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Consolidated Bioprocessing of AFEX-Pretreated Corn Stover to Ethanol and Hydrogen in a Microbial Electrolysis Cell

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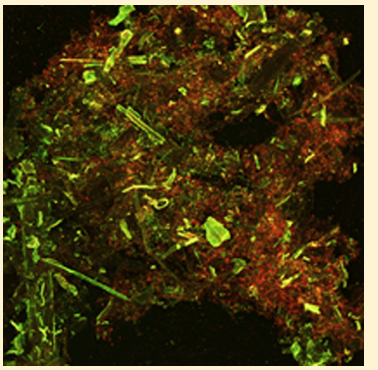
Supporting Information

ABSTRACT: The consolidated bioprocessing (CBP) of corn stover pretreated via ammonia fiber expansion (AFEX-CS) into ethanol was investigated in a microbial electrolysis cell (MEC) driven by the exoelectrogen *Geobacter sulfurreducens* and the CBP bacterium *Cellulomonas uda*. *C. uda* was identified in a screening for its ethanologenic potential from AFEX-CS and for producing electron donors for *G. sulfurreducens* fermentatively. *C. uda* produced ethanol from AFEX-CS in MECs inoculated simultaneously or sequentially, with the concomitant conversion of the fermentation byproducts into electricity by *G. sulfurreducens*. The fermentation and electrical conversion efficiencies were high, but much of the AFEX-CS remained unhydrolyzed as nitrogen availability limited the growth of the CBP partner. Nitrogen supplementation stimulated the growth of *C. uda*, AFEX-CS hydrolysis and ethanologenesis. As a result, the synergistic activities of the CBP and exoelectrogen catalysts resulted in substantial energy recoveries from ethanologenesis alone (ca. 56%). The cogeneration of cathodic H_2 in the MEC further increased the energy recoveries to ca. 73%. This and the potential to optimize the activities of the microbial catalysts via culturing approaches and genetic engineering or adaptive evolution, make this platform attractive for the processing of agricultural wastes.



INTRODUCTION

Ethanol is a promising biofuel that can be manufactured from lignocellulosic feedstocks by microbial fermentation of biomass sugars.[1] However, the high lignin content in these substrates limits its enzymatic digestibility[2] and biomass pretreatments are required to improve enzymatic hydrolysis.[3−5] The ammonia fiber expansion (AFEX) process shows promise as a cost-effective, scaled-up pretreatment of lignocellulose substrates because it recycles the pretreatment chemical (ammonia),[4] improves the enzymatic digestibility of the substrate,[6] and generates a highly fermentable hydrolysate[7−9] that yields high ethanol titers without the need for biomass washing, detoxification or nutrient supplementation.[8] However, the enzymatic hydrolysis step and the inefficient fermentation of hemicellulose sugars remain major bottlenecks.[8]

Consolidated bioprocessing (CBP; [10]) technologies, that is, those in which a single microbe hydrolyzes the substrate and ferments the hexose and pentose sugars, have been proposed as the most cost-efficient industrial configuration for ethanol production.[11] While significant advances have been made to engineer CBP yeasts, challenges still remain to produce industrial strains that heterologously express saccharolytic enzymes and coferment cellulose and hemicellulose sugars.[12,13] Native lignocellulose degraders show promise as CBP catalysts[11,14] because their hydrolysis and fermentation efficiencies are naturally evolved to maximize cell growth yields from biomass.[15] However, these microorganisms are adapted to growing within specialized, synergistic consortia,[12] where fermentation products are rapidly removed to prevent feedback inhibition of biomass decomposition and fermentation using various electron acceptors as final electron sinks.[16] The possibility of mimicking CBP consortia in bioelectrochemical cells is attractive because an electrode can be used to replace the natural electron acceptors and model exoelectrogens such as *Geobacter sulfurreducens* are available that conserve energy for growth by transferring electrons from waste fermentation products such as acetate, formate, lactate, and H_2 to electrodes.[17−20] Furthermore, with sufficient electrical input the current generated in the anode can be converted into H_2 in the cathode chamber in a microbial electrolysis cell (MEC), thus producing H_2 fuel as a coproduct.[21]

