Supplementary Information

Efficient Solar-to-Fuels Production from a Hybrid Microbial | Water-Splitting Catalyst System

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Index *Page*

Materials and Methods

General Reagents. Cobalt nitrate hexahydrate ($Co(NO_3)_2 \cdot 6H_2O$, Alfa Aesar), potassium hydroxide (KOH, Sigma Aldrich) dihydrogen potassium phosphate (KH_2PO_4, Alfa Aesar) were purchased from the indicated supplier and used as received. Electrode materials consisted of 304 stainless steel foil (Strem Chemicals Inc.), 304 stainless steel mesh (McMaster-Carr) or nickel mesh (Unique Wire Weaving Co.) and were cut with scissors to the appropriate dimensions and sonicated in acetone, propanol and then water for 10 min.

Electrode Fabrication. Catalyst depositions were accomplished by using a CH Instruments model 760D potentiostat. The geometric area of submerged electrode was typically 2 cm^2 unless otherwise stated. The stainless steel mesh was the same for both CoPi and NiMoZn electrodes and had an effective surface area of ~ 1 cm^2 per geometric cm^2 of mesh. Catalyst deposition was conducted in solutions composed of water (18 MΩ cm resistivity, <3 ppb total organic carbon) provided by a Millipore Milli-Q water purification system.

The NiMoZn cathode was fabricated by electrodepositing onto a substrate of either plainer stainless steel, 304 stainless steel mesh or nickel mesh using a previously reported procedure.[1] The cobalt phosphate (CoP_i) catalyst was deposited by bulk electrolysis in a two-compartment electrochemical cell with a glass frit junction of fine porosity. For electrodeposition, the working compartment was charged with a ~50 mL solution consisting of 0.1 M KP_i and 0.5 mM Co^{2+}. The auxiliary compartment was charged with a ~50 mL solution of 0.1 M KP_i. Bulk deposition was also carried out in a single vessel containing 0.1 M KP_i and 0.5 mM Co^{2+}. In all cases the working electrode was either plainer stainless steel or 304 stainless steel (SS). Electrolysis was carried out at 0.85 V vs Ag/AgCl until the desired amount of charged was passed. Typically 100 mC of charge was passed for a 2 cm^2 electrode (50 mC/cm^2). Deposition times were typically ~1 h.

Preparation of heat-inactivated catalase. 20 mg of bovine liver catalase (Sigma, C1345) was dissolved in 50 mL minimal medium and sterilized by passage through a 0.45 μm SFCA filter. *R. eutropha* were then added to an appropriate OD and before addition of the medium to the H-cell. For experiments in which bovine liver catalase was heat-inactivated, the above procedure was repeated, but the filtered minimal medium was heated to 95 °C for 10 min and cooled to room temperature before addition of cells and transfer to the H-cell.

Bioelectrochemical reactors. For the H-cell experiments, *R. eutropha* pre-adapted to H_2 were suspended at an OD of 0.05 in 50 mL of minimal medium and added to each of two chambers in an electrochemical H-cell. Two 50 mL chambers were separated by a 0.45 μm

[1] Reece SY, Hamel JA, Sung K, Jarvi TD, Esswein AJ, Pijpers JJH, Nocera DG (2011) Wireless solar water splitting using silicon-based semiconductors and earth-abundant catalysts. *Science* 334:645–648.

SFCA filter to prevent exchange of microbes between them. Each chamber contained a 1.5 cm stir bar rotating at 200 rpm. Electrodes were suspended in the medium from above, and a plastic enclosure was fitted around the H-cell to prevent contamination from the air. In all other experiments, the center of a suba-seal white rubber septum (Sigma, Z167355) was punctured, and a medium-frit gas dispersion tube (Chemglass, CG-207-02) inserted through it. The septum and tube was then inserted into the top of a clean, dH_2O-rinsed 40 mL screw-top glass vial (Sigma, 27379) containing a 1 cm magnetic stir bar. The top of the gas dispersion tube was then fitted with rubber adapters, covered in foil, and autoclaved for 20 min. Once it cooled to room temperature, the foil was removed and a sterile 0.45 μm syringe filter (Thermo, 190-2545) was attached to the adapter. Sterile 18-gauge needles were then used to puncture temporary holes on different sides of the rubber septum, ethanol-sterilized electrodes inserted through them, and the needles removed. Finally, a sterile, truncated 20 μL pipette tip was inserted next to the dispersion tube to provide a gas outlet. 35 mL of minimal medium containing *R. eutropha* pre-adapted to growth on hydrogen was then injected through the gas outlet, and the electrochemical cell immediately attached to the appropriate electrodes and CO_2 gas line and submerged in a water bath maintained at 30 °C.

