Final Report of GCEP Project

Synthesis of Biofuels on Bioelectrodes

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Abstract

Microbial electrosynthesis of multi-carbon organic compounds is a promising novel technology for converting electricity into renewable organic molecules as well as for storing electrical energy. This project explored the breath of possible platform organisms to be used in this emerging technology and identified the potential as well as the limitations. Of the multiple microorganism and experimental settings tested, microbial electromethanogenesis is emerging as a strong technology platform, and we provided the first insights in the molecular mechanism of cathodic electron uptake as well as operation and resilience of the system. This project provided the basis for important follow-up studies on microbial electrosynthesis.

Introduction

Petroleum and other fossil hydrocarbons are primarily used as energy source for liquid (transportation) fuels as well as raw material for organic syntheses of commodity and fine chemicals. These uses represent the largest contribution to a net release of CO_2 and global warming. Development of novel and alternative energy technologies to reduce or eliminate net CO_2 release are urgently needed but often limited by their incompatibility with the current liquid hydrocarbon-based infrastructure (e.g. H_2 or electricity) in storage, transport, and use. Currently, solar and wind energy are the most promising sources for renewable energy, and, similarly as nuclear energy, produce electricity as the primary energy form. In the absence of better electricity and distribution technologies (e.g. battery), new approaches are needed to connect electrical energy to the infrastructure advantages of hydrocarbon fuels. This project explored ideas and examined the bottlenecks of a new technology linking electricity to synthesis of fuels and other useful chemicals at cathodes using microorganisms (Fig. 1).
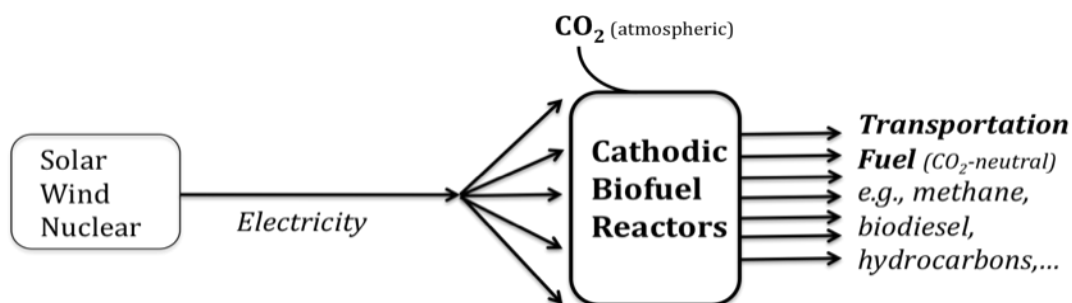

Figure 1 Basic approach and impact biofuel production on bioelectrodes (electrofuels) as proposed here as an innovative strategy to mitigate global warming and to minimize green house gas emission.

Biofuels encompasses a broadly defined class of relatively reduced gaseous or liquid organic molecules, and includes methane, ethane, long chain alcohols, oils, fatty acid esters, and isoprenes. While chemically diverse, they are biosynthetically typically derived from acetyl-CoA or related small molecule intermediates, with the exception of methane. For the purpose of this project and the limited scope that could be addressed in a three year research program, we focused on synthesis of methane, acetate, and fatty acids *in vitro*. However, because of the choice of experimental system including the specific microorganisms, the platform can be adopted to drive the autotrophic synthesis

of isoprenes and other hydrocarbons that can be easily separated from the reactor and represent energy-dense biofuels.

Background

Microbial life is inherently coupled to redox chemistry and typically involves transfer of electrons (or reducing equivalents) from soluble electron donors to electron acceptors that are extracted and returned to a cell's external environment [1]. However, some microorganisms are capable of transferring cellular electrons to insoluble compounds, in particular to iron-oxide mineral surfaces, such as in hematite or goethite [2-4]. These dissimilatory metal-reducing microorganisms, such as *Geobacter* or *Shewanella*, mediate such electron transfer through an ill-defined network of c-type cytochromes that eventually mediates electron transport to the insoluble mineral surface. This mechanism evolved under geological constrains multiple times independently, and is today the key microbial feature in microbial fuel cells (MFC) [5-7]. In MFCs the solid anodic electrode serves as electron acceptor, whereas the cathode is oxidized typically by molecular oxygen [8]. In this way, MFCs are being used successfully to convert inexpensive organic waste or biomass into electricity [8, 9].