Previous studies[22] with cocultures of *G. sulfurreducens* and the CBP bacterium, *Clostridium cellulolyticum*, demonstrated that cellulose degradation can be coupled to electricity generation in a microbial fuel cell (MFC). The direct coupling of cellulose to electricity was also demonstrated in MFCs driven by strains of *Enterobacter cloacae* and mixed cultures.[23] Fermentation inhibitors derived from the pretreatment of lignocellulose substrates can also be converted into electricity in a MFC powered by a microbial consortium enriched on the anode.[24] Additionally, untreated and steam-exploded corn

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stover supported current in an MFC driven by a CBP consortia enriched from soil samples.[25] However, energy recovery from corn stover or from cellulose in MFCs is much lower than that of cellulosic bioethanol production, suggesting that considerably more power needs to be produced to make the platform competitive.[25] This will require significant increases in the efficiency of corn stover saccharification and electricity generation.

MECs are attractive as CBP platforms for ethanol because the electrical input can be used to simultaneously produce H_2 in the cathode at much higher yields than those achieved fermentatively.[21] Furthermore, the applied potential removes cathodic limitations[22,23,25] and promotes the growth of exoelectrogenic biofilms on the anode electrode.[26] This maximizes the conversion of fermentation byproducts to cathodic H_2 while preventing the accumulation of feedback inhibitors. However, it is important to minimize electron losses by selecting CBP strains that produce fermentation byproducts that serve as electron donors for the exoelectrogen. Here we describe the identification of a CBP strain, *Cellulomonas uda*, which hydrolyzed and fermented AFEX-pretreated corn stover (AFEX-CS) to ethanol and produced fermentation byproducts that served as electron donors for *G. sulfurreducens* in a MEC. The synergistic interactions between the CBP catalyst and the exoelectrogen stimulated ethanol production and minimized electron losses through the conversion of all the fermentative byproducts into H_2 in the cathode, thereby increasing the total energy recovery from the AFEX-CS. This provides a competitive CBP platform for bioenergy production from lignocellulosic substrates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Geobacter sulfurreducens* PCA was routinely cultured at 30 °C in anaerobic DB medium[20] with 20 mM acetate and 40 mM fumarate. Native CBP strains (Table S1) were cultured at 35 °C in anaerobic GS2 media[27] supplemented with 0.2% cellobiose (GS2-CB). When indicated, 0.2% (w/v) corn stover (processed and pretreated with the AFEX method[8] and ground and sieved (0.75 mm pore size) to a homogeneous powder with ca. 8% moisture content) (AFEX-CS) was used as a carbon source. Growth of the CBP strains with AFEX-CS was evaluated by inoculating late exponential-phase GS2-CB cultures into anaerobic DB medium with AFEX-CS to a final OD_{660} 0.04. The cultures were incubated at 35 °C and spectrophotometric readings (OD_{660}) were taken every 12 h after resuspending the cultures by inversion and allowing the solids to settle for 20 min.

Batch cultures with fumarate. Late-exponential phase cultures of *C. uda* ATCC 21399 and *G. sulfurreducens* grown at 30 °C in DB medium with cellobiose (0.2%) or acetate (20 mM) and fumarate (40 mM), respectively, were inoculated to an OD_{660} of 0.02 in the same (coculture) or separate (monocultures) tubes containing anaerobic DB medium with 0.2% (w/v) AFEX-CS and 40 mM fumarate. Control monocultures of *G. sulfurreducens* contained AFEX-CS and 40 mM fumarate or 20 mM acetate. When indicated, *G. sulfurreducens* was also grown with or without AFEX-CS in the presence of acetate (20 mM) and fumarate (40 mM). All cultures were incubated at 30 °C and planktonic growth (OD_{660}) was periodically monitored of undisturbed cultures. Three replicates were sacrificed every 48 h for pH measurements of the fermentation broth and for GC and HPLC analyses, as described in the Supporting Information (SI). Cells in the cocultures were differentially stained with the SYTO 9 (green, Gram-negative, *G. sulfurreducens*) and hexidium iodide (red, Gram-positive, *C. uda*) dyes in the BacLight Gram Stain kit (Invitrogen), as recommended by the manufacturer. The stained cells were adsorbed onto glass slides, imaged at random locations using a fluorescence microscope, and counted to calculate the relative percentage of each strain.

