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INNOVATIVE BIOLOGICAL SOLUTIONS FOR NITROGEN FIXATION

by

Mathangi Soundararajan

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biochemistry

Approved:

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UTAH STATE UNIVERSITY  
Logan, Utah

2019

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## ABSTRACT

Innovative Biological Solutions for Nitrogen Fixation

by

Mathangi Soundararajan, Master of Science

Utah State University, 2019

Major Professor: Lance Seefeldt, Ph.D.

Department: Chemistry and Biochemistry

Nitrogen fixation is an essential process supporting all life on Earth. Industrial nitrogen fixation using the Haber-Bosch process supports over half the human population, but is not sustainable as it requires extreme temperatures and pressures, which come from fossil fuels. Biological  $N_2$ -fixation is a viable alternative, particularly for extraterrestrial applications where resources are scarce and more precious. However, scalable biohybrid technologies for nitrogen capture are not well developed. Another challenge for extraterrestrial applications is that the nitrogen available is dilute ( $\sim 2\text{-}3\%$  on Mars compared to  $78\%$  on Earth) and this needs to be addressed as well. This thesis presents our initial steps towards the development biological nitrogen fixation technologies for minimalistic growth conditions.

To establish a sustainable biohybrid system for nitrogen reduction, a microbial electrosynthetic system for  $N_2$  and  $CO_2$  fixation, using IR light ( $\sim 850\text{ nm}$ ) was developed with *Rhodopseudomonas palustris* TIE-1. The bacteria were shown to utilize IR photons centered around  $850\text{ nm}$  to support growth under both photoheterotrophic, non-diazotrophic and photoautotrophic, diazotrophic conditions with growth rates

similar to standard light conditions. They were also grown successfully in a bioelectrochemical system with in situ produced  $H_2$  as the electron source and IR radiation as the sole energy source under  $N_2$ - and  $CO_2$ -fixing conditions with high Faradaic efficiencies ( $\sim 8.5\%$ ).

Further, to address the challenge of low concentrations of  $N_2$ , the bacterial strain was adapted to grow with 2%  $N_2$  as the sole nitrogen source. The strain was observed to grow well with 2%  $N_2$ , 20%  $CO_2$  and either  $H_2$  or thiosulfate as an electron source. Maximal cell densities achieved were similar to growth with standard  $N_2$  (50%  $N_2$ ) while no growth was observed with wild type under the same conditions. Additionally, the adapted strain was found to respond differently to nitrogen starvation compared to wild type.

Together, these results provide the the basis for the development of robust nitrogen fixation technologies under resource-limited conditions. Further studies could provide sustainable systems for production of fertilizers at scale for use on Earth and in Space.

## PUBLIC ABSTRACT

## Innovative Biological Solutions for Nitrogen Fixation

Mathangi Soundararajan

Nitrogen is an important part of biological molecules like proteins and DNA and hence is essential for life as we know it. The most commonly found form of nitrogen is dinitrogen gas ( $N_2$ ) in the atmosphere, which is not accessible to most organisms. Conversion of  $N_2$  into a usable form (ammonia), occurs through an energy-demanding reaction called nitrogen fixation. As we think about sending humans to Mars, there is a need to develop technologies for nitrogen fixation to produce fertilizer and other valuable compounds to support life in those harsh conditions.

The Haber-Bosch process is used in industries on Earth to produce ammonia from  $N_2$  and  $H_2$  under 400-500 °C temperature and 150-250 atm pressure. While this has enabled low-cost fertilizer production on Earth, it is not sustainable and is impractical for manned missions to outer space. Some microorganisms can perform a similar reaction using an enzyme called nitrogenase. The biological process typically occurs under standard temperature and pressure and so is a more feasible and sustainable option. However, scalable biohybrid technologies are not well developed, especially for applications in outer space.

The overarching aim of this thesis is to lay the groundwork for biological technologies that can replace the industrial process and function in conditions where resources and nutrients are scarce. The work presented here will demonstrate a proof-of-concept system that can support bacterial growth by capturing both  $N_2$  and  $CO_2$  using low energy infra-red light, and  $H_2$  produced from water electrolysis. The bacterial culture

generated can be utilized as a fertilizer for plant growth and food production, as well as support other microbes for production of bioplastics and pharmaceuticals.

Further, we developed a bacterial strain that can grow under low nitrogen concentrations (2%  $N_2$ , compared to 78% in the Earth's atmosphere). To our knowledge, this is the first time bacteria have been observed to grow with such low concentrations of  $N_2$ . This strain will not only allow us to increase efficiencies of the hybrid system under conditions similar to Mars, but can also reveal genetic targets for further improvements. Taken together, these studies can help develop sustainable nitrogen fixation technologies for applications in outer space and may also provide a competitive alternative to the current industrial process on Earth.

To my father, mother and sister for their inspiration, encouragement and boundless support.

## ACKNOWLEDGMENTS

If it's one thing I have learned in my time at graduate school, it is that every success, small or big, is a collaborative effort. This thesis is no exception and it would be incomplete without thanking all those whose encouragement and support was crucial in my journey over the last two years.

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Mathangi Soundararajan

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## CHAPTER 1

### INTRODUCTION

#### 1.1 Supporting human exploration of Mars

Mars is a fascinating planet for a number of reasons, including its potential similarity to Earth, and the possibility of finding water and even life-forms. Martian exploration so far has been performed remotely using satellites and robots, but the inability of these systems to adapt to unexpected circumstances limits what can be studied. Manned missions to Mars, on the other hand, would allow us to explore the planet even further, enabling us to investigate hypotheses that would not be possible otherwise.

Supporting life outside Earth, however, is not a trivial task. A major concern is the production of sufficient food to support the astronauts for the entire period of the mission. The duration of a trip from Earth to Mars is about 9 months, which makes restocking from Earth a non-viable option. Growing and eating fresh fruits and vegetables also provides a number of psychological benefits, especially in extreme conditions ([Smith et al., 2009](#)). Thus, understanding how to grow plants and produce food on site is a critical part of the mission. Space travel is also associated with a number of physiological issues like radiation exposure, bone loss, vitamin deficiencies, etc., ([Smith et al., 2005](#)) that need to be treated by nutritional supplements and drugs. These pharmaceuticals have to be produced on site as they are not stable for extended periods of time ([Cooper et al., 2017](#)). Additionally, the mission has to support nitrogen fixation needed to support on-demand production of fertilizer, tools and equipment that cannot be anticipated or may be too expensive to transport.

All of these tasks require complex catalysts and chemical transformations to be performed *in situ*. Microorganisms are ideal as they are self-replicating, highly selective and can be transported in relatively small quantities (thereby reducing the weight needed for transport) (Menezes et al., 2015). The diversity of their metabolisms and enzymes allow for stable production of complex molecules like glucose, bioplastics, pharmaceuticals, etc.

