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Project: Synthesis of Biofuels on Bioelectrodes

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Abstract
The proposed research explores the opportunities and bottlenecks of a novel approach for carbon-neutral synthesis of energy-dense transportation fuel from electrical energy (electrofuels). The technology is based on microbial CO_2 fixation and biofuel production at cathodes in modified fuel cells by naturally occurring and genetically modified microbes. This approach takes advantage of known microbial enzymes, pathways, and organisms, but requires the engineering of novel pathways and communities for the production of biofuels as well as the engineering of the cathodic process. 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 proposal and the limited scope that can be addressed in a three year research program, we will focus on synthesis of acetate, fatty acids, and surrogate compounds enable to study uptake of cathodic electons. However, because of the choice of experimental system including the specific microorganisms, the platform can be adopted to drive the autotrophic synthesis of isoprenes, methane, and other hydrocarbons that can be easily separated from the reactor and represent energy-dense biofuels.
In the first approach, we have been exploring the use of well developed microbial organisms as platforms for metabolizing cathodic electrons. Using *Cupriavidus necator* we were able to demonstrate for the first time direct uptake and metabolism of electrons form cathodic surfaces. On the other hand, experiments using *E. coli* in conjunction with low redox potential electron shuttle compounds were not successful.

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 as well as to sustainably produce fuels and other organic compounds from electricity and CO_2 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 proposal explores ideas and proposes to test the bottlenecks of a new technology linking electricity to synthesis of fuels and other useful chemicals at cathodes using microorganisms. In the past year research has focused on exploring two microorganisms whose general metabolism and physiology has been studied extensively in the past: *Cupriavidus necator*, *E. coli*, and *Shewnella oneidensis* MR1.

Background

1) *Cupriavidus necator* H16

Cupriavidus necator H16 (also known as *Ralstonia eutropha* H16) has great potential to be a bacterium that can utilize current since it contains multiple pathways that have been linked to electrical activity at a cathode. *C. necator* H16 is a β-proteobacteria and has been studied extensively since the 1960's for its diverse metabolism (Wilde, 1962). *C. necator* H16 can grow as an organotroph utilizing carbon compounds like fructose, N-acetyl glucosamine, fatty acids, amino acids, citric acid cycle intermediates, aromatic acids and formate as an energy source, or as a lithotroph utilizing the inorganic compound hydrogen as an energy source. Also, *C. necator* H16 can grow as a chemotroph using the reduced carbon compounds listed above as a carbon source, or as an autotroph using carbon dioxide as the carbon source (Pohlmann et. al., 2006). Another metabolic pathway that has been studied extensively in *C. necator* H16 is poly-hydroxybutyrate (PHB) production, a plastic polymer that the cells use as an energy storage compound. PHB is a compound of commercial interest because it can be stored up to 90% of the cell's dry weight and then be harvested as a bioplastic (Schlegel et. al., 1961 and Verlinden et. al., 2007), and C. *necator* H16 has already been genetically engineered to produce biofuel compounds by rerouting the carbon flux from the PHB storage pathway into biofuels (Li et. al., 2012. We are exploiting here on consumption of electrical current under

autotrophic conditions to determine whether carbon dioxide can be reduced with the cathode for an overall carbon neutral fuel production process using this microorganisms.

C. necator H16 shares many similarities with other electrically active studies that makes it a promising system to study. One method of delivering electrons from the cathode to *C. necator* H16 is through direct electron transfer from the cathode. Previous studies have shown that the hydrogenase enzyme of *C. necator* H16 can be used to coat an electrode to consume hydrogen and produce current (Vincent et. al., 2005). *C. necator* H16 contains three hydrogenases: a regulatory hydrogenase, an energy conserving hydrogenase and a soluble hydrogenase. The soluble hydrogenase is the only reversible hydrogenase that can be used to both produce and consume hydrogen by the cell. The energy conserving hydrogenase and the regulatory hydrogenase can only consume hydrogen. The energy conserving hydrogenase allows hydrogen to be used as an energy source for respiration in the cell (Burgdorf et. al., 2005). In the previous work, the energy conserving hydrogenase was coated on a graphite electrode, the same kind that is used in our studies, and shown to produce current when hydrogen was bubbled through the liquid electrolyte (Vincent et. al., 2005). In our studies, we plan on coating the surface of the electrode with a biofilm of *C. necator* H16 to determine if the hydrogenase will remain electrochemically active *in vivo*. If the electrons from the cathode could be channeled into respiration then the energy that was generated could be used to drive other cellular processes such as carbon dioxide fixation.

As part of direct electron transfer a biofilm would need to form on the cathode. *C. necator* H16 contains a fln-like adhesion gene cluster which is used for tight, non-specific adhesion to surfaces. (Pohlmann et. al., 2006). Therefore, *C. necator* H16 has to capability of adhering to the surface of the cathode to be in close contact in order to perform direct electron transfer.