MECs. Dual-chambered, H-type MECs, set up as previously described,[20] were autoclaved before adding 90 mL of sterile, anaerobic DB medium to the anode and cathode chambers. The reference electrodes (3 M Ag/AgCl, Bioanalytical systems Inc.) were sterilized by Tyndallization in anaerobic Luria−Bertani medium (four cycles, each comprising 30 min in boiling medium and 24 h at 30 °C) and then in 70% ethanol for 1 min before rinsing with sterile water. The anode electrode was poised to 0.24 V with a VSP potentiostat (BioLogic) and the MEC chambers were sparged with filter-sterilized N_2:CO_2 (80:20) gas until the current stabilized. Cells were harvested by centrifugation (6000*g*, 6 min, 25 °C) from a 40% (v/v) inoculum of an early stationary-phase culture of *C. uda* or *G. sulfurreducens* grown at 30 °C in DB medium with cellobiose or acetate and fumarate, respectively. The cells were washed once, and resuspended in 10 mL of DB medium before inoculating them separately (monocultures) or together (coculture) into an anode chamber containing 0.2% (w/v) AFEX-CS. Alternatively, a sequential inoculation strategy was followed in which *G. sulfurreducens* anode biofilms were first grown with 1 mM acetate until the acetate was depleted and the current declined. The medium of the anode chamber was then replaced with fresh DB-AFEX-CS medium with or without 35 mM NH_4Cl supplementation inside a glovebag (Coy Laboratory Products, Inc.). When indicated, the anode chamber was also inoculated with *C. uda* cells. All MECs were incubated at 30 °C with stirring and without sparging of the anode chamber. The cathode chamber was sparged continuously to prevent crossover of H_2 into the anode chamber. The percent of cathodic H_2 recovered in our system was determined by discontinuing the sparging of the cathode chamber, sampling the headspace and analyzing the gas composition by GC, as described in the SI. Fermentation products in the anode broth were analyzed by HPLC and the AFEX-CS was also collected and used to estimate the hydrolysis efficiency, fermentation efficiency, and energy recovery, as described in the SI. When indicated, nitrogen assimilation was monitored over time by determining the concentration of NH_4^+ in the fermentation broth. Briefly, 60 μL of culture supernatant fluids were mixed with 120 μL of Nessler's reagent (Fluka) and the optical density of the solution at 425 nm was measured and compared to a standard curve generated with NH_4Cl as a standard.

RESULTS

CBP of AFEX-CS coupled to fumarate reduction by *G. sulfurreducens*. Fifteen CBP strains grew at 35 °C with the AFEX-CS substrate anaerobically in GS2 medium over the course of approximately two weeks (Table S1). Four actinobacterial strains had the highest ethanologenic yields (ca. 50% of the maximum theoretical yield) and robust growth (SI Table S1) and produced acetate, formate, lactate and succinate as fermentation byproducts (SI Table S2). Acetate accounted for 80.2% (±1.8) of all of the electrons potentially available as electron donors for *G. sulfurreducens*, whereas the remaining electrons were distributed between formate (9.0% ± 0.7), succinate (6.4% ± 2.0) and lactate (4.4% ± 0.6). Less than

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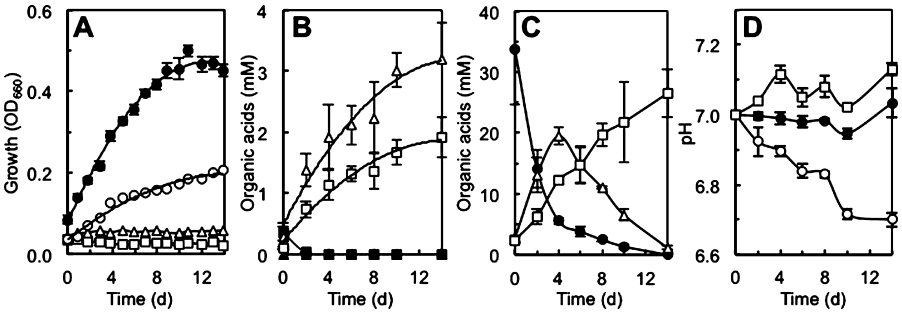