However, very recently a much underappreciated and understudied microbial reaction at the cathode is gaining significant interest for production of electrofuels [10]. Rather than transferring electrons to high potential electrodes from low potential organic matter, microbes can also access and uptake low potential electrons directly or indirectly from a cathode[8, 11]. Those electrons are used to drive catabolic processes as electron donor. For example, solid phase microbial iron oxidation with nitrate has been observed in anaerobic systems [11-13]. Strycharz et al reported that cathodic electrodes served as direct electron donors for microbially catalyzed reductive dehalogenation, enabling *Geobacter sulfureducens* to grow with fumarate as electron acceptor on cathodic electrons [14]. More recently, Logan and coworkers reported methanogenesis at a low potential cathode, where most likely cathodic electrons were used directly by *Methanobacterium palustre* without cathodic H_2 as intermediate [15]. These data collective provide strong evidence that cathodic, low potential electrons can be used by several microorganisms, and that the resulting catabolic reactions can support growth of the microbes and an associated microbial ecosystem. As cathodic electrons support autotrophic growth, this implies that biofuels could be synthesized from CO_2 and electricity electrons by metabolic engineering novel autotrophic pathways for biofuel synthesis in addition to, or rather than, cell mass synthesis. Such CO_2-based biofuels can serve a very critical function in a new energy economy: As electrical energy can currently not be stored well in light vehicles, conversion of electricity into biofuels to be used as transportation fuel can be employed in a decentralized system for local production of transportation fuel. Such scenario would take maximal advantage of the current (and expensive to change) fuel and car infrastructure, and bring important innovation in liquid transportation fuel technology enabling to convert solar wind and nuclear electric energy carbon-neutral into useful transportation fuel.

In this proejct, we investigated a broad suite of microbial systems to identify the most promising platform for microbial electrosyntheses. We investigated anaerobic and aerobic microorganisms.

Results

For assessing the most optimal platform for microbial electrosynthesis of fuels, anaerobic and aerobic microorganisms were explored:

1. Microbial electromethanogenesis by cathode-immobilized redox-active enzymes

We investigated microbial electromethanogenesis in two methanogenic archaea: *Methanococcus maripaludis* and *Methanosarcina acetivorans*.

1.1 Microbial electromethanogenesis in Methanococcus maripaludis

Methanococcus mariplaudis was found to be an ideal microbial platform for electromethanogenesis. Cultures grown in mineral medium on formate, harvested in early stationary phase and introduced into the anoxic, bicarbonate-containing MOPS buffer immediately formed methane at a rate of approximately 0.38 mmol/h when the cathode was set at a potential of −600 mV (vs a standard hydrogen electrode) (Fig. 1a). Based on the current and methane recovery, the coulombic efficiency was 70-80%. This activity
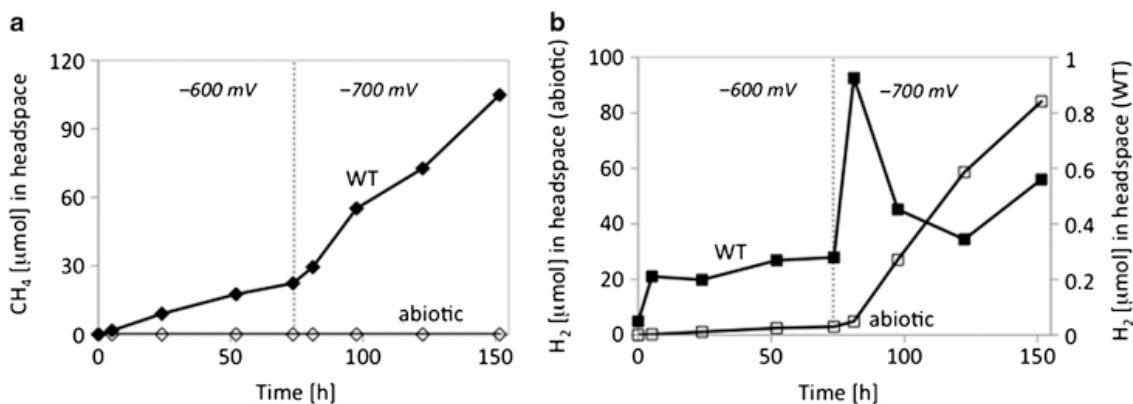

Figure 1. Bioelectrochemical methane formation in *M. maripaludis* wild-type cells. (**a**) Potential-dependent methane formation was observed in the wt strain (◆) but not in the abiotic control (◊) when tested in bioelectrochemical reactors with cathode potentials of −600 and −700 mV. (**b**) Hydrogen concentrations in the abiotic control (□) were much higher and potential dependent compared with the wt strain (▪). Results shown are a representative example of replicate experiments (*n*=4). The potential was decreased from −600 mV to −700 mV at *t*=73 h. Electron recovery in the form of methane and hydrogen, that is, the coulombic efficiency under those conditions was in the range of 70–80%.

was observed for at least 7 days (Fig 1). Methane formation was not detected in controls without cells (abiotic control; Fig. 1a) or in the absence of a cathodic potential (data not shown). At a potential of −600 mV, molecular hydrogen was formed abiotically at a rate of 0.04 mmol/h (Fig. 1b). The concentration of hydrogen did not increase in the presence of *M. maripaludis* wt cells, but reached a low steady state concentration during the experiment.