Another requirement for direct electron transfer is the surface of the microbe is electrically active. Previous studies have shown that electrically active microbes are able to interact with metal surfaces like *Geobacter* and *Shewanella* (Gregory et. al., 2004 and Ross et. al., 2011). *C. necator* H16 has been shown to precipitate soluble Pd(II) to solid Pd(0) on the surface of the cell when fed formate as an electron donor and oxygen as an electron acceptor (Søbjerg et. al., 2009). This demonstrates that *C. necator* H16 is capable of performing reduction reactions on the surface of the cell, demonstrating potential for interaction of the cell surface with the cathode.

Work with denitrifying microbial fuel cells has also demonstrated microbes are able to accept electrons from a cathode. A typical microbial fuel cell uses microbes to donating electrons to the anode to create electricity. When there are only microbial catalysts in the anode compartment, a toxic substance like hexacyanoferrate solution is required to catalyze the reaction in the cathode compartment. To eliminate the need for this toxic

material, studies were done enriching for electrically active microbes that could denitrify in the cathode compartment (Clauwaert et. al., 2007). When these environmental samples were enriched with current as the only electron donor, carbon dioxide as the only carbon source and nitrate as the only electron acceptor, electrical conversion efficiencies between 44% and 100% percent were achieved (Desloover et. al., 2011, Puig et. al., 2011 and Virdis et. al., 2008).

All of the fuel cell experiments (with microbes on the cathode and anode side) were done with mixed cultures. While there are advantages with looking at mixed culture (for example to discover new diversity) it would be easier to determine the mechanism of electron entry with a pure culture where controlled manipulations are performed. Previous work with pure cultures has shown *Geobacter metallireducens* in the cathode compartment can stoichiometrically convert NO_3^- to NO_2^-, the first step in denitrification (Gregory et. al., 2004). *C. necator* H16 would also be an interesting pure culture to investigate current consumption for denitrification. *C. necator* H16 has the metabolic capabilities of denitrifying and fixing carbon dioxide, so if it was also able to utilize electrons from the cathode it would also be able to grow under the same experimental conditions as the fuel cell experiments. Also, previous work has created molecular techniques for *C. necator* H16 (Li et. al., 2012). Therefore, molecular techniques could be used to show if the presence or absence of certain genes are linked to current consumption.

2) *E. coli*

E. coli is one of the best studied microorganism and used in biotechnology extensively. To test whether this microbe could be a useful platform for microbial electrosynthesis the use of the mediator compounds methyl viologen and neutral red as electron shuttles was explored.

Methyl viologen (MV), also known as paraquat, is a redox dye capable of accepting electrons at potentials less than -440mV versus standard hydrogen electrode (SHE). In its oxidized form, methyl viologen is colorless when dissolved in water, but when reduced, the color changes to violet. Neutral Red (NR) is a redox dye capable of accepting electrons at potentials less than -330mV versus standard hydrogen electrode (SHE). In its oxidized form, NR is red, but when reduced, the color changes to yellow.

Results

1) *C. necator*

Initial Current Consumption Experiments with C. necator

Preliminary experiments were performed to see if current consumption was observed while *C. necator* H16 was grown in the reactors. A constant potential of -503 mV vs. SHE applied between the reference electrode and the cathode. Two different electron donors

were used to help *C. necator* H16 adapt to growth on the cathode. Growth of *C. necator* as biofilms on the cathode was selected for by exchanging the media and removing the planktonic cells. These experiments demonstrate that current consumption occurs and current consumption stops when the electron acceptor is absent.

Abiotic Controls

Abiotic controls were conducted to determine the rates of abiotic interaction were higher or lower than the rates due to cellular processes for chemical species present in the reactors. It appears small amounts of hydrogen can be produced at the potential of -503 mV vs. SHE, as shown by the blank media current control from the fructose adaptation experiment. This small amount of hydrogen could potentially be used as an electron donor by the cell instead of the electrons from the cathode. Also, when fructose was spiked into the reactors after a sustained period of only having the cathode as the electron donor, the no current reactor with cells had a spike in nitrite but the current reactor with cells did not have a nitrite spike. This could be a result of the adapted cells being better able to utilize nitrite or an abiotic process utilizing the nitrite. The abiotic reaction rate of hydrogen formation will reveal if the abiotic process was dominating the reaction rate compared with the metabolic process.