## 1.2 Biological nitrogen fixation and nitrogenase

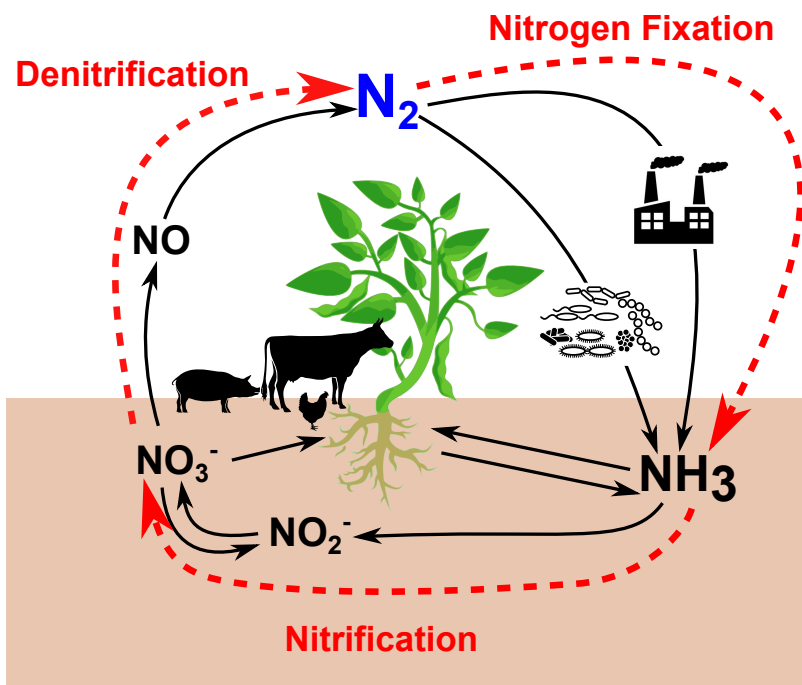


Fig. 1.1: Simplified schematic representation of the global nitrogen cycle.

The nitrogen cycle is a major biogeochemical cycle that supports all life on Earth (Figure 1.1). It allows for the incorporation of the element nitrogen into biomolecules like proteins and DNA which are essential for life as we know it. The majority of nitrogen available on Earth is in the form of dinitrogen ( $N_2$ ) which is inaccessible to

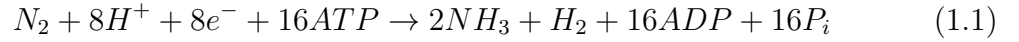
most organisms. In order to support life on Mars, we have to provide bioavailable nitrogen for plants and bacteria that perform other necessary functions like bioplastic or acetate production. Nitrogen fixation is the process by which dinitrogen ( $N_2$ ) gets reduced to ammonia ( $NH_3$ ). Currently, about half of all the nitrogen in humans is derived from the Haber-Bosch process used in industries to produce ammonia from  $N_2$  and  $H_2$  (Erisman et al., 2015; Smil, 2001). Although this process has almost reached its theoretical maximum efficiency, it requires extreme temperatures and pressures (400-500 °C, 150-250 atm) (Bosch, 1911; Haber, 1910, 1922). It also uses about 2% of all fossil fuel consumed and is not sustainable (Erisman et al., 2015).

The other half of the nitrogen comes from biological sources, where microorganisms (diazotrophs) catalyze the reduction of  $N_2$  under less extreme physical conditions. Some diazotrophs are symbiotically associated with plant roots (*Rhizobium* and *Frankia*). But these associations require complex microbe-plant interactions which is difficult to recreate in plants that don't inherently have this property like rice, wheat, etc. On the other hand, free-living diazotrophs are well studied with established culture conditions, don't require complex associations for survival and so are a viable solution to develop sustainable nitrogen reduction systems.

The microbial reduction of  $N_2$  is catalyzed by an enzyme called nitrogenase. It is a two component system consisting of a dinitrogenase reductase (more commonly known as the Fe protein) and a dinitrogenase protein (commonly known as the *M*Fe protein) (Hoffman et al., 2014). There are three known isozymes of nitrogenase based on the metal found at a particular position in the catalytic cofactor of the dinitrogenase protein (Mo, V or Fe) (Eady, 1996). The Mo-dependent nitrogenase is the most commonly found isozyme and hence the best studied. All known nitrogen-fixing organisms contain the Mo-dependent nitrogenase and may contain one or both of the other isozymes as alternative nitrogenases which are expressed when the Mo-

nitrogenase cannot be expressed or is non-functional.

The three isozymes are encoded by different genes and require different proteins for cofactor biosynthesis, expression and regulation (Eady, 1996). Equation 1.1 represents the equation for  $N_2$  reduction by the Mo-nitrogenase under ideal conditions. Despite being similar in their overall mechanism, they differ significantly in their rates and efficiencies of  $N_2$  reduction (Harris et al., 2019).



Nitrogen fixation technologies developed for application outside Earth are subject to even more constraints. Any inputs into the system (either as energy or material) that need to be processed are very costly and so must be minimized. Based on equation 1.1 it is evident that  $N_2$  reduction by nitrogenase requires  $N_2$ ,  $H^+$ , ATP and reducing equivalents. Due to the limitations of the biological system,  $H^+$  availability cannot be altered, but the other three inputs can be modulated for optimal functioning.

### 1.3 Optimizing energy source: Light

Even under ideal conditions, the nitrogenase enzyme catalyzes a highly energy-demanding reaction, hydrolyzing 16 ATP molecules for every  $N_2$  reduced. In reality, the conditions are less than optimal and there is wasteful consumption of ATP that goes into  $H_2$  generation which is not accompanied by the reduction of  $N_2$ , further increasing the ATP demand of the reaction. Thus, by identifying sustainable sources of energy for the bacteria, high energy demands of  $N_2$  fixation can be supported under minimalistic conditions.

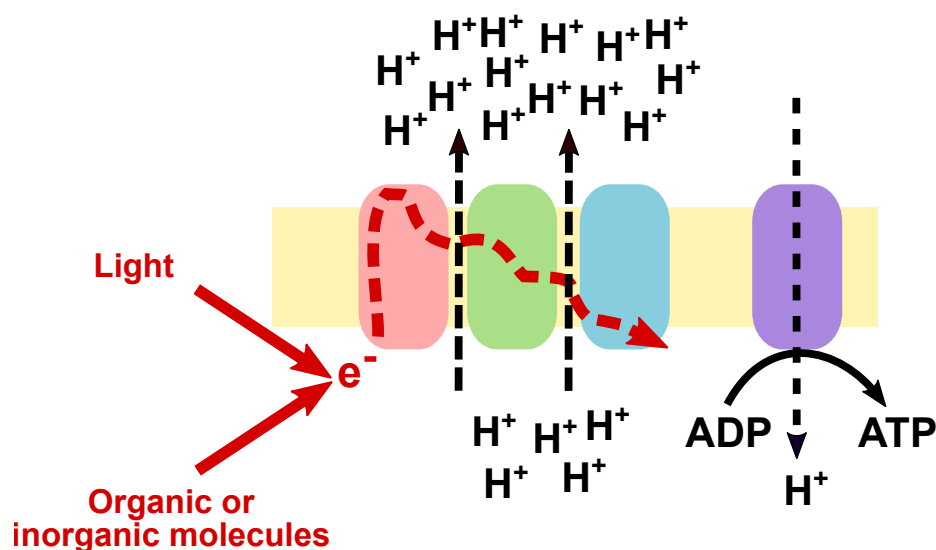


Fig. 1.2: ATP synthesis in living organisms uses energy from light or chemical compounds which mobilizes electrons through an electron transport chain. This allows protons or other ions to be pumped across the cell or organelle membrane. These excess protons are then allowed to flow back through the ATP synthase into the cell or organelle which uses the energy released by the ion transport to drive ATP synthesis.

Some free-living diazotrophs contain photosystems and use light to drive ATP production (phototrophs) (Figure 1.2). Since there is sufficient sunlight on Mars ( $\sim 50\%$  of light on Earth) (Williams, 2019), this is a viable strategy for supporting the energy demands of  $N_2$  fixation. A number of them like *Rhodopseudomonas palustris*, *Rhodobacter capsulatus* and other purple non-sulfur bacteria can also fix their own carbon (autotrophs) from  $CO_2$ . This eliminates the need for additional processes required to produce complex organic substrates. The genomes of several species and strains are fully sequenced which allows us to be able to generate mutants with desired characteristics for the system if needed.