Figure 1. Syntrophic growth of *G. sulfurreducens* and *C. uda* in batch cultures with AFEX-CS and fumarate at 30 °C. (A) Growth (OD_{660}) of the coculture (solid circles) and *C. uda* (open circles) or *G. sulfurreducens* (open squares) monocultures. (B) AFEX-CS fermentation products in the coculture (solid symbols) and *C. uda* monoculture (open symbols). Only formate (triangles) and acetate (squares) were detected. (C) Reduction of fumarate (solid circles) to succinate (open squares) with the transient accumulation of malate (open triangles) in cocultures. (D) pH profile in the coculture (solid symbols) and *C. uda* (open circles) or *G. sulfurreducens* (open squares) monocultures.

10 μM of H_2 was detected in the culture headspace. *C. uda* 21399 was selected for further studies based on its robust anaerobic growth,[28] cofermentation of hexose and pentose sugars,[29−31] and well-characterized cellulase and xylanase enzymes[29,32−34] and physiology.[28]

The syntrophic growth of *C. uda* and *G. sulfurreducens* was investigated in batch cultures using DB medium (the standard MEC medium) with AFEX-CS as the sole carbon and energy source for *C. uda* and with fumarate serving as the terminal electron acceptor for *G. sulfurreducens* (Figure 1A). The increases in optical density of the coculture and the *C. uda* monoculture both followed a polynomial biphasic distribution (R^2 = 0.991 and 0.975, respectively) as expected of cells that first grow exponentially and then enter stationary phase. Growth rates during the exponential phase of growth (approximately the first 4 days) were similar in the coculture (0.42 ± 0.03 d^{-1}) and the *C. uda* monoculture (0.48 ± 0.08 d^{-1}). However, growth yields were 2.4-fold higher in the coculture (0.50 ± 0.01, OD_{660}) than in the *C. uda* monoculture (0.21 ± 0.01, OD_{660}), as growth was stimulated when the two strains grew syntrophically. At the end of the coculture experiment, *G. sulfurreducens* accounted for 42% (±7%) of the cells in the coculture, suggesting that the growth of the two strains was syntrophically maintained at constant ratios (50:50) throughout the incubation period. No growth was observed in the *Geobacter* monocultures (Figure 1A) or in monocultures of *G. sulfurreducens* in which fumarate had been replaced with acetate (data not shown). Thus, AFEX-CS was not used as either an electron donor or acceptor by *G. sulfurreducens*. Furthermore, doubling times (8.7 ± 0.5 h) and growth yields (0.77 ± 0.05, OD_{660}) in *G. sulfurreducens* monocultures with acetate and fumarate and supplemented with AFEX-CS were similar to cultures without the AFEX-CS (8.7 ± 0.3 h and 0.79 ± 0.04 OD_{660}, respectively), thereby ruling out any growth inhibition or stimulation by the AFEX-CS.

The maximum ethanol concentrations detected over the duration of the experiment were similar in the coculture (1.8 ± 0.1 mM) and the *C. uda* monocultures (1.6 mM ± 0.4). No fermentation byproducts were detected in the coculture broth during the course of the experiment (Figure 1B), nor was H_2 detected in the headspace of the coculture vessel. In contrast, acetate and formate accumulated in the *C. uda* monocultures (Figure 1B) following the same biphasic polynomial distribution (R^2 = 0.966 and 0.952, respectively) as the optical density of the *C. uda* monoculture (Figure 1A), as expected of a metabolic process coupled to cell growth. The removal of waste fermentation products by *G. sulfurreducens* in the coculture was coupled to the reduction of fumarate to succinate and the transient accumulation of malate (Figure 1C). In contrast, fumarate levels remained constant (ca. 40 mM) in the *C. uda* monocultures, and only fermentative succinate was produced (0.5 mM ± 0.1). The removal of the organic acids by *G. sulfurreducens* also prevented the acidification of the coculture medium (Figure 1D).