When the cathode potential was lowered to −700 mV a transient increase in H_2 concentration coupled to an increased rate of methane formation was observed (Fig. 1b). As the cathodic potential affects the rate of abiotic hydrogen release, the lower potential led to a faster production of hydrogen, which was presumably consumed subsequently by the cells, indicative of an indirect electron uptake via hydrogen as an intermediate (Fig.

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1b). Consequently, the rate of methane formation increased to about 1 mmol/h at -700 mV (Fig. 1a).

In order to investigate whether a H^+/H_2 cycling between cathode and this archaeon might be involved in the apparent direct cathodic electron uptake, we used *M. maripaludis* mutant MM1284, which carries markerless in-frame deletions of all five catabolic hydrogenase genes, *fru*, *frc*, *hmd*, *vhu*, and *vhc*, plus a deletion in the anabolic *ehb* hydrogenase [16, 17]. These deletions rendered MM1284 strain defective in methane formation from H_2 and CO_2, and unable to grow by this catabolic reaction. The only hydrogenase present, the energy conserving hydrogenase Eha that is needed to reduce ferredoxin for anabolic reactions under the consumption of proton motive force, does not enable growth or methane formation from H_2/CO_2 [16,17]. When cells of strain MM1284 were tested in the bioelectrochemical reactor with a cathode potential at -600 mV, methane was formed at a rate of 0.04 mmol/h, which was about 1/10 of the rate of methane formation in wt at a coulombic efficiency of 50-60% (Fig. 2a). The onset of
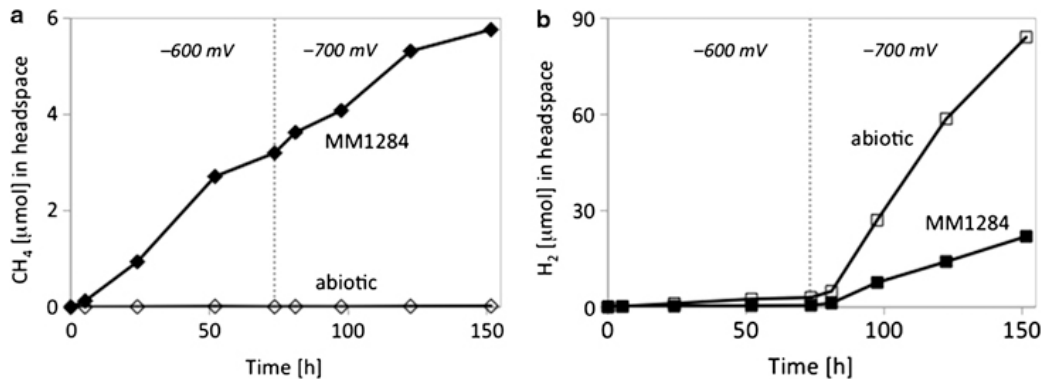

Figure 2. Hydrogen-independent methane formation in the *M. maripaludis* strain MM1284. (**a**) Bioelectrochemical methane formation was observed in mutant strain MM1284 (◆) but not in the abiotic control (◊), and it was independent of the set cathode potential in bioelectrochemical reactors at cathode potentials of -600 and -700 mV. (**b**) Hydrogen did accumulate in tests with either strain MM1284 (▪) or the abiotic control (□), and in both the cases formation rates were dependent on the set cathode potential. The potential was decreased from -600 mV to -700 mV at t=73 h. Results shown are a representative example of replicate experiments (n=2).

methanogenesis was immediate after setting this potential. When the cells of the mutant strain in the electrochemical reactor were subjected to the same downshift in cathodic potential to -700 mV, no change in the rate of methanogenesis was observed, in contrast to the increase in methanogenesis rates observed for wt cells. A concurrent steady increase in H_2 was found, as expected from an abiotic reaction at the cathode, which is consistent with the inability of strain MM1284 to consume hydrogen (Fig. 2b).