Up to eight electrochemical cells were controlled potentiostatically using a Gamry Reference 600 potentiostat and ECM 8 multiplexer. A custom script was employed to control each channel's potential independently. Current data was recorded and analyzed using Gamry Framework software. Every day (20 - 28 h), 200 μL of culture was extracted from each electrochemical cell using sterile long-neck Pasteur pipettes (Kimble Chase, 63B95P), diluted ten-fold in minimal medium, and the OD_{600} measured. For some experiments, these diluted cell suspensions were used to test viability via spot assays. For HPLC analysis, an additional 500 μL was extracted and frozen for later analysis. When necessary, dH_2O was resupplied to offset medium loss to evaporation. Average five-day volume loss and resupply was 17 ± 2%.

Bacterial strains and media composition. *R. eutropha* H16 (wild-type) and Re2133-pEG12 were obtained from the Sinskey laboratory at MIT and the latter is described in Grousseau *et al*.[2] Rich broth was prepared as described previously[3] (16 g/L nutrient broth, 10 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$), then sterilized by autoclaving for 30 min. Minimal medium was prepared as previously described[4] and filter sterilized. The final composition of the minimal medium was 6.74 g/L $Na_2HPO_4 \bullet 7H_2O$, 1.5 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$,

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[2] Grousseau E, Lu J, Gorret N, Guillouet SE, Sinskey AJ (2014) Isopropanol production with engineered *Cupriavidus necator* as bioproduction platform. *Appl Microbiol Biotechnol* 98:4277–4290.

[3] Li H, Opgenorth PH, Wernick DG, Rogers S, Wu TY, Higashide W, Malati P, Huo YX, Cho KM, Liao JC (2012) Integrated electromicrobial conversion of CO_2 to higher alcohols. *Science* 335:1596.

[4] Schlegel HG, Lafferty R (1965) Growth of 'knallgas' bacteria (*Hydrogenomonas*) using direct electrolysis of the culture medium. *Nature* 205:308–309.

80 mg/L $MgSO_4 \bullet 7H_2O$, 1 mg/L $CaSO_4 \bullet 2H_2O$, 0.56 mg/L $NiSO_4 \bullet 7H_2O$, 0.4 mg/L ferric citrate, and 200 mg/L $NaHCO_3$.

Pre-adaptation of *R. eutropha* to growth on H_2. To grow *R. eutropha* strains electrochemically, glycerol stocks of the strain of interest were first streaked on rich broth and 1.5% agar plates containing 10 μg/mL gentamicin (Gm10). Wild-type *R. eutropha* are naturally resistant to Gm10, which provides protection against contamination by other gram-negative microbes such as *E. coli*. For growth of Re2133-pEG12, 200 μg/mL kanamycin (Kan200) was also included in the plates to maintain the pEG12 plasmid. Bacteria were allowed to grow at 30 °C for 48 - 72 h. Individual colonies were picked and inoculated into 10 mL rich broth and Gm10 (+Kan200 for Re2133-pEG12) in a 15 mL Falcon culture tube. They were then shaken overnight (~16 h) at 250 rpm and 30 °C. Saturated cultures were centrifuged at 4,000 rpm for 10 min, then resuspended in 1 mL minimal medium and diluted to an OD of 0.2 in 10 mL minimal medium, 1% D-fructose and Gm10 (+Kan200 for Re2133-pEG12) in 15 mL Falcon culture tubes. These cultures were grown until saturated (1 - 2 days) at 30 °C, 250 rpm. The centrifugation was repeated, the pellet resuspended in 1 mL minimal medium and diluted into 20 mL minimal medium and Gm10 (+Kan200 for Re2133-pEG12) in 50 mL flasks to an OD of ~ 0.2. These cultures were then sealed in an air-tight jar containing air and 8 mm Hg H_2 and 2 mm Hg CO_2; the culture was magnetically stirred at 250 rpm, 30 °C, for 2 - 4 d. Once the cultures reached an OD_{600} between 1 and 2, they were centrifuged at 4,000 rpm for 10 min and resuspended in 35 mL fresh minimal medium and Gm10 (+Kan100 for Re2133-pEG12) to an OD between 0.17 and 0.22.