CBP of AFEX-CS to ethanol in a MEC. We investigated the ability of the binary culture to couple the fermentation of AFEX-CS into ethanol and electricity in a MEC in reference to monoculture MEC controls. Current started soon after the two strains were inoculated simultaneously into anode chambers supplemented with AFEX-CS and increased exponentially (6.3 ± 0.2 mA h^{-1}) until reaching a maximum of 1.0 (±0.1) mA (Figure 2A). The current then decreased slowly to <0.1 mA over a period of 4 days, suggesting that growth had become limiting in one or both of the strains. Although some acetate (0.24 ± 0.03 mM in uninoculated controls, Figure 2B) was provided in the AFEX-CS,[35,36] it was too low to support the growth of the anode biofilm of *G. sulfurreducens* and, as a result, no current was produced in MECs driven by *G. sulfurreducens* monocultures (Figure 2A). Similarly, no current was produced in the *C. uda* monocultures (Figure 2A).

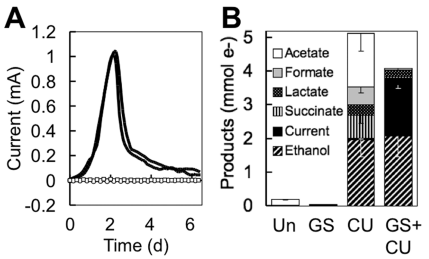

Figure 2. Simultaneous inoculation of *G. sulfurreducens* and *C. uda* in MECs with AFEX-CS. (A) Current production by the coculture in two representative MECs (solid lines) and in controls with *G. sulfurreducens* (open circles) or *C. uda* (open squares) monocultures. (B) Yields of current and fermentation products (expressed in electron equivalents, mmol e-) in the MECs described in (A). Shown are averages and standard deviations of three independent MECs for each. Un, uninoculated; GS, *G. sulfurreducens* monoculture; CU, *C. uda* monoculture; GS+CU, *G. sulfurreducens* and *C. uda* coculture.

Similar amounts of AFEX-CS were hydrolyzed in the *C. uda* monocultures (36 ± 8%) and in the coculture (42 ± 6%) and

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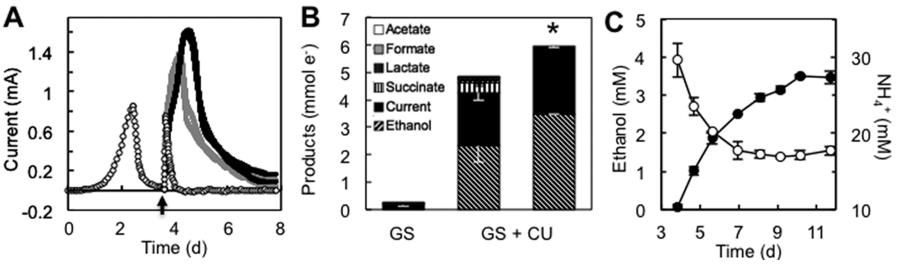

Figure 3. Sequential inoculation of *G. sulfurreducens* and *C. uda* in MECs. (A) Current production by a *G. sulfurreducens* monoculture driven by an initial acetate supplementation and then by the residual acetate in AFEX-CS (open circles), which was added while exchanging the medium (arrow). Inoculating the MEC with *C. uda* during the media exchange increased and further sustained current production (gray solid lines, two representative experiments shown). Supplementing the AFEX-CS media with 35 mM NH4Cl increased electricity production further production (black solid lines, two representative experiments shown). (B) Yields of current and fermentation products (expressed in electron equivalents, mmol e^-) measured in the anode chamber of MECs driven by the *G. sulfurreducens* monoculture (GS) and the cocultures (GS+CU) without or with (star) NH_4Cl supplementation. (C) Ethanol production (solid symbols) from AFEX-CS coupled to nitrogen assimilation (NH_4^+ equivalents, open symbols) in MECs supplemented with NH_4Cl.

fermentation efficiencies (~ 99% for glucose and ~98% for xylose) were comparable in both. Furthermore, ethanol concentrations increased over the duration of the experiment and reached a similar plateau in both the *C. uda* monoculture (1.8 ± 0.5 mM) and the coculture (1.9 ± 0.6 mM). Acetate (2.2 ± 0.8 mM), formate (2.9 ± 1 mM), lactate (0.3 ± 0.1 mM) and succinate (0.6 ± 0.2 mM) accumulated in the *C. uda* monocultures but were removed in the coculture (Figure 2B). Overall, 51% of the total electrons available through fermentation in the coculture were recovered as ethanol, 42% were diverted to current, and 7% remained as unutilized fermentation byproducts (Figure 2B). Of the fermentation byproducts removed by *G. sulfurreducens,* approximately 60% were utilized for current production with the remaining 40% being used to support the growth of the *G. sulfurreducens* anode biofilm. As a result, the net yield of moles of electrons recovered as current or fermentation products in the coculture MEC was lower than in the monoculture (Figure 2B).