In order to test whether metabolism of cathode-derived electrons in *M. maripaludis* is impeded when the central catabolic pathway and main electron sink (reduction of CO_2 to CH_4) is inhibited, we treated wt and MM1284 mutant cells with 7 mM 2-bromoethanesulfonic acid (BES), a specific inhibitor of methylcoenzyme M reductase, the key enzyme in the last step in methanogenesis. As expected, in both wt as well as the MM1284 mutant experiments, methane formation ceased upon introduction of BES (Fig. 3a,b). Bio-electrochemical reactors carrying wt cells previously grown on formate were found to accumulate both H_2 and formate, while the reactors with MM1284 mutant cells accumulated formate only (Fig. 3a,b). When wt cells, previously grown on H_2 and CO_2
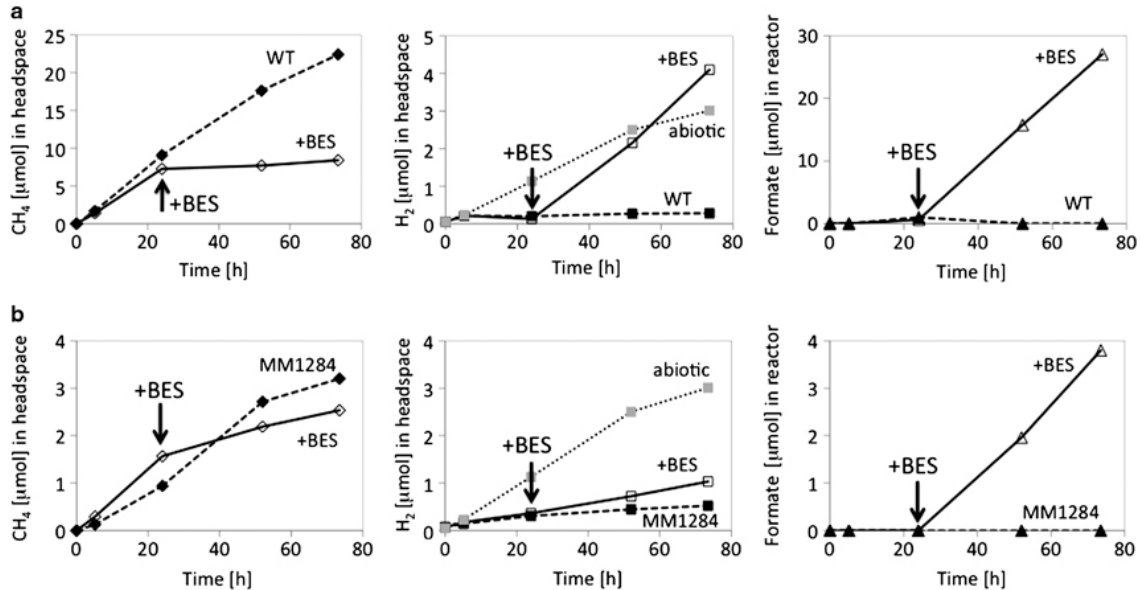

Figure 3. Inhibiting electron flow towards methanogenesis at a cathodic potential of −600 mV results in the formation of other reduced compounds. (**a**) When inhibited with 7 mM BES (solid line), wt *M. maripaludis* ceased forming methane (◊), increased hydrogen formation (□) compared with the abiotic control (▲), and produced formate (△). (**b**) Mutant strain MM1284 also showed inhibition of methane formation (◊) but no significant change in the rate of hydrogen production (□). Formate was observed when cells were inhibited with 7 mM BES (△). Black filled symbols indicate the respective non-inhibited controls results shown are a representative example of replicate experiments (*n*=2).

without formate, were tested in the electrochemical reactor under the same conditions, no formate but hydrogen accumulation was detected (data not shown).

As close contact of cells with the cathodic surface is a prerequisite for a direct electron uptake, we investigated whether the observed microbial activity was directly associated with electrode contact. We removed all planktonic cells from the cathodic chamber after a week of active electromethanogenesis, and the chamber was rinsed twice and subsequently refilled with fresh anoxic sterile medium. The subsequent rate of methane formation observed at a cathode potential of –600 mV was in the same range as the rate in the reactor before rinsing and even increased to a small extent for wt cells, indicating that most of the electromethanogenic activity was cathode associated.

We also found that the presence of *M. maripaludis* wt cells effectively lowered the cathodic overpotential for hydrogen evolution. Reactors containing wt cells started consuming current at more positive potentials (–400 mV to –450 mV) than the abiotic control where significant current consumption started at –600 mV. This ability of cells to

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form methane at a more positive cathode potential than that needed for H_2 production is also reflected by a H_2 production rate that is twice the abiotic rate in BES-inhibited wt cells on the cathode at –600 mV. When the hydrogenase deletion strain MM1284 was used in the reactors, the H_2 formation rate was lower than in the abiotic control. This reduction in the H_2 evolution rate in MM1284 cells shows that the presence of such cells on the surface reduces, rather than increases, the rate of abiotic electron release as H_2. Thus, microbial biomass may 'passivate' a cathodic graphite surface.
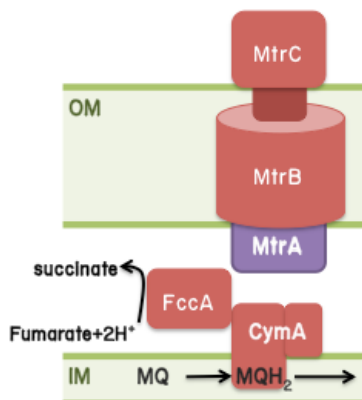
Figure 4: The Mtr pathway in *Shewanella oneidensis* MR-1.