Spot Assays. 100 μL of culture was diluted 1:10 in fresh minimal medium and vortexed. Four serial 10-fold dilutions were made of this sample, and 2 μL of each dilution spotted on rich broth agar plates and allowed to dry on the benchtop. Plates were typically grown for 2 d at 30 ºC before imaging.

H_2O_2 assay. To measure the H_2O_2 concentration in an electrochemical cell, 50 μL of electrolyte was transferred at a series of time points to a 96-well plate (Corning). The plate was kept on ice, in the dark, for no more than 1 h prior to measurement. Once all time points were collected, the 96-well plate was assayed using the Amplex Red H_2O_2 Detection Kit (Sigma-Aldrich) according to the manufacturers' instructions. The assay uses horseradish peroxidase to enzymatically convert H_2O_2 and a proprietary dye to resorufin (λ_{max} = 571 nm), and is therefore selective for H_2O_2 detection. Absorbance at 555 nm was measured in 200 μL 96 well flat bottom, non-treated, sterile polystyrene plates (Corning) on a Victor3V 1420 Multi-label Counter (Perkin Elmer) using a 555/38 filter. H_2O_2 was quantified by comparing A555 values from samples to a standard curve generated from H_2O_2 standards ranging from 0 to 40 μM. To calculate the initial rate of H_2O_2 production, a linear fit was performed to the data points from 0 to 8 min after the start of electrolysis.

HPLC analysis. Frozen 500 µL culture samples were thawed and passed through 0.45 µm SFCA filters, and 40 µL of each sample was analyzed by HPLC. HPLC was performed on an Agilent HPLC 1200 equipped with an Aminex HPX-87H column. The mobile phase, a filtered solution of 0.0025 N H_2SO_4, was run at a flow rate of 0.5 mL/min for 30 min. Column effluent was analyzed with a UV detector (210 nm) to identify pyruvate, and a refractive index detector to identify acetone and isopropanol. Known standards of sodium pyruvate, acetone and isopropanol were used to determine retention times and generate a standard curve from 0 to 500 mg/L for quantification.

Calculation of maximum biomass and isopropanol yields

Maximal thermodynamic yields of biomass and isopropanol were calculated from their Gibbs energies of formation under standard conditions. Grosz *et al.*[5] estimated the standard Gibbs free energy of formation of biomass in *E. coli* to be −46 kJ/mol carbon. Biomass composition is typically taken to be $CH_{1.77}O_{0.49}N_{0.24}$. Using the biomass synthesis equation $CO_2(g) + 0.24\ NH_3^+(aq) + 0.525\ H_2O(\ell) \rightarrow CH_{1.77}O_{0.49}N_{0.24} + 1.02\ O_2(g)$ we calculate that for biomass, ΔG^o = 479 kJ/mol carbon. As the molecular weight of biomass containing 1 mol carbon is 25 grams dry cell weight (gDCW), the maximum thermodynamic biomass yield is 25 gDCW/479 kJ ~ 52 mgDCW/kJ. Isopropanol has ΔG_f^o = −180 kJ/mol. As the combustion of isopropanol follows $CH_3CH_2OCH_3(aq) + 4.5\ O_2(g) \rightarrow 3\ CO_2(g) + 4\ H_2O(\ell)$, synthesis of a mole of isopropanol from CO_2 and water would require 1,950 kJ. Given that isopropanol has a molecular weight of 60.1 g/mol, the maximal yield of isopropanol from energy is 60.1 g / 1,950 kJ ~ 31 mg/kJ. Using a similar approach, for Table S1 we calculated the energies for synthesis of isobutanol and 3-methyl-1-butanol to be 2,590 kJ/mol and 3,220 kJ/mol respectively.