First, a cyclic voltammetry experiment was performed using a phosphate solution spiked with different concentrations of chemical species of interest to determine at what potentials these components interacted with the electrode. Nitrite showed a reductive peak around -300 mV vs. SHE. This demonstrates abiotic nitrite reactions could have produced current while cells were growing in the reactors. The amount of current seen at different potentials due to a cyclic voltammogram is not representative of the current produced during a constant voltage vs. time experiment because of capacitance. Therefore, constant voltage vs. time experiments were run using the same reactors that were set up for the cyclic voltammogram with the highest concentration of chemical species as reported on the cyclic voltammogram.

The rate of hydrogen production did not become significant until -700 mV vs. SHE and is a more negative potential then in the fructose adaption experiment. Therefore, the hydrogen contribution to the electron balance by abiotic processes is minimal. However, a significant current was produced at the highest concentration of nitrite expected in the reactors. This demonstrates the current consumption in the reactors may have been due to the abiotic nitrite reduction process. More abiotic constant voltage vs. time experiments will be performed for a constant voltage of -503 mV vs. SHE, the same voltage as in the reactors with cells, while varying the nitrite concentration to determine whether the current produced from abiotic nitrite reduction matches the current profiles observed in the reactors with cells producing nitrite from denitrification.

2) *E. coli*

The *E. coli* DSMZ 30083 strain was chosen as the experimental strain for its ability to grow aerobically on hydrogen gas and fumarate (Macy et al 1976).

Experiments were performed using cell suspensions of *E. coli*. To prepare for inoculation into the reactor, *E.coli* was grown aerobically on LB plates at 30C overnight. Colonies were picked and grown aerobically in LB overnight. 10mL of LB culture was inoculated into an anaerobic medium adapted from Macy, Kulla, and Gottschalk (1976). The pH was adjusted to about 7 with an 80%N_2/20%CO_2 gas mixture after autoclaving. Glycerol (0.8%) served as electron donor and fumarate (15 mM) as electron acceptor.

Cell suspensions were prepared from cultures in early stationary phase of anaerobic growth. Cultures were centrifuged at 5000 rpm for 15 minutes at 4C. The cell pellet was then washed with minimal media lacking electron donor and acceptor before use in the bioelectrochemical reactor. Resuspended cells were placed in the cathode chamber of the electrochemical cell with fumarate as electron acceptor and electrode-reduced shuttles as electron acceptor.

Neutral Red as electron shuttle

Initial results (reported last year) seemed to show that *E. coli* was able to receive electrons from reduced methyl viologen to stoichiometrically reduce fumarate to succinate with an efficiency of nearly 100%. However, it was later determined that in these experiments the cathodic potential of -600mV (vs SHE was sufficient to produce enough abiotic hydrogen to sustain fumarate reduction. Raising the cathodic potential to -500mV, while reducing hydrogen concentrations to background levels, resulted in little to no succinate formation as the fumarate was instead being converted into malate, a known product of stress response (Robert et al. 1977).

Additionally, it has been shown that compounds such as MV, must first cross the cytoplasmic membrane before donating electrons to the fumarate reduction pathway (Robert et al. 1977). It has thus been determined that methyl viologen is not a suitable mediator for fumarate reduction in *E. coli* under these conditions.

Neutral Red as electron shuttle

Experiments were done using Neutral Red; using cathodically Neutral Red to reduce fumarate to succinate in both cell suspension and in cell growth. Unfortunately, work with cell suspensions produced similar results to what was seen with methyl viologen. Rather than succinate formation, malate was accumulated.

A paper published by Park et. al. showing that *Actinobacillus succinogenes* is able to grow on cathodically reduced neutral red and fumarate, inspired the idea that perhaps *E. coli* could do the same (Park et al 1999). Attempts to replicate these experiments using *E. coli* rather than *A. succinogenes* did not result in reduction of fumarate with reduced NR, but, as in the case of cell suspensions, the accumulation of malate. Additionally, as before there was no observed current consumption.

Progress

The data show that *C. necator* utilizes cathodic electrons during metabolism of oxygen and nitrate. This is an important finding suggesting that this microbe can be used as a platform for microbial electrosynthesis. In contrast, our *E. coli* data show no evidence of cathodic electron utilization using electron shuttles. Data obtained in the experiments with *Shewanella oneidensis* indicate that this microbe might be a very useful and effective microorganism for further technology development.

Future Plans

1) *C. necator*

With future work we plan on exploring different growth conditions other than denitrification as well as denitrification at more positive cathodic redox potentials where abiotic nitrite reduction will not occur. Also, we plan on trying to evolve *C. necator* H16 to better utilize electrons provided by the cathode and characterize the associated genetic changes.

2) *E. coli*

Our results have shown that in the absence of significant metabolic engineering E. coli is not a viable useful platform for funneling cathodic electrons via mediators into cellular metablism. We will examine in the future direct electron transfer in cathodic biofilms of *E. coli.*

Publications and Patents

There are no publications and patents at this point.

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