## 1.2 Microbial electromethanogenesis in **Methanosarcina acetivorans**

Methanosarcina acetivorans C2A
It was investigated whether *Methanosarcina acetivorans* C2A could directly uptake electrons from the cathode. *M. acetivorans* is a cytochrome-containing methanogen which can grow on methanol, trimethylamine, acetate and carbon monoxide. The cytochrome b present in *M. acetivorans* C2A respirator chain is on the outside of the cytoplasmic membrane and could be a point of electron entry. In addition, *M. acetivorans* C2A does not contain active hydrogenases therefore hydrogen would not be a soluble shuttle for growth. There was minimal current uptake above background in *M. acetivorans* C2A cells grown on methanol. There is not a significant rate of electron uptake in *M. acetivorans* C2A.

2. Electron transfer into microbial cells via redox-active shuttles
Electron shuttles are chemical compounds that facilitate the transfer of electrons to and from bacteria. Some shuttling compounds such as flavins are produced naturally by some bacteria. Others, humic acids and metal ions, are naturally occurring and ubiquitous in soil. Finally, there are artificial mediators, like methyl and benzyl viologen, which are frequently used as an electron acceptor in enzyme activity assays. In a bioelectrochemical system, many of these shuttles have been shown to transfer electrons between bacteria and an electrode [18]. These compounds thus allow for the use of the entire reactor volume rather than strictly relying on an attachment of the bacteria to the electrode surface. The purpose of this project was to examine the use of these redox-active electron shuttles on cathodic electron uptake by *Shewanella oneidensis* MR-1.

Shewanella oneidensis MR-1 is a facultative aerobe, which is able to derive energy by coupling the oxidation of organic matter to the reduction of a wide range of extracellular organic and inorganic electron acceptors. Electrons are transferred out of *S. oneidensis* by the Mtr pathway shown in Figure 1. There are five primary components of the Mtr
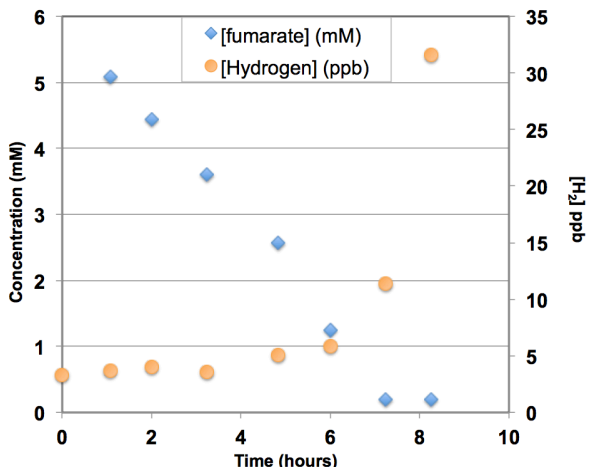
Figure 5: In the absence of fumarate (blue), hydrogen gas (orange) is produced.

pathway: four c-type cytochromes, OmcA, MtrC, MtrA, and CymA and one integral membrane scaffolding protein, MtrB. The current model of electron transfer through this pathway assumes that electrons from the oxidation of organic carbon sources are passed via the menaquinone pool to CymA, MtrA and finally MtrC or OmcA. This pathway has been shown to be essential for the reduction of iron and electrodes, by direct attachment with MtrA and MtrB acting as the critical components of electron transfer in the pathway.

We investigated three questions:
1. Can mediator-dependent cathodic electron transfer be utilized to form biofuel and biofuel precursors such as H_2 and formate?
2. Can these shuttled electrons be used to bias pyruvate fermentation products to more reduced compounds.
3. What is the effect of electron transfer via shuttling compounds with an mtrA mutant?

To address these questions, cell suspensions of *S. oneidensis* MR-1 were placed in an h-cell electrochemical reactor with reduced mediators: AQDS (-180 mV), Neutral Red (-330 mV), and methyl viologen (-460 mV), provided as the sole electron donor and either fumarate, hydrogen ions, or bicarbonate (dissolved CO_2) provided as an electron acceptor. In the case of fermentation, only pyruvate was added. The depletion of fumarate or pyruvate and the formation of succinate, formate, lactate, and acetate were recorded and monitored by HPLC. The formation of hydrogen gas was observed using a Hydrogen Analyzer from Peak Laboratories. The corresponding utilization of cathodic electrons was observed using a potentiostat.

The formation of hydrogen and formate are exciting products of *S. oneidensis MR-1*. Hydrogen, itself, is a biofuel. And formate, while being a precursor to biofuels and other compounds, is also a form of fixed carbon. It was observed that mediated cathodic electrons were consumed by the cell in a semi-hierarchical fashion where fumarate reduction occurred first, followed by the production of formate and hydrogen gas (Figure 2). In the absence of bicarbonate, no formate was formed and in the absence of cells, almost no hydrogen was produced.

Electrofermentation is a hybrid metabolism where cathodic electrons are utilized as reducing equivalents in addition to those derived from the carbon source. Studies have shown that, in *Clostridium* this method results in a bias of fermentation products to more reduced compounds such as propionate, glutamate, butyrate, and butanol [19].