## 1.4 Optimizing electron source: Microbial electrosynthesis

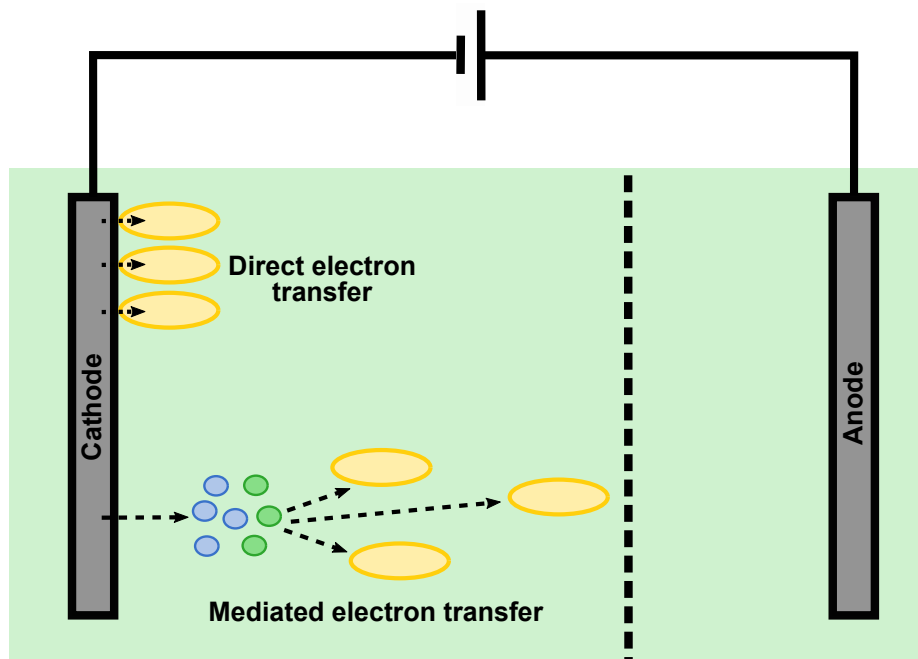


Fig. 1.3: Mechanisms of electron transfer between electrodes and microorganisms.

Nitrogenase also requires a large amount of electrons for the reaction (equation 1.1). This electron demand of the bacteria further increases when they have to fix their own  $\text{CO}_2$  as well, and so sustainable delivery of electrons is another important factor. Microbial electrosynthesis is a relatively new field that couples the efficiency of electrochemical systems with the diversity of microbial metabolisms (Rabaey and Rozendal, 2010; Schröder et al., 2015). The electrons from the electrode are transferred to bacteria through a variety of mechanisms and are used to drive reductive biosynthetic pathways for the production of valuable compounds like acetate (Liu et al., 2017) and biopolymers (Ranaivoarisoa et al., 2019).

Electron transfer can either be direct or mediated by diffusible compounds (Liu et al., 2018) (Figure 1.3). Direct electrochemistry has been well established and

studied only with a few organisms like *Geobacter sulfurreducens* (Bond and Lovley, 2003; Gregory et al., 2004). The cells typically associate closely with the electrode as a biofilm, and can transfer electrons through protein complexes on the cell surface. Other mechanisms of direct electron transfer include nanowires or electroactive pili produced by the bacteria on electrode surfaces. Mediated electron transfer uses diffusible compounds that are either added externally or produced by the bacteria themselves to shuttle electrons from the electrode to the cells. The bacteria are able to remain planktonic, and generate a lot more biomass when compared to direct electron transfer systems. Some externally added mediators commonly used in electrochemical studies include  $H_2$ , formic acid, methyl viologen, methylene blue and humic acids (Liu et al., 2015).

Although several electrosynthesis systems describe the reduction of  $CO_2$  to higher value carbon compounds, not many have extended them to study  $N_2$  reduction. One of the focuses of this thesis will be on the development of an electrosynthesis system that uses a non-toxic mediator ( $H_2$ ) to drive  $N_2$  and  $CO_2$  fixation.

### 1.5 Optimizing $N_2$ utilization under Mars-like conditions: Laboratory evolution

Although nitrogenase is efficient, it is still a slow enzyme and has a relatively high  $K_m$  for  $N_2$  binding (0.1 atm partial pressure for the purified *A. vinelandii* Mo-nitrogenase (Harris et al., 2018)). This can also change with other conditions like temperature (Dilworth et al., 1993), ATP availability (Davis et al., 1975) and the isozyme (Harris et al., 2018). Concentrations of  $N_2$  at or below the  $K_m$  can further reduce the rates and efficiencies of  $N_2$  reduction. This is not much of an issue on Earth but it can have significant impacts for extraterrestrial applications where the nitrogen is a lot more dilute. For example, the Martian atmosphere is composed of

2-3% nitrogen at less than 1% of the atmospheric pressure on Earth ([Williams, 2019](#)). This considerably limits the amount of dissolved nitrogen available for the enzyme and could reduce the yield of fixed nitrogen obtained.

One way to deal with this challenge would be to purify and concentrate the  $N_2$  gas on Mars. However, this increases the energy demands associated with the process. Another approach would be to adapt the bacteria for growth and nitrogen fixation under very low partial pressures of  $N_2$ . The metabolic versatility of purple non-sulfur bacteria make them an ideal candidate for laboratory adaptation methods. A second focus of this thesis will be on our efforts towards developing and characterizing a laboratory-adapted strain that can grow well under low partial pressures of  $N_2$ .

## 1.6 Acknowledgements

Thanks to Kyle Valgardson for his comments and discussion related to this work.

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## CHAPTER 2

Phototrophic N<sub>2</sub> and CO<sub>2</sub> Fixation Using a *Rhodopseudomonas palustris*-H<sub>2</sub>  
Mediated Electrochemical System With Infrared Photons**2.1 Abstract**

A promising approach for the synthesis of high value reduced compounds is to couple bacteria to the cathode of an electrochemical cell, with delivery of electrons from the electrode driving reductive biosynthesis in the bacteria. Such systems have been used to reduce CO<sub>2</sub> to acetate and other C-based compounds. Here, we report an electrosynthetic system that couples a diazotrophic, photoautotrophic bacterium, *Rhodopseudomonas palustris* TIE-1, to the cathode of an electrochemical cell through the mediator H<sub>2</sub> that allows reductive capture of both CO<sub>2</sub> and N<sub>2</sub> with all of the energy coming from the electrode and infrared (IR) photons. *R. palustris* TIE-1 was shown to utilize a narrow band of IR radiation centered around 850 nm to support growth under both photoheterotrophic, non-diazotrophic and photoautotrophic, diazotrophic conditions with growth rates similar to those achieved using broad spectrum incandescent light. The bacteria were also successfully cultured in the cathodic compartment of an electrochemical cell with the sole source of electrons coming from electrochemically generated H<sub>2</sub>, supporting reduction of both CO<sub>2</sub> and N<sub>2</sub> using 850 nm photons as an energy source. Growth rates were similar to non-electrochemical conditions, revealing that the electrochemical system can fully support bacterial growth. Faradaic efficiencies for N<sub>2</sub> and CO<sub>2</sub> reduction were 8.5% and 47%, respectively. These results demonstrate that a microbial-electrode hybrid system can be used to achieve reduction and capture of both CO<sub>2</sub> and N<sub>2</sub> using low

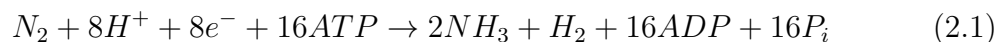
energy IR radiation and electrons provided by an electrode.