Tafel Analysis

Anodes were compared to benchmark catalyst function in minimal medium. Working electrodes with a geometric area of ~2 cm^2 consisted stainless steel 304 mesh with 100 mC electrodeopositied CoP_i. Tafel analysis was conducted using multi-current step measurements between 5 mA - 1 µA allowing between 300 - 1200 s for equilibration between steps. Solution resistance was measured and resistive voltage contributions were subtracted from the total measured OER overpotential. All Tafel measurements were conducted in a two-compartment electrochemical cell with a glass frit junction of fine porosity. Both working and auxiliary compartments were charged with ~50 mL minimal medium for the comparison. Auxiliary and reference electrodes were platinum and Ag/AgCl respectively. The Tafel slope for SS304 + CoP_i was 75 mV/dec in good agreement with previously reported values.

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[5] Grosz R, Stephanopoulos G (1983) Statistical mechanical estimation of the free energy of formation of E. coli biomass for use with macroscopic bioreactor balances. *Biotechnol Bioeng* 25:2149–2163.

Additional Tafel analysis was conducted to compare CoP_i performance in growth media and 0.5 M phosphate buffer. In these experiments the working and auxiliary compartments were charged with a ~50 mL solution consisting of 0.5 M KP_i or minimum growth medium. Tafel slopes for KP_i and minimum growth medium were 71 and 79 mV/dec, indicating no significant change in OER performance between solutions.

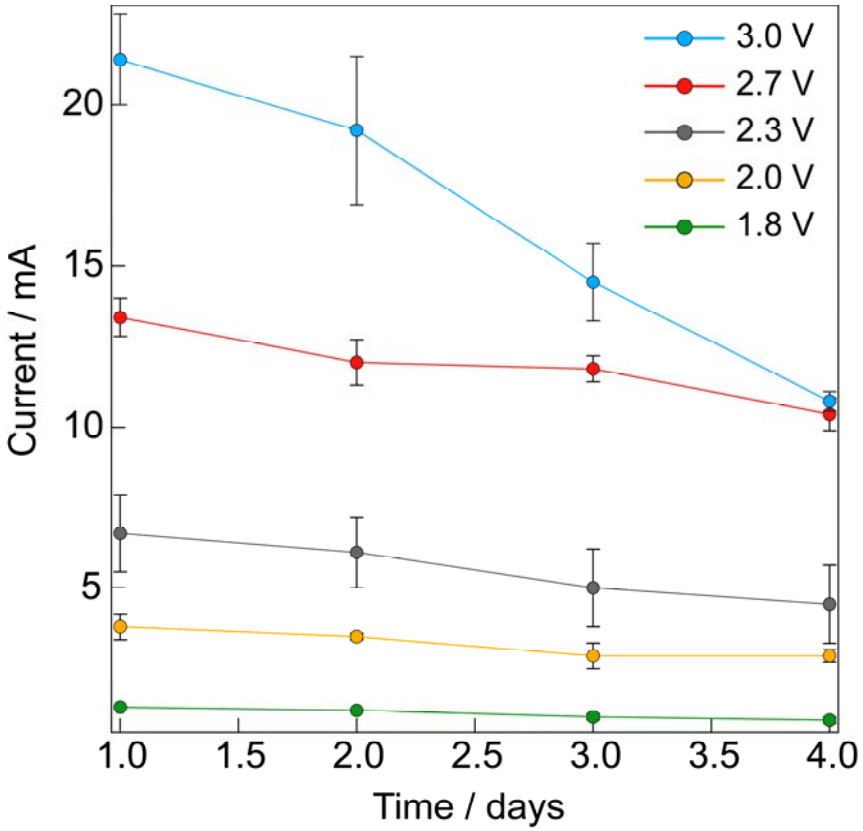

Fig. S1 | CoP_i | NiMoZn electrode cell current in minimal medium as a function of E_{cell}. The data shown correspond to the experiment shown in Fig. 2A. Mean daily current is plotted as a function of time for each cell potential tested. Error bars represent S.E.M. for N = 3-5 independent experiments.