Shewanella can ferment pyruvate to acetate, lactate, and formate. We wanted to see if supplementing fermentation with additional reducing equivalents from continually reduced methyl viologen. As can be seen by figure 3, the proportion of lactate, the most reduced product of pyruvate fermentation increases with the addition of cathodic current and further increased by the addition of reduced methyl viologen. Additionally, the fraction of unrecovered carbon—most likely CO_2—is increased with the addition of electrostimilation.

Previous studies have shown that flavin reduction is diminished by 95% without the mtrA or mtrB genes in *S. oneidensis* MR-1 [20]. We hypothesized that we would also see diminished reduction of fumarate in strains of *S. oneidensis* MR-1 lacking the mtrA gene, using methyl viologen, AQDS or riboflavin as electron donor rather than electron acceptor. While it was observed that reduction of fumarate to succinate occurred faster when methyl viologen or neutral red were used as mediators, there was no evidence that the mtrA mutant was in any way lacking the ability to use any of the three mediators as electron donor.
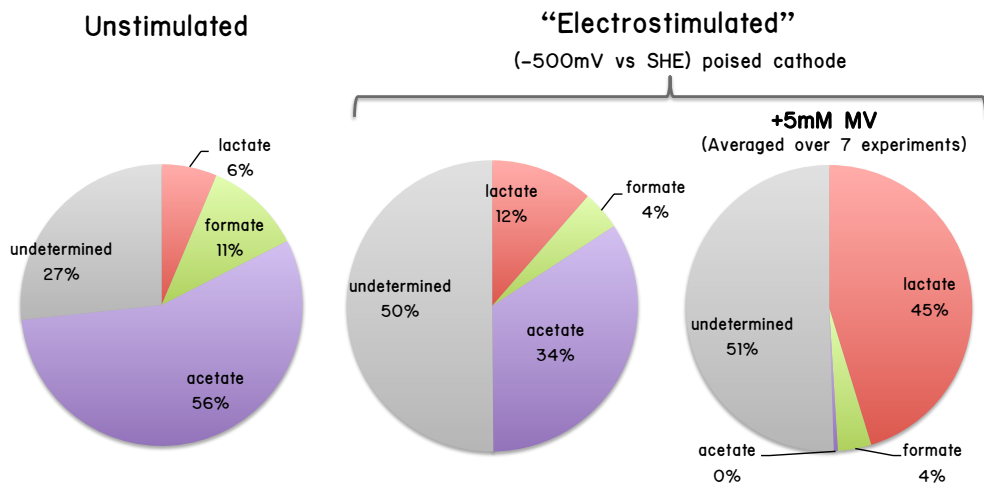
Figure 6: Carbon Balance of fermentation products
During normal pyruvate fermentation (unstimulated), the majority product is acetate. However, with the addition of a poised cathode and reduced methyl viologen, the majority product becomes lactate.

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3. Electron uptake in aerobic Ralstonia eutropha H16
3.1 Ralstonia eutropha

This work investigated the capacity of *Ralstonia eutropha* H16 to uptake electrons directly from a graphite cathode. Previous work showed that an engineered strain of *R. eutropha* H16 grown on hydrogen produced isobutanol at a cathode [21]. However, in order to overcome kinetic limitations of hydrogen production, in that work the cathode potential was set at a -1400 mV vs. SHE, a much lower potential than is required for hydrogen production. To reduce the overall energy required to operate a biocathode, we investigated whether *R. eutropha* H16 could directly uptake electrons from the cathode at a higher potential of -500 mV vs. SHE. The higher potential would reduce the overall energy required to operate the biocathode but the electrons from the cathode would still be at a low enough potential to perform any desired cellular reaction. In addition, the electron acceptor was changed to nitrate to eliminate the toxic by product of hydrogen peroxide being produced at the cathode from oxygen.