The energy recovery from the fermentation of AFEX-CS to ethanol by the *C. uda* monocultures, which considered the energy outputs from the heat of combustion of ethanol and the energy inputs from the AFEX pretreatment of corn stover, was 32 (±2) % (Figure 4). Despite the electrical input resulting from applying the MEC potential, energy recoveries from fermentation alone were similar (30 (±9) %) in the MEC-driven by the coculture. Furthermore, approximately 72% of the moles of H_2 that are theoretically possible from the measured current were also recovered as H_2 fuel in the headspace of the cathode chamber of the coculture MEC. When the energy output from the heat of combustion of the cathodic H_2 was included in the calculations, the energy recovery from the AFEX-CS in the MEC increased to 45 (±10) % (Figure 4).

We also investigated the performance of the MEC platform when the microbial catalysts were sequentially inoculated, as previously reported for a binary culture of *C. cellulolyticum* and *G. sulfurreducens*.[37] *G. sulfurreducens* anode biofilms produced some current from AFEX-CS in the MECs (Figure 3A), due to the availability of AFEX-CS-derived acetate as an electron donor (0.17 ± 0.02 mmol e^-). However, substantially more current (2 ± 0.3 mmol e^-) was produced in the MECs inoculated with *C. uda* due to the syntrophic growth of the strains (Figure 3A). Approximately, 28% (±6) of the AFEX-CS was degraded in the coculture-driven MEC, which is lower than the hydrolysis efficiencies measured with the simultaneous strategy. Fermentation efficiencies (~ 99% for glucose and ~96% for xylose) were also similar. Maximum ethanol yields (2.1 ± 0.6 mM) were similar to those obtained in the simultaneous inoculation, and approximately half of the succinate (0.3 ± 0.1 mM) produced in the *C. uda* monocultures accumulated in the fermentation broth (Figure 3B). Overall, the ethanol:current ratio (48:41% of all the electron equivalents available from fermentation) was similar as in the simultaneously inoculated platform (51:42%), but more electrons (11%) were lost to unutilized fermentation byproducts, mostly succinate (Figure 3B). Succinate is not an electron donor for *G. sulfurreducens* but can be assimilated for carbon. As the anode biofilms were pregrown with acetate, the carbon demands of the biofilms were low and less succinate was removed. Yet a higher percentage of the electrons in the fermentation products removed by *G. sulfurreducens* were converted into electrical current (69%) in the sequentially inoculated MEC compared to the simultaneous inoculation strategy (60%), with the remaining (31%) being used for cell growth. As a result, the energy recovery as ethanol and H_2 in the MEC (49% ± 12) was within the ranges calculated for the simultaneously inoculated MECs and was almost twice the energy recovery calculated for the fermentation to ethanol alone (29% ± 12) (Figure 4).

Stimulation of ethanol production in a MEC supplemented with nitrogen. Interestingly, the inoculation strategy did not affect the composition of the anode biofilms, which had confluent biofilms of mostly *G. sulfurreducens* cells (SI Figure S1A and B), but increased the biofilm biomass compared to the *G. sulfurreducens* monocultures (SI Figure S1C). *C. uda* cells did not attach to the bare anode electrodes in the *C. uda* monocultures (SI Figure S1D), and preferentially grew planktonically or as biofilms on the AFEX-CS solids (SI Figure S1E). Acid hydrolysates of the AFEX-CS biofilms also contained glucose levels 1.25 fold higher than the glucan content provided in the initial amount of AFEX-CS. This is consistent with the acid hydrolysis of the Curdlan (*β*-1,3 glucan) biofilm matrix of *C. uda*. As the Curdlan matrix that enables *C. uda* cells to specifically attach to cellulosic substrates is induced when nitrogen is growth-limiting,[38] we investigated if nitrogen availability limited the growth and metabolism of *C. uda* in the MEC driven by the coculture. For these experiments, we used a sequential inoculation strategy and grew *G. sulfurreducens* anode biofilms with the standard low nitrogen medium and with acetate as electron donor. Once the current declined, the anode medium was replaced with AFEX-CS medium supplemented with 35 mM NH_4Cl (ca. 10-times the

