Based on: Li et al. “Integrated Electromicrobial Conversion of CO2 to Higher Alcohols”. *Science*, 2012.

Dear Richard, and John, Jane and Bill,

We are writing you to summarize our recent work on our project to directly convert CO_2 to higher alcohols. Below, we summarize the current standing of the project and recommend how you can pursue this work.

Salutations.

As you know, a man-made photovoltaic device is relatively efficient in converting sunlight to electricity, but the electrical energy generated is difficult to store. The biological photosystems, on the other hand, are limited by the intrinsic design and biomaterials available, for which no near-term improvements are in sight (*1*). Our objective was to circumvent both problems **by linking a man-made solar cells to biological CO_2 fixation and fuel production**. Theoretically, H_2 generated by solar electricity can drive CO_2 fixation in lithoautotrophic microorganisms engineered to synthesize high energy-density liquid fuels. However, the low solubility, low mass transfer rate, and safety issues of H_2 in microbial cultures limit the efficiency and scalability of such processes.

Introduction and statement of general objectives (in bold).

Compared with H_2, formic acid is a favorable energy carrier. Electrochemical production of formic acid from CO_2 and H_2O can achieve relatively high efficiency (*2*, *3*). Formate is highly soluble and is readily converted to CO_2 and NADH in the cells, providing a safe replacement for H_2. However, the high solubility of formate increases the cost of separation. Accumulated formate will decompose at the anode, decreasing the yield of the process (*2*). Therefore, simultaneous electrochemical formate production and biological formate conversion to higher alcohols is desirable (Fig. 1A) and was our first targeted deliverable. Unfortunately, introduction of electricity to microbial cultures may impede cell growth.

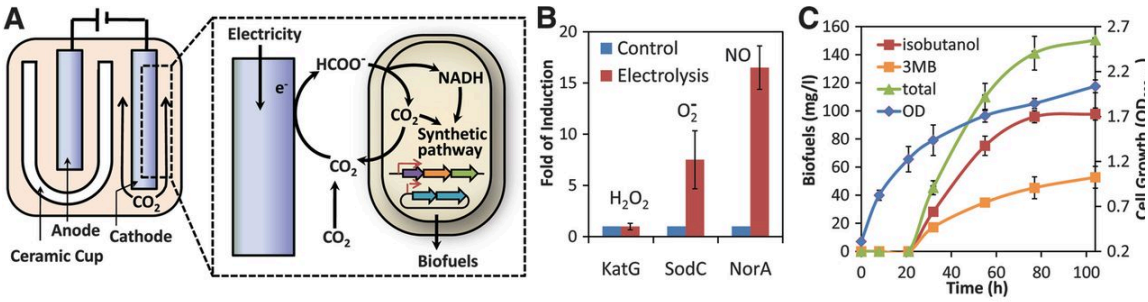

Fig. 1. An integrated electromicrobial process to convert CO_2 to higher alcohols. (**A**) Electricity powered the electrochemical CO_2 reduction on the cathode to produce formate, which is converted to isobutanol and 3MB by the engineered *R. eutropha.* (**B**) *R. eutropha* strains harboring a reporter gene driven by promoters that respond to reactive oxygen and nitrogen species showed increased reporter activity upon electrolytic exposure. (**C**) Engineered strain *R. eutropha* LH74D (supporting online material) showed healthy growth and produced over 140 mg/l biofuels in the integrated electromicrobial reactor with electricity and CO_2 as the sole energy and carbon sources, respectively. Error bars indicate SD (n = 3).

As such, an integrated process for reduction of CO_2 to liquid fuel powered by electricity requires (i) metabolic engineering of a lithoautotrophic organism to produce liquid fuels (*deliverable 1*), (ii) electrochemical generation of formate from CO_2 in fermentation medium (*deliverable 2*), and (iii) enabling microbes to withstand electricity (*deliverable 3*). In this work, we chose *Ralstonia eutropha* H16 as the production host and isobutanol and 3-methyl-1-butanol (3MB) as the target fuels, which can be used in the internal combustion engines. We introduced the set of genes previously reported (*4*, *5*) for isobutanol and 3MB production into *R. eutropha* H16. We also disrupted polyhydroxybutyrate synthesis and used the heterologous isobutanol and 3MB

Deliverables and discussion. Notice the explicit description of deliverables (highlighted in italics) and how their completion or non-completion is always addressed in

production pathway as the new metabolic sink for carbon and reducing equivalents. In a pH-coupled formic acid feeding fermentor, the engineered strain LH74D produced fuels with the final titer of over 1.4 g/l (~846 mg/l isobutanol and ~570 mg/l 3MB) thus satisfying *deliverable 1*.

Next, we used an In foil cathode and a Pt anode in the culture medium bubbled with air containing 15% CO_2 to produce formate electrochemically (Fig. 1A). This satisfied *deliverable 2*. However, growth of *Ralstonia* was inhibited by electric current. Studies using *Escherichia coli* showed transient inhibition of electric current on cell growth. When the current stopped, cell growth resumed, suggesting that unstable compounds such as reactive oxygen or nitrogen species might be responsible for the growth inhibition.