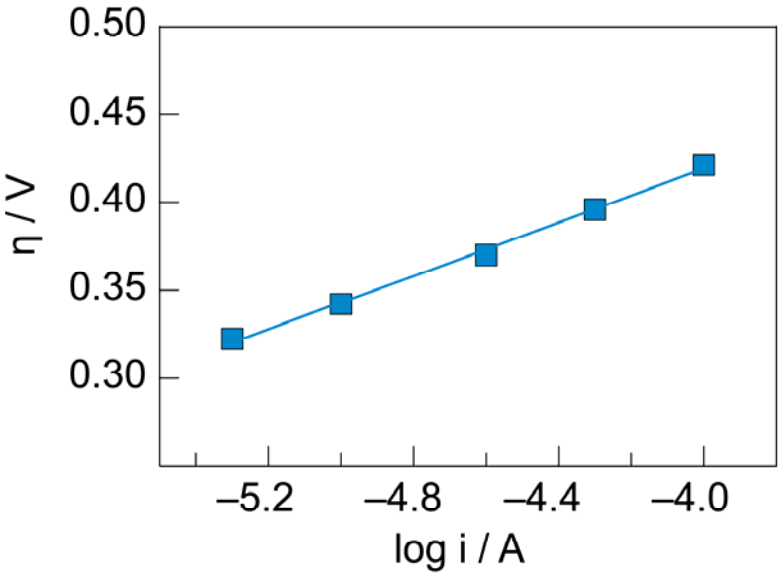

Fig. S2 | Tafel slopes. Tafel slope of CoP_i on stainless steel for OER operating in minimal medium.

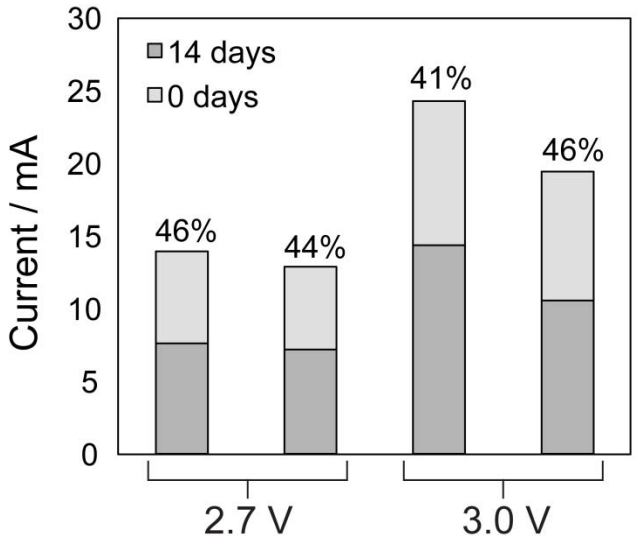

Fig. S3 | Current stability in cell cultures over two weeks. Four of the electrochemical cultures from Fig. 2A, two at 2.7 V and two at 3.0 V, were continued beyond the original 4 d electrolysis period to test the stability of CoPi/NiMoZn electrodes in the presence of minimal medium and *R. eutropha* cells. The overall height of the bar indicates the current at 0 h, while the height of the darker bar indicates current after fourteen days. The number above the bar indicates the decrease in current after two weeks of continuous operation.

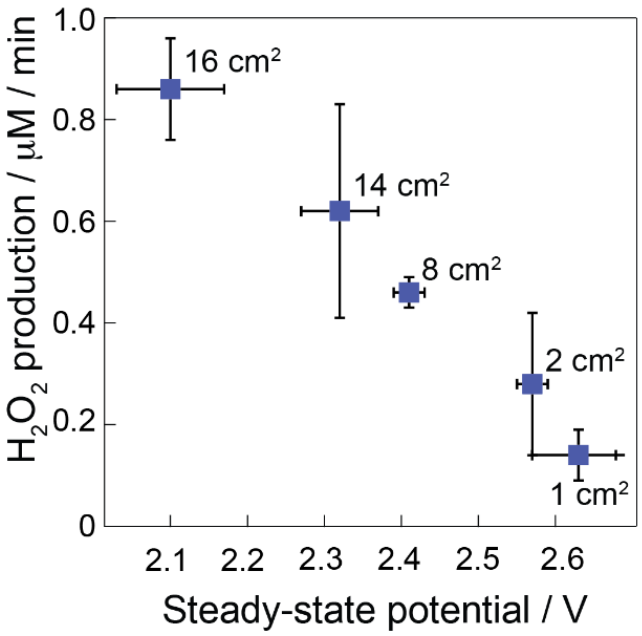

Fig. S4 | H_2O_2 production rate as a function of cell potential and electrode size. Initial H_2O_2 production rate in an electrochemical cell run galvanostatically at 5 mA as a function of total cell potential E_{cell}. Two equal-sized stainless steel mesh electrodes were used, and their geometric area (indicated in the plot area) was varied to alter the steady-state potential. H_2O_2 production rate was measured using the amplex red assay (Methods). Error bars represent S.E.M., N = 3 independent experiments for each potential.