## 2.2 Introduction

Coupling microbes to electrodes is a frontier area for specialty chemical production. This approach combines the advantages of a sustainable and efficient source of electrons with the varied and designable metabolism of microbes. In microbial fuel cells, oxidation of reduced carbon compounds (such as found in the wastewater or the environment) by bacteria coupled to an electrode (anode) results in a flow of electrons to the anode, either directly or indirectly through mediators, providing a usable electric current ([Schröder et al., 2015](#)). In the other direction, electrons delivered by an electrode (cathode), either directly or through mediators, can be used to drive the biosynthetic machinery of a microbial cell for the synthesis of compounds of interest (microbial electrosynthesis) ([Rabaey and Rozendal, 2010](#); [Schröder et al., 2015](#)). Mediators commonly used to shuttle electrons between the bacteria and the electrode in microbial electrochemical systems include  $H_2$ , formate, certain dyes (e.g., neutral red, methyl viologen) and other organic compounds (e.g., hydroquinone, anthraquinone-2,6-disulfonate) ([Liu et al., 2018](#)).

A number of microbial electrosynthetic systems have been used to fix  $CO_2$  (autotrophy) and upgrade the C to an array of value added compounds such as acetate, precursors for polymers, and precursors for pharmaceuticals ([Liu et al., 2018](#); [Rabaey and Rozendal, 2010](#)). While these bacteria can obtain all C from  $CO_2$  reduction, a source of reduced N is also required to sustain life. Nitrogen is abundant in the Earth’s atmosphere as dinitrogen ( $N_2$ ), but accessing it requires energy intensive  $N_2$  fixation. This can be achieved by the industrial Haber–Bosch reaction for reduction of  $N_2$  using  $H_2$  over Fe based catalysts, which is efficient, but dependent on fossil fuels ([Erisman et al., 2015](#)). Alternatively, a number of bacteria and archaea contain

nitrogenase, the enzyme that catalyzes the ATP-dependent reduction of  $N_2$  to ammonia ( $NH_3$ ) according to the minimal reaction stoichiometry for the Mo-dependent enzyme of:



Nitrogenase requires a minimum of 16 ATP for each  $N_2$  reduced. For many nitrogen fixing bacteria, this energy comes from the consumption of reduced carbon compounds. However, this energy can come exclusively from light for nitrogen fixing, phototrophic bacteria.

Given the absolute need for fixed N to support bioelectrosynthesis and the high energy demand for conversion of  $N_2$  to fixed N, there would be value in developing microbial-electrode systems that could achieve both  $CO_2$  and  $N_2$  fixation. An earlier report showed that a complex microbial community from the environment could support both  $CO_2$  and  $N_2$  fixation driven by an electrode ([Rago et al., 2019](#)). While such a complex system has advantages, it does not easily work for production of desirable reduced N and C compounds since there could be symbiotic relationships within the community where the compounds produced by one organism may be used by another, and vice versa. In another system, the bacterium *Xanthobacter autotrophicus* could be grown on electrochemically produced  $H_2$  to achieve both  $CO_2$  and  $N_2$  fixation ([Liu et al., 2017](#)). Since *Xanthobacter* is not a phototroph, electrons abstracted from  $H_2$  by hydrogenase were required for both biosynthesis (e.g.,  $CO_2$  reduction and  $N_2$  reduction) as well as energy generation via the electron transport chain. As a result, the currents needed to drive  $CO_2$  and  $N_2$  fixation were high (10–12 mA for a 100 mL reactor), since both processes (reductive biosynthesis and energy generation) consume electrons. The use of photoautotrophic  $N_2$ -fixing bacteria in an electrosynthetic system could provide an energetic advantage since light energy captured by the

bacteria could provide the energy needed to drive both  $\text{CO}_2$  and  $\text{N}_2$  fixation while the electrons abstracted from  $\text{H}_2$  will be used for biosynthesis among other processes.

Further, there would be value in utilizing very low energy (long wavelength, greater than 750 nm) photons to support such a system. For example, plants absorb radiation minimally beyond 750 nm ([McCree, 1971](#); [Nelson and Bugbee, 2015](#); [Noda et al., 2014](#)), and thus these longer wavelength photons are not used in intensive food production scenarios. In conditions where plants and the bacteria would have to be grown in the same place (e.g., indoor food production, on deep space missions, etc.) and sunlight is the most efficient light source (in terms of both energy and economy), making maximal use of wavelengths available is critical. Fiber optics can be used effectively to capture and transmit radiation between 400 to 800 nm with much higher energy efficiencies than artificial lighting. Since light (or energy source) could very well limit the growth of both plants and the bacteria, separating wavelengths that may be used for either process would be an added advantage. Earlier studies have indicated that some phototrophic bacteria can utilize these longer wavelengths of electromagnetic radiation ([Kuo et al., 2012](#); [Qi et al., 2017](#); [Xiao, 2017](#); [Zhou et al., 2014](#)).

Here, a system is reported that couples the phototrophic bacteria *Rhodospirillum rubrum* TIE-1 to an electrode through the mediator  $\text{H}_2$  and demonstrates bioelectrosynthetic  $\text{N}_2$  and  $\text{CO}_2$  fixation supported by low energy infrared (IR) photons.

## 2.3 Materials and Methods

### 2.3.1 Culture Media

*Rhodospseudomonas palustris* TIE-1 cells were used for all our experiments because the type strain CGA009 does not have a functional uptake hydrogenase. The bacteria were grown in a defined mineral medium containing 12.5 mM of  $\text{Na}_2\text{HPO}_4$  and 12.5 mM of  $\text{KH}_2\text{PO}_4$  as the buffer components, 0.002 mg  $\text{mL}^{-1}$  of p-aminobenzoic acid and 0.1% (v/v) final concentration of the concentrated base solution [stock solution containing the following components (concentration in mM): nitrilotriacetic acid (105),  $\text{MgSO}_4$  (240),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (45),  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$  (0.015),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (2.51), EDTA (0.855),  $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$  (3.808),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (0.911),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.157),  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  (0.086),  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  (0.046)] as described before (Kim and Harwood, 1991). The medium was also supplemented with 200 mM NaCl, 1  $\mu\text{M}$   $\text{NiSO}_4$ , and 0.5% (v/v) final concentration of Wolfe’s vitamins stock solution [stock solution containing (in g  $\text{L}^{-1}$ ): p-aminobenzoic acid (0.005), folic acid (0.002), lipoic acid (0.005), riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)]. The 200 mM NaCl was added in order to improve the electrical conductivity of the medium and was included in all other growths for consistency. Further, 1  $\mu\text{M}$   $\text{NiSO}_4$  was added as a supplement when hydrogen was provided as the electron donor since the uptake hydrogenase of *R. palustris* requires Ni as a cofactor.

For growing the cells photoautotrophically, but in the presence of fixed nitrogen, photosynthetic medium (PM) was prepared as described above, but with 0.1%  $(\text{NH}_4)_2\text{SO}_4$  as the nitrogen source and 30 mM  $\text{NaHCO}_3$  as the carbon source added after degassing the medium. Photoheterotrophic growth medium was the same as PM medium with 20 mM sodium acetate as the carbon source. For photoautotrophic,

diazotrophic growth, nitrogen-fixing (NF) medium was prepared as described above and 30 mM NaHCO<sub>3</sub> was added after degassing of medium. The pH of the medium was maintained at ~6.8–7.0, unless otherwise specified.

