate of only 0.004 L-H$_2$/L-day from cellulose [34], which is considerably lower than *C. thermocellum* by several orders of magnitude. The higher rate observed in *C. thermocellum* could be due to more favorable kinetics at a higher temperature.

The hydrogen molar yields reported with cellobiose and lignocellulose are comparable with those in the fermentation literature using *C. thermocellum*, with values of 1.65 for delignified wood [23], and 1.6 [31] and 1.9 [35] for z-cellulose. The concentrations of end-products reported in the literature are highly varied and depend on the types of substrate and loading rates, pH$_2$, and pH$_2$ controls. The types of fermentation end-products obtained here were comparable to those reported by other labs using *C. thermocellum* 27405 [36,37]. The only exception is that we routinely detected a trace amount of succinic acid in the fermentation effluent. This is consistent with the presence of putative genes encoding the succinic-acid biosynthesis pathways in the sequenced genome of *C. thermocellum*. Earlier studies using a novel strain *C. thermocellum* strain 1.1.1 [38] and 0.5% (w/v) corn stover lignocellulose yielded same profile yet elevated levels of VFA and ethanol [39], which form the basis for the single substrate and synthetic fermentation effluent studies via MEC.
4.1. MEC performance

Acclimation of the inoculum to the individual substrates was needed to obtain consistent performance of the MECs with the synthetic fermentation effluent (Table 3). The maximum hydrogen production rate \( Q_{H_2,max} \) increased by 88% (to 1.11 L-H\(_2\)/L-d) with SSI compared to FEI, and batch cycle times were reduced (3–4 days for SSI, compared to 5–7 days for FEI) [26]. The performance of the MECs with the synthetic effluent was also consistent with predictions made on the basis of the reactors with individual substrates (Table 3) in terms of observed: predicted ratios of 270:270% for electrical energy efficiency, 86.89% cathodic hydrogen recovery, 91.93% COD removal, and 1.11:1.02 L H\(_2\)/L-d for hydrogen production.

The results obtained with synthetic effluent in the MEC were less than we predicted based on experiments with pure chemicals. For example, the hydrogen yield (980 mL H\(_2\)/g-COD) was only 82% of that predicted for the synthetic effluent based on its composition and the performance of the single substrates. Similarly, the hydrogen recovery was 16% less than that predicted. The reduced performance in terms of these variables likely was a result of the longer batch cycle times needed for the fermentation effluent (3–4 days) than those for the single substrate experiments (1–2 days). In the single substrate tests each reactor received 1 g/L of substrate, whereas the fermentation effluent received a mixture of the substrates at a total concentration 3.6 g/L (synthetic effluent) to 5.3 g/L (corn stover effluent). The longer cycle time thus led to a lower hydrogen production rate, and allowed more time for growth of methanogens. It has been found in other studies that high organic loading and longer cycle times adversely affect reactor performance [9,28]. The effect of the cycle time was not included in our predicted results for reactor performance.

An examination of several different performance factors for the MEC shows that overall the performance was better using the cellobiose effluent than the corn stover lignocellulose effluent. Comparing these factors a series of ratios of cellobiose: lignocellulose: synthetic effluent shows results in 900:750:980 mL-H\(_2\)/g-COD for hydrogen production, 220:230:279% for electrical energy efficiency, and 2.9:2.4:3.2 mol H\(_2\)/mol substrate for hydrogen yield.

The MECs were designed to metabolize specific VFAs and ethanol, and there was good removal of these chemical species in all samples tested. While total COD removal was very high using the synthetic effluent (91%), there was relatively less COD removal using both lignocellulose and cellobiose fermentation effluents (65%) (Fig. 6). The COD that is not removed likely originated from the cellulosomes of C. thermocellum. It is known that cellulosomes are attached to cell surface during early log phase of growth, but they detach and become freely suspended in the late exponential growth phase and thus are routinely recovered in the culture supernatant [40,41]. Cellulosomes are composed of multi-subunit protein complex that the MECs were not acclimated to degrade. It is possible that there was some additional fermentation of these materials in the MECs, as shown by a decrease of the “other” part of the COD in some tests. For example, in one test with the cellobiose there was an increase in lactic acid during one cycle (Fig. 6). Fermentation would likely result in soluble compounds that could be removed in the MEC.

It was observed that the coulombic efficiency exceeded 100% in tests with the synthetic effluent and FEI inoculum (CE = 110%). This was likely due to hydrogen oxidation by exoelectrogens on the anode that effectively “cycled” the electrons produced at the cathode. Exoelectrogenic bacteria, such as Shewanella sp., are able to oxidize both lactate and H\(_2\) under anaerobic conditions [10,42–44]. This cycling of electrons could lead to CEs larger than 100%. Of course it is also possible for hydrogen to be oxidized without current generation, for example for methane production, which would lower CEs. This recycling of electrons and methane consumption could both contribute to the low cathodic recovery (49%) observed in FEI tests.