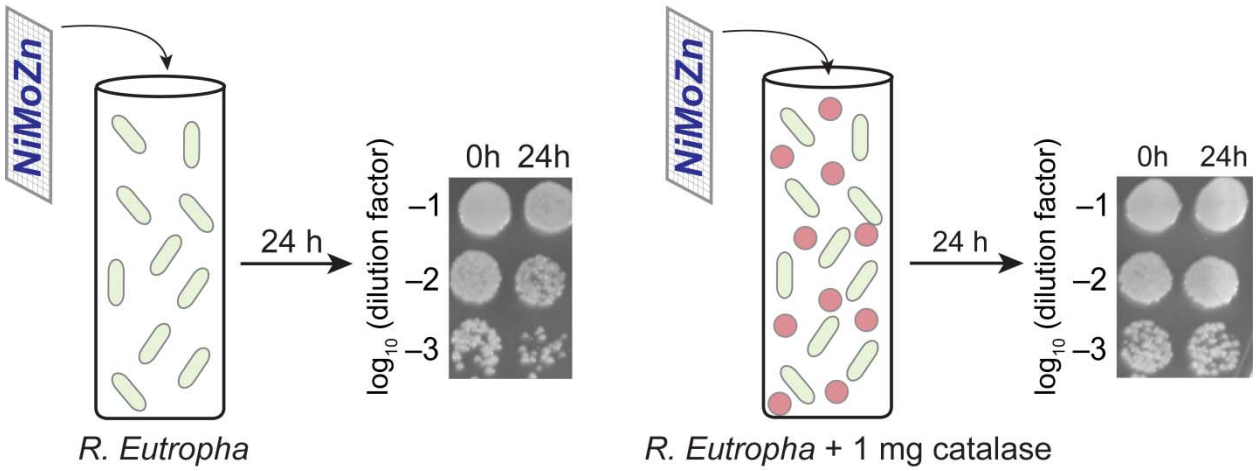

Fig. S5 | Cell viability with NiMoZn cathode at open circuit potential. Low level toxicity is observed from the NiMoZn cathode in the absence of applied current. Cathodes were incubated with *R. eutropha* for 24h with and without exogenous bovine catalase. *R. eutropha* cultures incubated with catalase were noticeably healthier compared to samples without catalase.

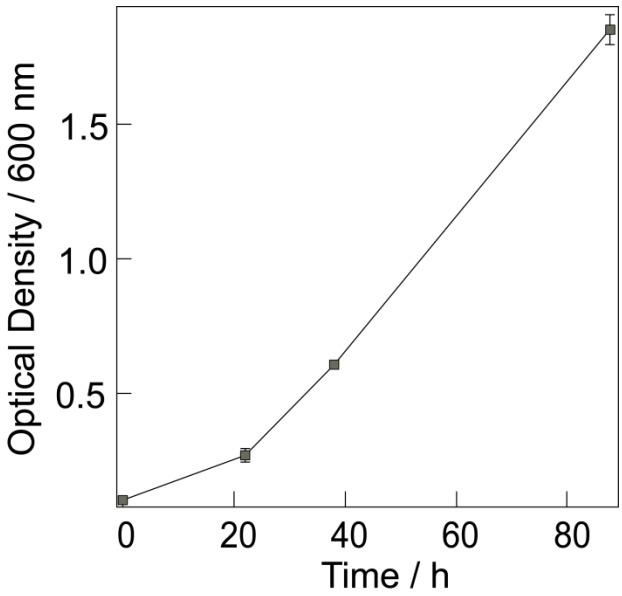

Fig. S6 | Biomass production time course using a SS cathode. Time course of biomass production by *R. eutropha* using a CoP_i anode and SS304 mesh 60 cathode. The average current passed over 88 h was 5.1 ± 0.3 mA. Error bars represent S.E.M., N = 3 independent experiments.