### **2.3.2 Measurement of *R. palustris* TIE-1 Whole Cell Absorbance and Leaf Absorption Spectra**

*Rhodopseudomonas palustris* TIE-1 whole cell absorbance spectra were measured using a Cary 50 UV-visible spectrophotometer (Varian Instruments, CA, United States). Bacteria grown with either incandescent or IR light were pelleted and either used immediately or stored at 20 °C until measurement. The cells were resuspended in NF medium until the absorbances at 660 nm were approximately 0.21. An empty cuvette was used to blank the instrument and the absorbance spectra were measured with a scan step of 1 nm and a scan rate of 250 nm min<sup>-1</sup>.

Soybean was used to obtain a percent absorption spectrum of a single leaf. Leaf transmission and reflectance measurements were made with halogen lamps using a spectroradiometer (Apogee Instruments, Model PS-200, Logan, UT, United States). Measurements were made from 400 to 850 nm at 1 nm intervals. Transmission was measured through the leaf 90 ° from the abaxial side. Reflectance was measured from the adaxial side over black felt (a highly absorbent material) so that only reflectance, and not trans-reflectance, was measured. Absorptance was calculated as 1 - reflectance - transmission.

### 2.3.3 Growth Conditions

#### Photoheterotrophic Growths to Analyze Effect of Wavelength on Growth of *R. palustris* TIE-1

Glass vials with photoheterotrophic growth medium (with acetate and ammonium sulfate) were prepared and degassed by sparging argon gas through the medium for 30 min and the headspace for 15 min. The vials were sealed using rubber stoppers, and autoclaved prior to inoculation. After cooling, the headspace was again sparged with argon gas for at least 10 min and equilibrated to 1 atm using a syringe. Then, a volume of H<sub>2</sub> gas equal to the headspace volume of the vial was added to obtain a headspace gas composition of 1:1 of Ar:H<sub>2</sub>, at a pressure of 2 atm. NaHCO<sub>3</sub> was added to a final concentration of 30 mM. *R. palustris* TIE-1 cells grown photoheterotrophically with a 60 W incandescent bulb was used as the inoculum. The cells were first pelleted and the pellets were washed twice with NF medium, to remove all traces of previous media and extracellular components prior to inoculation.

The cultures were then grown at room temperature (20–25 °C) with light emitting diodes (LED) with peak wavelengths of 665 nm (Fluence Bioengineering, Model RAY22 custom spectra LED, TX, United States), 735 nm (Fluence Bioengineering, Model RAY22 custom spectra LED, TX, United States) and 850 nm (bought from Amazon.com), or broad spectrum cool white 6500 K LED (Fluence Bioengineering, Model RAY22 custom spectra LED, TX, United States). Cardboard boxes were used to exclude ambient light, and care was taken to minimize ambient light exposure while sampling. Light-source distances were adjusted to provide about 200  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  to the culture vials. The cultures were not stirred or shaken at any point during the experiment except during sampling. The cultures were sampled using a disposable syringe that was made anaerobic by purging with argon gas three times

prior to sampling, and the optical densities were measured using a Cary 50 UV-visible spectrophotometer (Varian Instruments, CA, United States) approximately every 4 h. All growths were performed in duplicates and the error bars represent standard deviations of the optical density measurements. Detailed description of the calculation of doubling times and specific growth rate are provided in the Supplementary Material (Appendix C).

### **Photoautotrophic, Diazotrophic Growth of *R. palustris* TIE-1 Using Incandescent or IR Photons**

Photoautotrophic, diazotrophic growths were performed in glass vials. The NF medium was added to glass vials, and degassed by bubbling N<sub>2</sub> gas through the medium for ~20 min, and ~10 min through the headspace. The vials were sealed with rubber stoppers and autoclaved. After cooling, the headspace was bubbled for another 20 min with N<sub>2</sub> gas and equilibrated to 1 atm pressure. Then a volume of H<sub>2</sub> gas equal to the volume of the headspace was added to obtain a headspace composition of 1:1 of N<sub>2</sub>:H<sub>2</sub>, at a final pressure of 2 atm. NaHCO<sub>3</sub> was added for a final concentration of 30 mM. *R. palustris* TIE-1 cells grown under photoautotrophic, diazotrophic conditions with incandescent or IR light were used as inocula for the incandescent and IR growth conditions, respectively. The cells were pelleted and re-suspended in NF medium prior to inoculation into the cultures.

The cultures were then grown at room temperature (20–25 °C) with 60 W incandescent light or IR LED (with a peak of 850 nm). Cardboard boxes were again used to exclude ambient light. The light conditions were not standardized for the photon flux density since it was not possible to obtain a full spectrum of the incandescent light that typically extends to well beyond 1000 nm. Also, due to the very broad spectrum of the incandescent light, it would not be possible to identify which of the

photons provided would be photosynthetically useful, and hence any measurement of the photon flux density would not be relevant to understanding its effects on the growth of the bacteria. The cultures were sampled at  $\sim 24$  h intervals as described above for photoheterotrophic growth and all growths were performed in duplicates.

### 2.3.4 Growth in the Hybrid System

A microbial fuel cell (Adams and Chittenden Scientific glass, Berkeley, CA, United States) was used for bacterial growth with electrochemically produced  $\text{H}_2$ . It was a modified H-cell with 150 mL cathodic and anodic compartments connected by half-inch glass flanges with corresponding seals and wraparound knuckle clamps. There were also two sampling ports on each compartment to sample either the media or the headspace. After autoclaving the glassware, a Nafion 117 cation exchange membrane was sandwiched between the flanges to separate the anodic and cathodic halves. Rubber stoppers were used to seal the sampling ports on the sides of the half cells as well as the top. The anode and cathode were made of 16-gauge platinum wires ( $\sim 3$  cm) extended with copper wires. Electrodes were cleaned prior to each experiment by soaking in 5% HCl for 5 min and then wiping with ethanol. 100 mL of the autoclaved growth medium was used as the electrolyte in both the anode and the cathode compartments (NF medium for  $\text{N}_2$ - and  $\text{CO}_2$ -fixing growth and PM medium for the  $\text{CO}_2$ -fixing growth). The electrolyte was made anaerobic by bubbling Ar or  $\text{N}_2$  gas through the anolyte and catholyte for 30 min and the headspace for 15 min.  $\text{NaHCO}_3$  was added at a final concentration of 30 mM after the medium was degassed. The electrolyte was constantly sparged with an 80:20 mixture of  $\text{N}_2$ : $\text{CO}_2$  gas in order to maintain anaerobicity, provide  $\text{N}_2$  and  $\text{CO}_2$  for bacterial growth as well as to maintain the pH at  $\sim 7.5$ . *R. palustris* TIE-1 cells grown photoheterotrophically with 60 W incandescent or halogen light were used as the inoculum. The cells

were pelleted, washed three times with NF medium, resuspended in 1 mL of degassed catholyte prior to inoculation and transferred to the cathodic compartment.

Electrolysis was performed at a constant potential difference of -3 V vs. the counter electrode (around -1.4 V vs. SHE) using a MultiEmStat (PalmSens BV, Netherlands) potentiostat to enable hydrogen evolution. This potential was chosen in order to achieve the currents necessary to support CO<sub>2</sub> and N<sub>2</sub> reduction. The cell was maintained at constant potential since the potentiostat did not have the capability to maintain constant current. An 850 nm LED light source (bought from Amazon.com) was used as the light source. The pH was checked regularly using pH strips and additional CO<sub>2</sub> gas was sparged through the catholyte to reduce it to  $\sim 7.5$  if it was found to be  $\sim 8.0$  or above. The cultures were maintained at room temperature (20–25°C). Under CO<sub>2</sub>-fixing conditions, the cultures were not mixed at any time except during sampling. Additional 850 nm light was provided for the N–2-fixing growth in the form of an additional, smaller IR LED (bought from Amazon.com), and the electrolyte was stirred. In our system, we found that this was important to support N<sub>2</sub>-fixation. We observed modification of the electrode surface during the course of the experiment which probably contributed to the slowly decreasing current over time (Supplementary Figure C.1).