There was methane production in all MEC tests, and production of methane continues to be a problem in MECs with mixed cultures [17,28,45]. The production of methane lowers hydrogen production either through conversion of acetate to methane or through hydrogenotrophic methanogenesis via

\[
\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \quad (8)
\]

Assuming four moles of hydrogen are consumed for each mole of methane produced, we can estimate the amount of hydrogen that would have been recovered based on the amount of methane production. The ratio of hydrogen recovered on this basis (i.e. from the methane produced), compared to that based on the actual hydrogen recovery, is 48.82% for lignocellulose; 66.128% for cellobiose; and 52.78% (FEI-Syn) or 63.94% (SSI-Syn). One value is larger than 100%, either due to electron recycling as discussed above, or to methane originating from acetic acid or precursors other than hydrogen. Methane production varied for the different feeds due to different COD loading (higher loadings lead to longer cycle times and more methanogen growth and methane production), differences in substrates (certain substrates such as acetic acid can result in more methane than others), and the different inocula (which likely contained different types and numbers of methanogens).

### Table 3 – Comparison between the results with synthetic effluent (Synth-FEI and Synth-SSI) and the predicted behavior (SSI-Predicted) in MEC (Standard deviations are calculated for duplicate reactors over 3 cycles: n = 6).

<table>
<thead>
<tr>
<th></th>
<th>FEI-Syn</th>
<th>SSI-Syn</th>
<th>SSI-Pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen yield, ( Y_{H_2} ) (mL H(_2)/g-COD)</td>
<td>800 ± 290</td>
<td>980 ± 110</td>
<td>1200</td>
</tr>
<tr>
<td>Methane yield, ( Y_{CH_4} ) (mL CH(_4)/g-COD)</td>
<td>100 ± 83</td>
<td>120 ± 18</td>
<td>15</td>
</tr>
<tr>
<td>Cathodic recovery, ( r_{cat} ) (%)</td>
<td>49 ± 16</td>
<td>86 ± 7</td>
<td>89</td>
</tr>
<tr>
<td>Coulombic recovery, ( r_{C} ) (%)</td>
<td>110 ± 20</td>
<td>73 ± 3</td>
<td>90</td>
</tr>
<tr>
<td>Hydrogen recovery, ( R_{H_2} ) (%)</td>
<td>52 ± 19</td>
<td>63 ± 7</td>
<td>79</td>
</tr>
<tr>
<td>Maximum hydrogen production rate, ( Q_{H_2,max} ) (mL(_2)/h/m²/d)</td>
<td>0.59 ± 0.21</td>
<td>1.11 ± 0.13</td>
<td>1.02</td>
</tr>
<tr>
<td>COD removal, COD (%)</td>
<td>89 ± 5</td>
<td>91 ± 2</td>
<td>93</td>
</tr>
<tr>
<td>Electrical energy efficiency, ( n_e ) (%)</td>
<td>150 ± 50</td>
<td>270 ± 20</td>
<td>270</td>
</tr>
</tbody>
</table>
Table 4 – Comparison of this study’s results for acetic acid, lactic acid and synthetic fermentation effluents with data from Cheng and Logan, 2007 [23].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\text{H}_2$ (%)</th>
<th>$Q_{\text{H}_2, \text{max}}$ (L/L-d)</th>
<th>$\eta_{\text{elec}}$ (%)</th>
<th>$\eta_{\text{elec}}$ (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>91</td>
<td>1.10</td>
<td>260</td>
<td>82</td>
<td>This study</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>93</td>
<td>1.17</td>
<td>280</td>
<td>84</td>
<td>This study</td>
</tr>
<tr>
<td>Cellulose</td>
<td>67</td>
<td>1.04</td>
<td>250</td>
<td>63</td>
<td>This study</td>
</tr>
<tr>
<td>SSI-Syn</td>
<td>68</td>
<td>0.11</td>
<td>270</td>
<td>63</td>
<td>This study</td>
</tr>
<tr>
<td>FEI-Syn</td>
<td>63</td>
<td>1.11</td>
<td>270</td>
<td>62</td>
<td>This study</td>
</tr>
<tr>
<td>SSI-cellulose effluent</td>
<td>66</td>
<td>0.96</td>
<td>220</td>
<td>61</td>
<td>This study</td>
</tr>
</tbody>
</table>

The MEC results obtained here with single substrates and fermentation effluents are generally in agreement with previous results (Table 4). The results for acetic acid compare well with data obtained by Cheng and Logan [12] in terms of rates, efficiencies and yields, but for lactic acid we obtained lower hydrogen and energy recoveries. One reason for the better performance by Cheng and Logan [12] is that they used a higher applied voltage ($0.6 \text{ V}$) than that used here ($0.5 \text{ V}$). A higher voltage increases the current density and shortens the reactor run-time, reducing the potential for methane production and electron cycling. However, we did not see higher yields for other substrates, so other factors such as the different inoculum in the tests could have been a factor. The results with fermentation effluents in MEC reactors showed comparable hydrogen recoveries, electrical and overall energy efficiencies to those obtained by Cheng and Logan using cellulose [12]. The major improvement obtained here was in terms of hydrogen production rate in the MEC. Using the fermentation effluent increased the production rate by an order of magnitude compared to that in the MEC. Using the fermentation effluent increased the overall hydrogen molar yield to favor its technoeconomic feasibility. Thus, the two-stage process is a promising approach for hydrogen production from the more abundant and renewable cellulose and lignocellulosic materials.

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[41] Lynd LR, Grethlein HE. Hydrolysis of dilute acid pretreated mixed hardwood and purified microcrystalline cellulose by cell-free broth from Clostridium thermocellum. Biotechnol Bioeng 2004;92:100.