R. eutropha H16 could not uptake electrons directly from the cathode with hydrogenases. An experiment was done which first adapted *R. eutropha* H16 to grow on the surface of a cathode and then determined if it was capable of direct electron uptake. To adapt the cells, a reactor inoculated with *R. eutropha* H16 was fed hydrogen and nitrate to allow for sufficient biomass to form. Then the liquid medium was replaced to select for the cells capable of direct electron transfer attached to the cathode. After two rounds of transferring the medium hydrogen was flushed out of the reactor and the cathode was the only electron donor. The current consumption stopped when hydrogen was no longer added indicating the cells were not adapted for direct electron uptake (Figure 7).
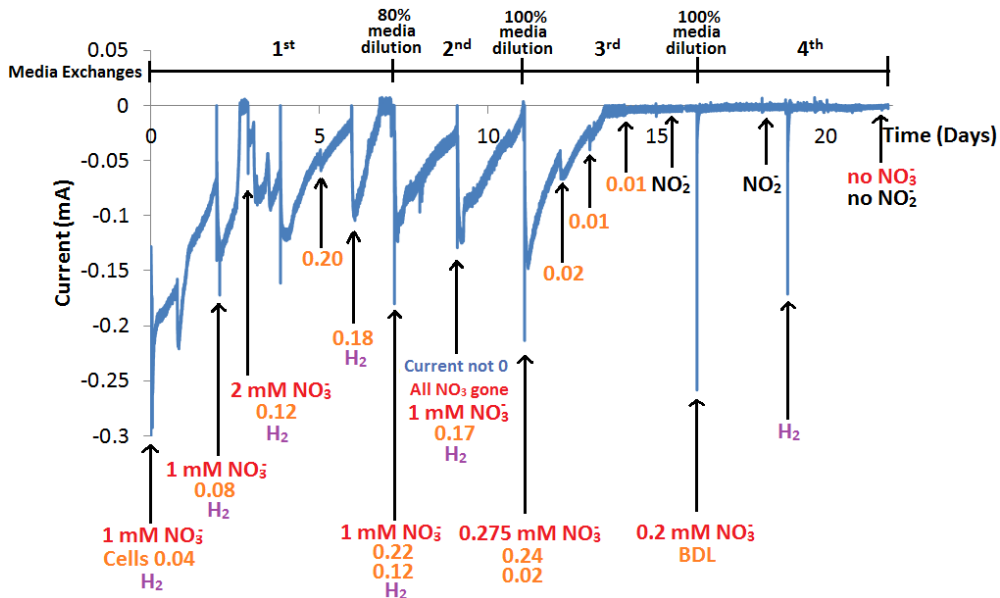

Figure 7: Electron Uptake for *R. eutropha* H16 grown on hydrogen and nitrate.

The current consumption in the *R. eutropha* H16 reactor could be due to a soluble shuttle. Not only did the experiment in Figure 1 show that *R. eutropha* H16 could not accept electrons directly, but only a fraction of the electrons were accounted for when an electron balance was performed. One possibility is a short circuit is present in the system where one chemical reacts directly with the cathode and not flowing through microbial metabolism. This sort circuit was tested by running abiotic control of all of the important compounds present in the system. While nitrate did not react directly with the cathode, nitrite, a common intermediate released during the denitrification metabolism did react at -500 mV vs. SHE (Figure 8). Therefore, all of the current production in Figure 1 could be due to abiotic nitrite reduction. This is a significant finding because many papers propose that the current they find in their experiments are due to direct electron uptake by microbes without eliminating the possibility of abiotic reactions of other compounds present in the system.
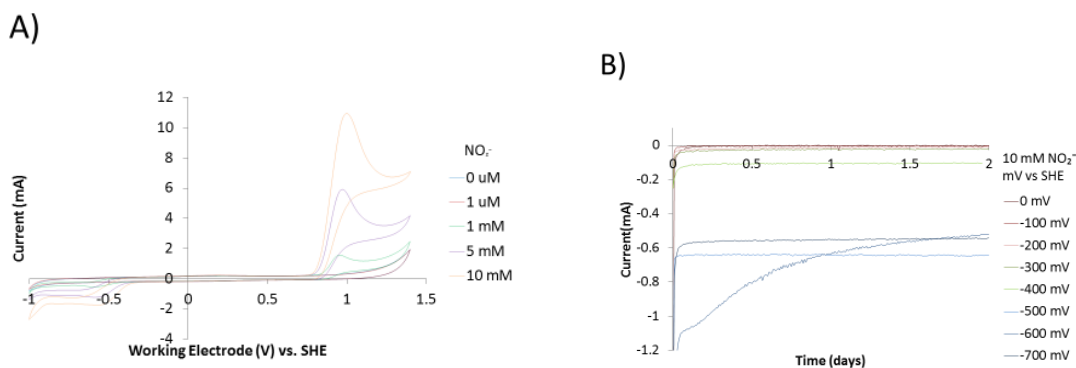

Figure 8: Abiotic nitrite reaction with graphite cathode. A) Cyclic voltammetry B) Constant Potential

3.2 Thiobacillus denitrificans

It was investigated if *Thiobacillus denitrificans* could directly accept electrons from the cathode. Previous work showed that *Geobacter sulfurreducens* and *T. denitrificans* could perform interspecies electron transfer using magnetite as an intermediate to transfer electrons. Part of these experiments reported that *T. denitrificans* could interact directly with a In_2O_3 cathode set at -400 mV vs SHE [22]. *T. denitrificans* grows on thiosulfate and nitrate. The first experiment that was performed was an abiotic control to see where thiosulfate interacts with the graphite cathode (Figure 9). The range to poise the cathode where there is no abiotic reaction is between -250 and +350 mV vs. SHE. When *T. denitrificans* was inoculated into a reactor with a graphite cathode poised at -250 mV vs. SHE so there would be no abiotic reaction, there was not significant current uptake above background. Therefore, it is unclear if carry over thiosulfate from the inoculum resulted in the current uptake in [22] or if it was truly bacterial interaction with the cathode and other differences in the experimental set up allowed for *T. denitrificans* to uptake electrons directly.