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NH_4Cl concentration in the standard MEC medium, SI Figure S2). After inoculating the anode chamber with *C. uda*, current increased to approximately 1.6 mA and then slowly decreased as all the fermentation byproducts were utilized (Figure 3A). Hydrolysis efficiencies increased (46 ± 1%) to levels comparable to those measured in the simultaneously inoculated MECs. Furthermore, ethanol yields were almost twice than those measured in any of MEC platforms and all of the fermentation byproducts were converted into current (Figure 3B). Ethanol production was coupled to nitrogen assimilation and reached a plateau once nitrogen assimilation stopped (Figure 3C). The data thus support our original hypothesis that nitrogen availability limited the growth and metabolism of *C. uda* in the MECs. As a result, nitrogen supplementation alleviated the growth limitation of *C. uda* and promoted the hydrolysis of AFEX-CS and ethanologenesis. This resulted in 2-fold increases in the energy recoveries from the fermentation of AFEX-CS to ethanol (56 ± 1%) (Figure 4). Furthermore, by

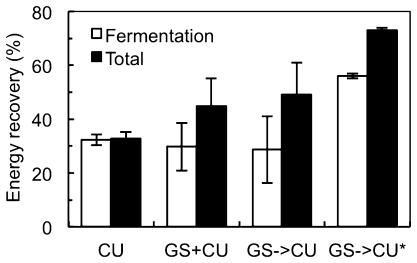

Figure 4. Energy recoveries from AFEX-CS as ethanol (fermentation, open columns) or ethanol and cathodic H_2 (total, solid columns) in MECs driven by *C. uda* (CU) or by cocultures of *G. sulfurreducens* and *C. uda* inoculated simultaneously (GS+CU) or sequentially (GS→CU). The sequential coculture labeled with a star (*) was grown in medium supplemented with 35 mM NH_4Cl.

stimulating the electrical conversion of all the fermentation byproducts, nitrogen supplementation also minimized electron losses and resulted in high (73 (±1) %) energy recoveries as ethanol and cathodic H_2 (Figure 4).

DISCUSSION

The results show that the CBP of AFEX-CS to ethanol can be achieved with high energy recoveries in a MEC driven by a defined binary culture selected for its robust saccharification of AFEX-CS, ethanologenesis and electrochemical removal of waste fermentation products. The identification of a range of native CBP microorganisms from the Actinobacteria and Firmicutes groups with robust growth and high yields of ethanol with AFEX-CS is consistent with previous studies indicating that the AFEX pretreatment increases the digestibility of lignocellulose substrates[8,39,40] while minimizing the release of toxic byproducts.[36] *C. uda*, in particular, had robust growth and produced high yields of ethanol with AFEX-CS. In addition, it produced fermentation byproducts (acetate, formate, and lactate) that serve as electron donors for the exoelectrogen *G. sulfurreducens*.[18−20] As a result, a MEC driven by the defined binary culture composed of *C. uda* and *G. sulfurreducens* converted AFEX-CS into ethanol while minimizing electron losses to waste fermentation products that limited the performance of previous MFC platforms.[37,41,42] The removal of waste organic acids from the fermentation broth by *G. sulfurreducens* also prevented the acidification of the media and the accumulation of feedback inhibitors. Acetate, in particular, is a noncompetitive inhibitor of cellobiose metabolism in *C. uda*, presumably because it interferes with cellobiose uptake.[43] Hence, its removal by the exoelectrogen promotes the uptake of cellobiose by *C. uda* and also prevents cellobiose accumulation, which would otherwise feedback-inhibit cellulase synthesis.[44]