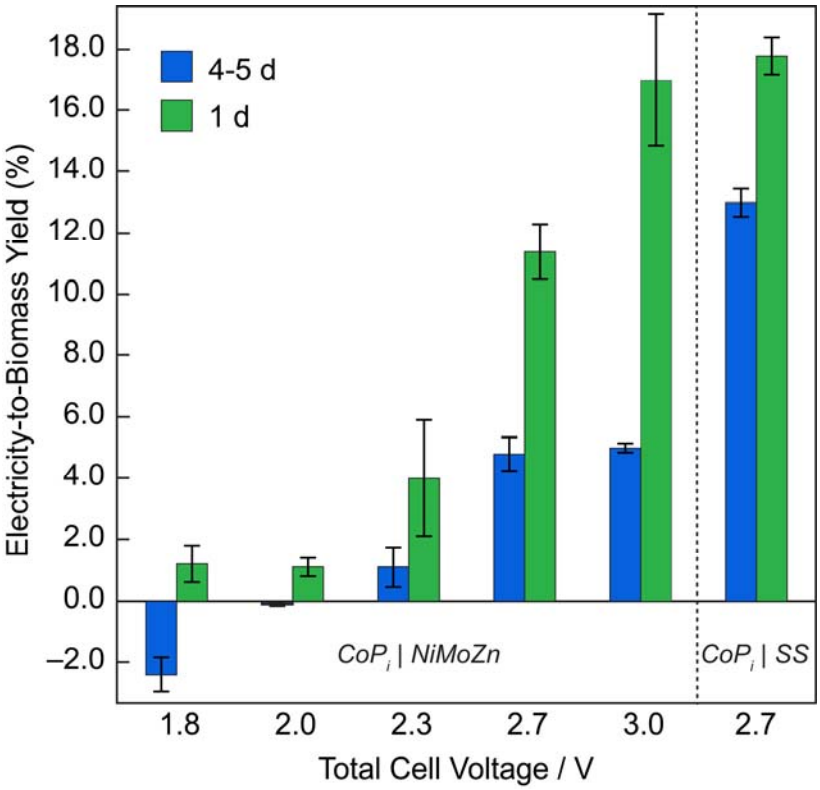

Fig. S7 | Biomass yields. Electricity-to-biomass yield as a percentage of thermodynamic maximum, plotted as a function of E_{cell} applied to a CoP_i cathode and NiMoZn or SS304 anodes. Yields were measured either over the entirety of a 4- to 5-day experiment (4-5 d), or as the highest yield observed in any given 24-hour period within the course of a given experiment (1 d). Error bars represent S.E.M., N = 3 – 5 independent experiments for each potential.

Table S1. Comparison of electrolysis conditions and biomass and fuel yields for previous studies of integrated bioelectrochemical systems

	this work	this work	Li *et al.* [a]	Schlegel *et al.* [b]
Organism/Strain	*R. eutropha* H16	*R. eutropha* Re2133-pEG12	*R.* eutropha LH74D	*R. eutropha* H16
Anode | Cathode:	CoP_i | NiMoZn	CoP_i | SS	Pt | In	Pt | Pt
Volumetric Current (mA/mL)	0.15	0.38	0.71	0.60
Total Cell Potential, E_{cell} (V)	2.7	3.0	~4	~5
Operation Time (days)	3.7	5.0	4.4	2.9
Volumetric Energy Use (kJ/mL)	0.1	0.5	1.1	0.8
Total Fuels Produced (mg/L)	–	216	150	–
Alcohol 1	–	isopropanol	isobutanol	–
Alcohol 1 Amount (mg/L)		216	~100	
Alcohol 2	–	–	3-methyl-1-butanol	–
Alcohol 2 Amount (mg/L)		–	~50	
Electricity-to-Biomass Efficiency [c]	13.0%	4.6%	1.8%	4.8%
Electricity-to-Fuel Efficiency [c]	-	1.5%	0.5%	–
Equivalent Solar-to-Biomass Efficiency [c]	2.3%	0.8%	0.3%	0.9%
Equivalent Solar-to-Fuel Efficiency [c]	–	0.3%	0.1%	–

[a] Li H, Opgenorth PH, Wernick DG, Rogers S, Wu TY, Higashide W, Malati P, Huo YX, Cho KM, Liao JC (2012) Integrated electromicrobial conversion of CO_2 to higher alcohols. *Science* 335:1596. [b] Schuster E, Schlegel HG (1967) Chemolithotrophes wachstum von hydrogenomonas H16 im chemostaten mit elektrolytischer knallgaserzeugung. *Arch Microbiol* 58:380–409. [c] These values were calculated on the basis of a full experiment, rather than as one-day maximum yields.