To test this hypothesis, we constructed three *Ralstonia* strains with different reporter plasmids to detect the presence of reactive oxygen and nitrogen species. The plasmids each contained a *lacZ* gene driven by a promoter of *Ralstonia katG*, *sodC*, or *norA* gene, genes which have been shown to be induced by hydrogen peroxide (H_2O_2), superoxide free radicals (O_2^-), and nitric oxide (NO), respectively (*6*–*8*). When the plasmid-bearing strains were exposed to electrolysis, expression of β-galactosidase from *sodC* and *norA* promoters were induced but not from the *katG* promoter (Fig. 1B). These results suggested that O_2^- and NO trigger a stress response in *Ralstonia* cells and inhibit growth. To circumvent this toxicity problem, a porous ceramic cup was used to shield the anode (Fig. 1A). This inexpensive shield provides a tortuous diffusion path for chemicals. Therefore, the reactive compounds produced by the anode may be quenched before reaching the cells growing outside the cup. Using this approach, we satisfied *deliverable 3* by achieving healthy growth of *Ralstonia* strain LH74D and production of over 140 mg/l biofuels with the electricity and CO_2 as the sole source of energy and carbon, respectively (Fig. 1C).

Deliverables and discussion. Continued.

This integrated process to convert CO_2 to liquid fuels does not depend on biological “light reactions.” Electricity generated from photovoltaic cells, wind turbines, or off-peak grid power sources can be used to drive CO_2 fixation and fuel production. Thus, this initial process provides a way to increase photosynthetic efficiency by coupling man-made photoelectric generation device with biological CO_2 fixation and fuel production capability. Our initial experiments demonstrated here could also be applied to produce other compounds, thus opening the possibility of electricity-driven bioconversion of CO_2 to a variety of chemicals including the bioplastic precursors lactic and succinic acid as was initially planned (*deliverables 4* and *5*, respectively). Furthermore, we realized that formate is also a by-product of chemical dehydration and conversion of biomass (*9*). Therefore, attempting to transform plant-derived formate into liquid fuel could allow us to expand this process to biorefineries and could be an interesting deliverable to add to the work plan.

Conclusions and recommendations for subsequent groups.

I hope you found this update useful. If you are interested, a description of the materials and methods are given below. Please don’t hesitate to contact us if you need additional information. Best regards,

Jack, Jill and Robert

Materials and Methods

Construction of the Ralstonia higher alcohol production strain LH74D

Escherichia coli XL-1 Blue (Stratagene, La Jolla, CA) was used in all the cloning work. The genes alsS (Bacillus subtilis), ilvC (E.coli), and ilvD (E.coli) were introduced by chromosomal integration into the Ralstonia eutropha H16 genome. The -200bp to +230bp DNA fragments relative to Ralstonia eutropha H16 phaB2 gene start codon and the last 639bp of phaC2 gene open reading frame were amplified from genomic DNA and assembled with splicing by overlap extension PCR (SOE-PCR) via a linker region containing the SacI restriction site between the two fragments. The SOE product was digested with MluI and XbaI and inserted into the conjugation vector pNHG1 to form pLH50. The artificial operon containing alsS, ilvC, and ilvD was amplified from plasmid pSA69 and assembled by SOE-PCR with the 836bp phaC1 promoter region amplified from R. eutropha H16 genomic DNA. This fragment was then inserted into the SacI site of pLH50 to form plasmid pLH63. The pLH63 was used to perform conjugation. After double-crossover selection on sucrose, the strain with alsS, ilvC, and ilvD overexpression was confirmed by PCR of genomic DNA and enzyme assays using cell lysate.

The genes kivd (Lactococcus lactis) and yqhD (E. coli) were introduced by a multiple-copy plasmid. The plasmid was used to transform LH67 strain by electroporation. Briefly, over night culture of R. eutropha in rich medium (16g/L nutrient broth, 10g/L Yeast extract, 5g/L) was inoculated into 20ml rich medium and allowed to grow to =0.8 in 30°C. The cells were harvested by centrifugation, washed twice with ice-cold 0.3M sucrose solution, and then resuspended in 2ml of ice-cold 0.3M sucrose solution. 0.1ml of this resuspended cells were mixed with ~50ng plasmid DNA and electroporated with 11.5kV/cm, 5.0ms, followed by rescuing with 0.2ml rich medium in 30°C for 2 hours and plated on rich medium plates containing 200mg/l kanamycin. Colonies from the transformation were confirmed by PCR. The strain was named LH74D.

Biofuel production by the integrated electro-microbial process

Ralstonia LH74D cells were grown in German minimal medium with 4g/l fructose to late log phase. Cells were then harvested and washed three times with minimal medium containing no carbon source. The washed cells were then inoculated into the electro-microbial bioreactors. The electrolysis was performed using the above-mentioned conditions. A porous ceramic cup was used to shield the anode. Evaporated alcohols in venting gas were condensed with a Graham condenser and collected. Daily, samples of culture broth and condensation liquid were taken and alcohols were quantified using gas chromatography (GC).

In this process, H_2 was produced electrochemically as a by-product. Both formate and hydrogen can serve as the energy source to support cell growth and biofuel production. Since electrolysis produces fine H_2 bubbles, mass transfer rate can be increased without mechanically dispersing large volume of gas, which is a significant energy cost in the conventional fermentation processes. Thus, hydrogen by-product will not be wasted.

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