Samples were obtained once a day using disposable syringes purged with Ar or N<sub>2</sub> gas as described for photoheterotrophic growths, through sampling ports on the sides of electrolysis cell. The optical density measurements were obtained as described for the photoheterotrophic growth. All growths were performed in duplicates.

### 2.3.5 Measurement of Spectroradiometric Traces of Light Sources

The spectra of the lights were obtained using a spectroradiometer (Apogee Instruments, Models PS-100 and PS-200, Logan, UT, United States). Spectral mea-

measurements were made at the same distance that the light sources were placed from the culture vials for the photoheterotrophic growths so that the area under the curve could be integrated to calculate the photon flux density received by each culture. The area was integrated using the software IgorPro 6 (Wavemetrics, Portland, OR, United States).

## 2.4 Results

### 2.4.1 Absorbance Spectra of *R. palustris* TIE-1 and of Plant Leaves

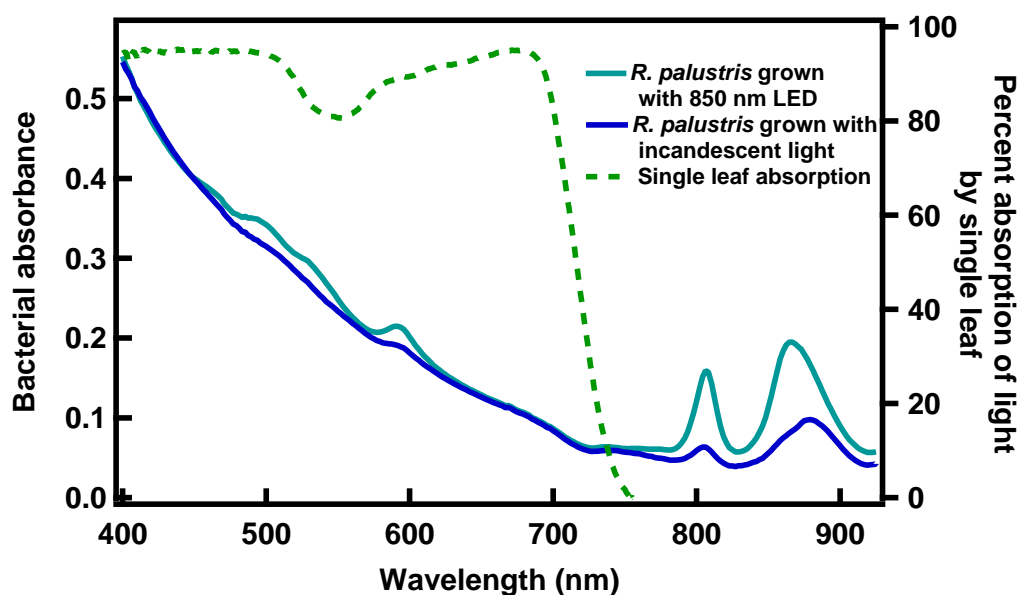


Fig. 2.1: Whole cell absorbance spectra of *R. palustris* TIE-1 and the absorption spectrum of a single leaf. The cell densities were normalized based on OD<sub>660</sub> prior to obtaining bacterial absorbance spectra.

In order to identify which wavelengths could potentially be used by the bacteria, but would not be used by plants, absorption spectra of leaves and of *R. palustris* TIE-1 were recorded (Figure 2.1). While the absorption of photons by leaves drops to zero beyond  $\sim 750$  nm, the bacteria still show some absorbance in the 750–900 nm

region. These spectra are similar to what has already been described for plant leaves (McCree, 1971; Nelson and Bugbee, 2015; Noda et al., 2014) and *R. palustris* (Gall and Robert, 1999; Hayashi et al., 1982; Qi et al., 2017). The absorption spectra for the bacteria were similar when grown with white, 665, 735, or 850 nm LED light (Supplementary Figure C.2). The two peaks at  $\sim 800$  and  $\sim 870$  nm in *R. palustris* have been ascribed to the two bacteriochlorophylls in purple non-sulfur bacteria (Gall and Robert, 1999; Hayashi et al., 1982). Thus, while these longer wavelengths are not used by plants, they might be used by the bacteria, a phenomenon that would be useful in cases where plants and the bacteria are grown in a closed system that has limited access to light.

It is noted that the intensities of the bacteriochlorophyll pigments varied between the two light conditions shown here (Figure 2.1) and that the peak corresponding to the second bacteriochlorophyll was slightly shifted when grown under incandescent light as compared to 850 nm LED. This could be a function of either the intensity or the spectrum of light used, and it is unclear which of the two factors play a role in causing the observed variations.

#### 2.4.2 Effect of Wavelength on *R. palustris* TIE-1 Growth

While IR photons have been used to culture purple non-sulfur photosynthetic bacteria in the past (Kuo et al., 2012; Qi et al., 2017; Xiao, 2017; Zhou et al., 2014), the effects of wavelength on the growth of the bacteria was not well described. The earlier studies quantified intensity as lux (Kuo et al., 2012; Qi et al., 2017; Zhou et al., 2014) or  $\text{W m}^{-2}$  (Xiao, 2017), which weight the photons in favor of human vision and total energy, respectively. Based on the Stark–Einstein law, intensity should be based on the number of photons delivered to the culture. ATP production by photophosphorylation is related to the number of absorbed photons and so normalizing

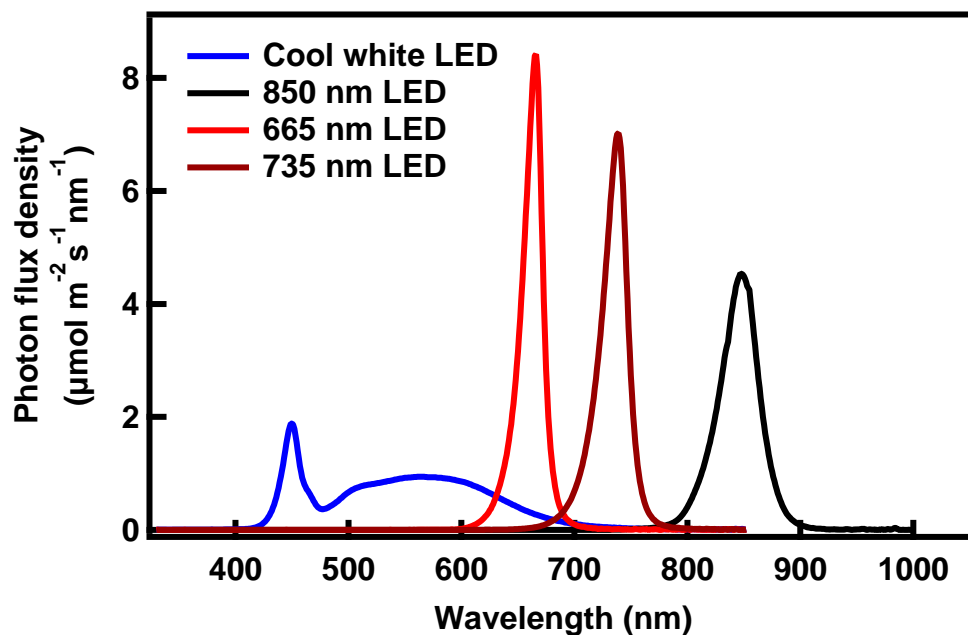


Fig. 2.2: The spectroradiometric traces of the different LED lights used in the experiments. The photon flux density as calculated as the area under the curve is  $\sim 200 \mu\text{mol m}^{-2} \text{s}^{-1}$  for all the conditions.

intensities based on photon flux density is essential to understanding the effects of different wavelengths on bacterial growth.