3.3 Acidithiobacillus ferrooxidans

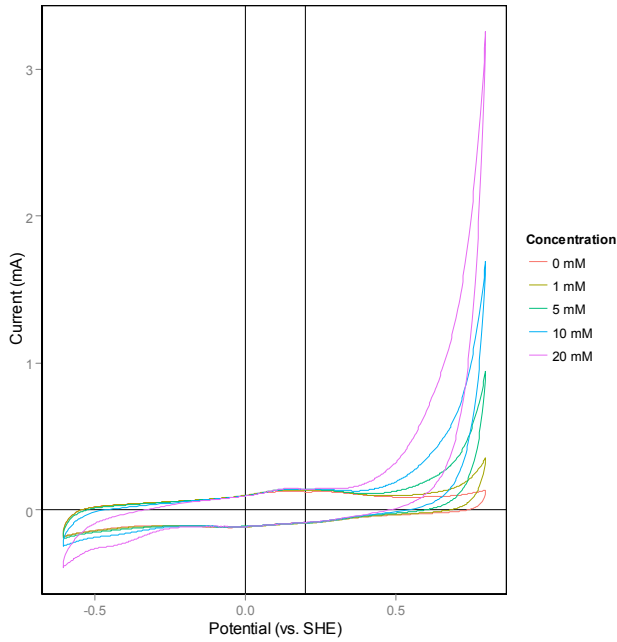Soluble electron transfer was investigated for *Acidithiobacillus ferrooxidans*.   A

Figure 9: Abiotic Cyclic Voltammetry with thiosulfate *Acidithiobacillus ferrooxidans*

ferrooxidans grows at pH 1.8 where Fe^{2+} is soluble.   Therefore, Fe^{2+} can be used as a soluble electron donor to shuttle electrons from the cathode to *A. ferrooxidans*.   When cultures were inoculated with a catalytic amount of Fe^{2+} the cells could still grow (Figure
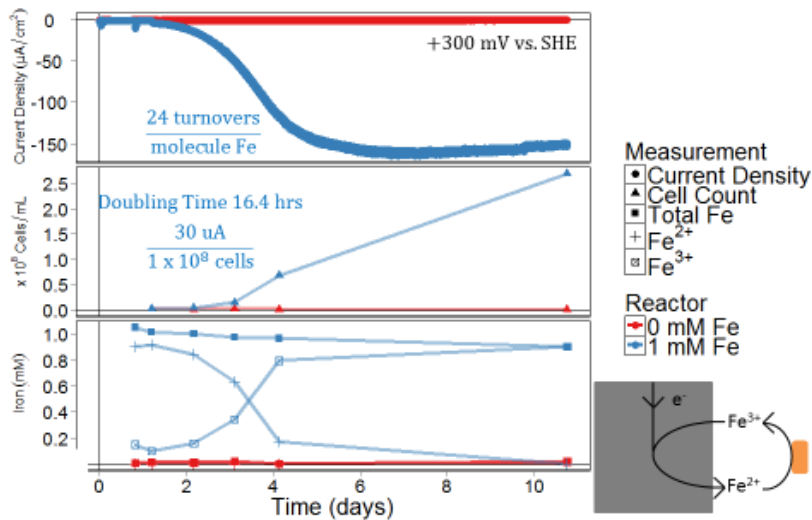

Figure 10: Current Density, cell concentration and iron concentrations for *A. ferrooxidans* growing in electrochemical system.

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10). In this experiment the cells turned over each molecule of Fe^{2+} over 20 times at the cathode.   In addition, when the biomass increased in the system, the cells were turning over Fe^{2+} as fast as it was produced at the cathode because all of the iron was in the Fe^{3+} form by the end of the experiment.   The only carbon source for *A. ferrooxidans* was CO_2 from the atmosphere so CO_2 had to be fixed in the process. Future work for metabolic engineering of *A. ferrooxidans* could make products from this fixed CO_2. Future work could be performed to increase the rate of iron turn over at the cathode since this is the limiting step in the system.

Conclusions

Of the multiple microbial platforms explored in this research, we identified one, *Methanococcus mariplaudis,* as the most promising. In fact, this is the most simple platform that enables the conversion of electricity plus CO_2 to a fuel, methane. Moreover, it can provide the technological basis for storing electrical energy in form of methane. Energy technologies based on methane (natural gas) is a mature technology. We observed a significant variability in electrosynthesis even within several methanogenic archaea.

On the other hand, a shuttle-based microbial electrosynthesis is not a promising platform. While aerobic autotrophic microorganisms in principle could be used for direct or indirect microbial electrosynthesis, our research showed that because of the oxidation of cathodes poised with a low redoxpotential by O_2, such system will be difficult to operate. Moreover, CO_2 reduction is anabolic and thus at lower rates, compared to methanogenesis. Therefore, the most promising platform investigated here is using methanogenic archaea with cathodes pised at -500 mV SHE.

Publications and Patents

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