Despite the electrical input in the MEC and the energy input required to pretreat the corn stover, total energy recoveries as ethanol and cathodic H_2 averaged 47% in systems run with the standard (low nitrogen) medium routinely used to support the growth and electroactivity of the exoelectrogen *G. sulfurreducens* on the anode electrode.[20] These recoveries are significantly higher than those reported in MFCs fed with raw (3.6%) or steam-pretreated (2%) corn stover, where only power is generated.[25] Thus, ethanol production from AFEX-CS in a MEC, with the added value of converting current into H_2 at the cathode, is a competitive platform. Ethanol yields were similar in MECs run with the standard medium when the strains were inoculated simultaneously (Figure 2) or sequentially (Figure 3). Each strategy has its own advantages. For example, more AFEX-CS was hydrolyzed in the simultaneous platform but fewer molar electron equivalents (ca. 86%) were recovered from the substrate that was hydrolyzed, as more of the fermentation byproducts were used as carbon sources and electron donors to sustain the growth of the anode biofilm. The simultaneous platform also simplifies MEC operation with the coinoculation of the strains, which reduces operational costs and the risk of contamination. On the other hand, a higher percentage of the electrons in the fermentation products were converted into electrical current (69%) in the sequentially inoculated MEC and less was diverted to support cell growth. However, fermentation byproducts such as succinate were left unutilized in the sequential platform, which could have had a feedback-inhibitory effect on sugar fermentation and hydrolysis efficiency.[43]

The observation that *C. uda* biofilms formed on the AFEX-CS solids (SI Figure S1) suggested that nitrogen availability in the MEC medium limited growth. This CBP bacterium secretes free cellulases to degrade lignocellulose substrates. However, cell-associated cellulases are expressed when nitrogen becomes growth-limiting and function as cell adhesins for the specific colonization of cellulosic substrates.[38] Although the biofilms continue to degrade the substrate, nitrogen limitation redirects fermentable sugars away from its fermentative metabolism and toward the synthesis of a Curdlan ($\beta(1-3)$ glucan) biofilm matrix.[38] Consistent with this, supplementing the MEC medium with excess nitrogen alleviated the growth limitation of *C. uda* and promoted the hydrolysis of AFEX-CS and ethanologenesis in the sequentially inoculated platform. Nitrogen supplementation of the growth medium was also reportedly necessary to increase ethanol yields and AFEX-CS hydrolysis efficiencies by the CBP microorganism *C. phytofermentans*, which the authors attributed to the high energy demand derived from the secretion of hydrolytic enzymes.[14] Overall, nitrogen supplementation in the MECs resulted in 2-fold increases in energy recoveries from ethanologenesis alone (Figure 4). It also promoted the removal and electrical conversion of all the fermentation byproducts and further increased the total energy recoveries as ethanol and cathodic H_2 to 73 (±1)% (Figure 4). Interestingly, nitrogen assimilation by *C. uda* stopped before all the available nitrogen was used (Figure 3C), suggesting that other factors limited the growth of the CBP partner in the MECs over time. As ethanol

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production was coupled to nitrogen assimilation (Figure 3C), further optimization of culturing parameters is likely to improve AFEX-CS hydrolysis and ethanol yields and the overall performance of the platform.

The MEC platform fed with AFEX-CS and described herein addresses the need to decouple bioenergy production from the food supply, to reduce processing costs through the use of lignocellulose substrates, and to carry out a single-step hydrolysis and fermentation while minimizing the accumulation of low-value fermentation byproducts that can also function as feedback inhibitors.[11] Relatively simple culturing approaches such as nitrogen supplementation were sufficient to improve the growth of the CBP partner and the electrical conversion of waste fermentation products by the exoelectrogen in the MEC. Further optimization of the culturing conditions shows promise to increase the activity of the microbial catalysts so as to improve the performance of the platform. This, and the possibility of genetically engineering and/or adaptively evolving the microbial catalysts for improved hydrolysis, saccharification, and electrical conversion, suggests that the processing of lignocellulose substrates in MECs can provide an economically and environmentally attractive CBP technology for ethanol and H_2.

■ ASSOCIATED CONTENT

Supporting Information

Additional materials and methods, figures and tables are included in the *SI*. This information is available free of charge via the Internet at http://pubs.acs.org/ .

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Notes

The authors declare no competing financial interest.

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