Light emitting diodes are a new technology that can provide light of narrow wavelength bands, enabling us to study the effects of specific wavelengths on bacterial growth. Longer wavelengths were chosen to facilitate identification of wavelengths that would be minimally used by plants, but may be used to grow bacteria. Spectra of the LEDs used in these studies showed that the red, far-red and IR sources provided a narrow spectrum, with peak wavelengths of 665, 735, and 850 nm (Figure 2.2). The cool white LED provided a broad spectrum light source including the lower wavelengths (Figure 2.2). It was not possible to compare the growth rates under LED lights to that under incandescent light, which is typically used to grow these bacteria, as it was not feasible to obtain a complete spectrum of the incandescent light due to the limited range of the spectroradiometer used. So calculation of the total number

of photons delivered to the bacteria was not possible.

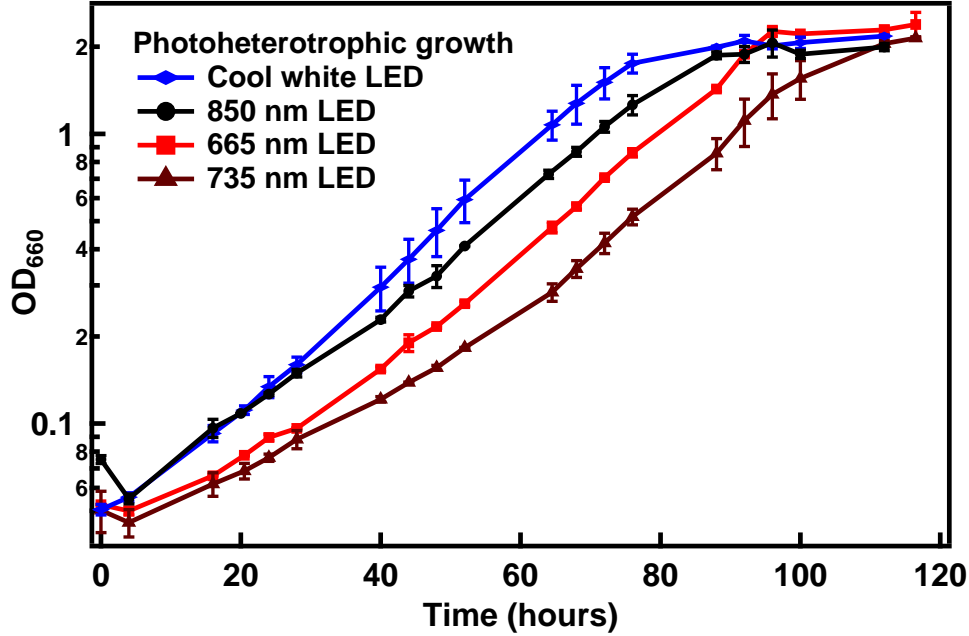


Fig. 2.3: Growth of *R. palustris* with different light sources under photoheterotrophic conditions. Doubling times were found to be  $\sim 16$  h under all light conditions except with 850 nm LED ( $\sim 20$  h). When calculated as specific growth rate, it was found to be  $0.5 \text{ h}^{-1}$  for all light conditions except under 735 nm LED ( $0.4 \text{ h}^{-1}$ ).

*Rhodospseudomonas palustris* TIE-1 was cultured under photoheterotrophic conditions with acetate and ammonium sulfate as carbon and nitrogen sources, respectively. Interestingly, it was found that the doubling time of the bacteria was the same for three wavelengths used in this study – broad-spectrum cool white, 665 or 735 nm ( $\sim 16$  h) – and was only slightly longer under 850 nm ( $\sim 20$  h) (Figure 2.3). This difference in growth rate was not due to higher absorption of IR light by medium components compared to the lower wavelengths since absorption of 400–900 nm wavelengths by the medium was negligible over the path length of the culture vial (data not shown). Since bacterial growth is exponential, the growth rates were also calculated based on the equation for exponential growth after 40 h until the end of log

phase for each growth condition (see Appendix C, Supplementary Material for more detailed calculations of specific growth rate and doubling times). The specific growth rates were very similar under all wavelength conditions ( $0.05 \text{ h}^{-1}$ ), while marginally slower under 735 nm LED ( $0.04 \text{ h}^{-1}$ ). It is unclear if this difference in specific growth rate is due to the error within the measurement or due to differences in the light conditions tested.

### 2.4.3 Growth Under Photoautotrophic, Diazotrophic Conditions Using IR Radiation

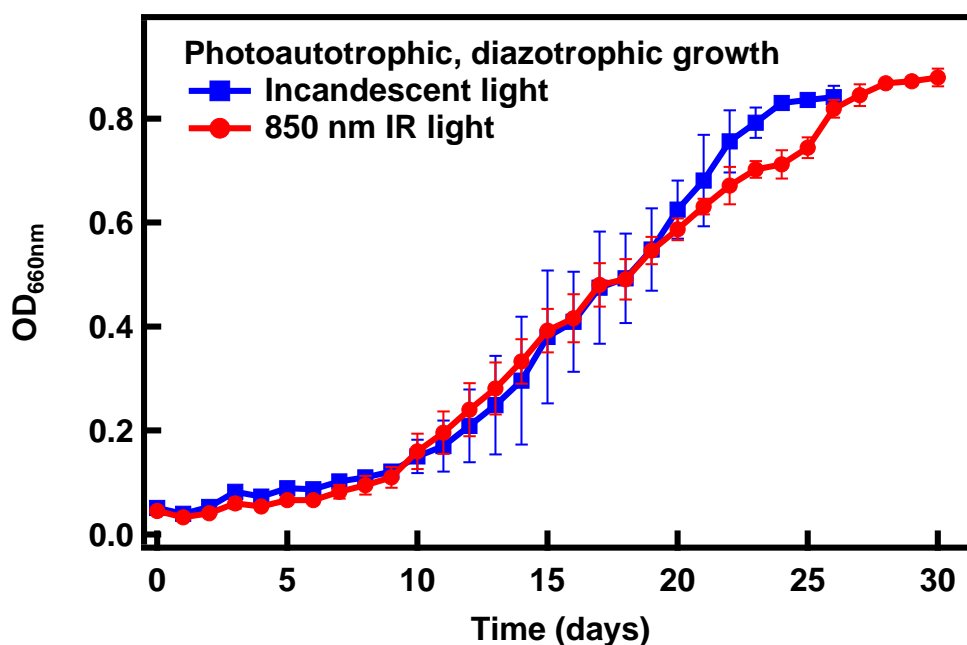


Fig. 2.4: Photoautotrophic, diazotrophic growth of *R. palustris* TIE-1 under non-electrochemical conditions. The doubling time was found to be  $\sim 4$  days under both light conditions.

$\text{N}_2$  fixation by *R. palustris* is an energy intensive process, and simultaneous  $\text{CO}_2$  fixation requires additional energy. Given the lower energy of light beyond 800 nm, it was important to study if IR light would support growth similar to the standard

light source (incandescent light) when grown under  $N_2$ - and  $CO_2$ -fixing conditions. *R. palustris* TIE-1 was cultured under photoautotrophic, diazotrophic conditions using either 850 nm or standard incandescent light. As can be seen (Figure 2.4), the cells grow equally well under both conditions, revealing that 850 nm photons can support the energy demands for both  $CO_2$  and  $N_2$  fixation.

#### 2.4.4 Growth in a *R. palustris*-Electrode Hybrid System

After revealing that 850 nm photons could be used to support *R. palustris* TIE-1 grown under  $CO_2$  and  $N_2$  fixation conditions in the presence of  $H_2$ , cells were grown in the cathodic side of an electrolysis cell with in situ generation of  $H_2$ . Electrolysis of water can provide a sustainable and regular supply of  $H_2$  for bacterial growth (Figure 2.5). The working electrode was maintained at around -3 V vs. the counter electrode using a potentiostat. This potential difference was chosen in order to provide the currents necessary to produce the amount of  $H_2$  that would support bacterial growth. The experiments were performed at constant potential and not constant current as this was beyond the capability of the potentiostat used.  $H_2$  evolution was ob-

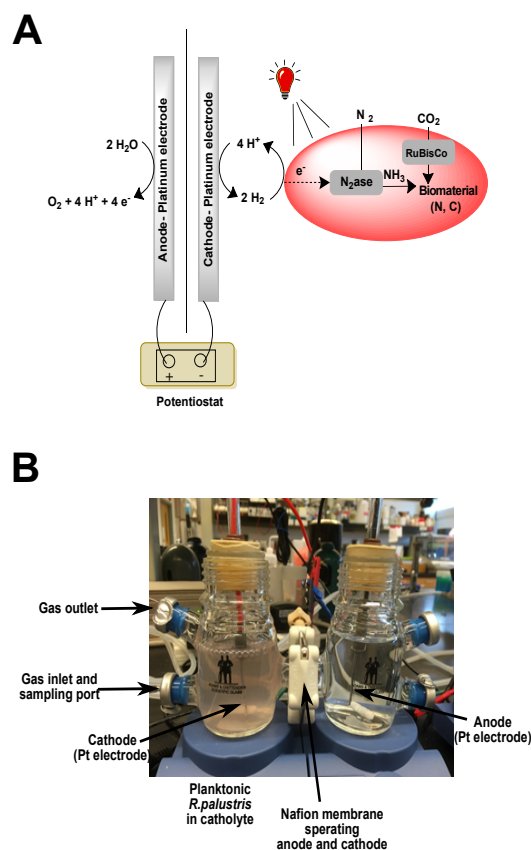


Fig. 2.5: A schematic version of the bio-electrochemical system (A) and the actual bioelectrochemical setup used in the experiment (B).

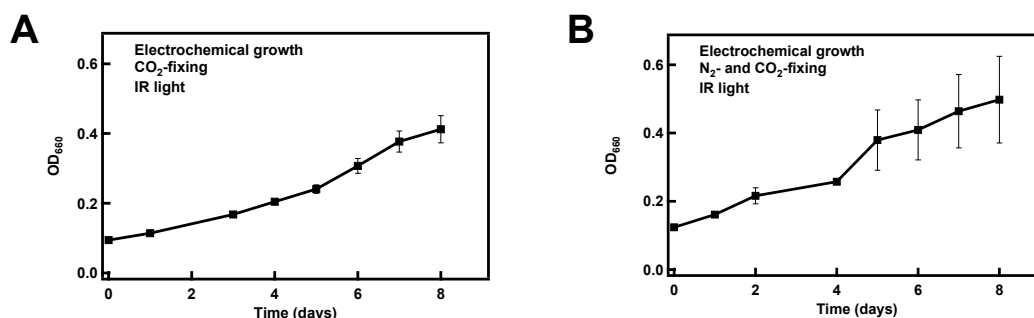


Fig. 2.6: Growth of *R. palustris* TIE-1 in a hybrid system under CO<sub>2</sub>-fixing (A) or N<sub>2</sub>- and CO<sub>2</sub>-fixing (B) conditions. The doubling time was  $\sim 4$  days under both growth conditions.

served as bubbles formed at the platinum cathode. The bacteria grew under CO<sub>2</sub>-fixing or N<sub>2</sub>- and CO<sub>2</sub>-fixing conditions in this hybrid system using 850 nm LED as the light source and the electrocatalytically produced H<sub>2</sub> as the electron source (Figure 2.6). The average doubling time was  $\sim 4$  days under both growth conditions, which was similar to growth under non-electrochemical, N<sub>2</sub>- and CO<sub>2</sub>-fixing conditions. No growth was observed over a period of 7 days when light or current was turned off (data not shown). Intriguingly, in the hybrid system, no lag phase was observed (Figure 2.6), although a significant lag phase ( $\sim 10$  days) was observed under non-electrochemical conditions (Figure 2.4).

The extracellular medium was tested for the presence of fixed nitrogen to confirm that growth of the bacteria in the hybrid system with NF medium was indeed under diazotrophic conditions. A modified o-phthalaldehyde fluorescence method previously described (Allison et al., 1984) was used to monitor the extracellular medium for the presence of primary amines and/or ammonia over the course of the diazotrophic bioelectrochemical growth. No signal above background was observed during the growth period (data not shown) indicating the absence of sufficient fixed nitrogen sources in the medium that could have supported their growth.

The amount of H<sub>2</sub> produced per day in the hybrid was estimated using the charge

passed through the system (Supplementary Figure C.1). It should be noted that the actual amount of  $\text{H}_2$  produced might be lower than what was calculated since the electrons driven into the solution from the cathode may also be used to drive reactions other than proton reduction. Since the electrolyte used was the growth medium, composed of a number of compounds including several metal salts, it was not possible to identify which (if any) other electrochemical reactions were occurring at the electrode. Assuming an ideal situation where all the electrons are used to reduce protons, the amount of hydrogen produced was estimated to be  $\sim 288 \text{ mol day}^{-1}$ .

At the end of the 8 day electrochemical  $\text{N}_2$ -,  $\text{CO}_2$ -fixing growth, the total cell dry weight fixed was found to be  $0.13 \text{ g } 100 \text{ mL}^{-1}$ . Assuming that 14% of the cell dry weight is N and 50% is C,  $16.25 \text{ } \mu\text{mol}$  of N atoms and  $67.7 \text{ } \mu\text{mol}$  C atoms were fixed per day for the  $100 \text{ mL}$  bioreactor. In order to calculate the efficiency of the system, the percentage of electrons delivered by the cathode that were converted into fixed N and fixed C products was calculated. The nitrogenase reaction produces 1  $\text{H}_2$  for every  $\text{N}_2$  reduced under ideal conditions. Knowing this minimal stoichiometry (assuming 0% recycling of  $\text{H}_2$  produced by the nitrogenase reaction), it was calculated that 11% of electrons were used for fixing N. If it is assumed that 100% of the  $\text{H}_2$  produced by nitrogenase was recycled, the faradaic efficiency for  $\text{N}_2$  fixation is 8.5%. Similarly, the reduction of  $\text{CO}_2$  by the Calvin cycle requires a minimum stoichiometry of four electrons per  $\text{CO}_2$  reduced. Using this assumption, it was calculated that 47% of the electrons were used for direct fixation of  $\text{CO}_2$ . The remaining electrons delivered at the cathode may be used for other reductive reactions by the bacteria, lost as  $\text{H}_2$  gas to the atmosphere, or be used to electrochemically reduce other medium components. Using similar assumptions, the faradaic efficiency was calculated for the  $\text{CO}_2$ -fixing growths and was found to be  $\sim 38\%$  for reduction of carbon (see Appendix

C, Supplementary Material for the complete calculations).

## 2.5 Discussion

Bacterial-electrochemical hybrid systems offer great potential for sustainable upgrading of both CO<sub>2</sub> and N<sub>2</sub> to higher value chemicals. The use of *R. palustris* TIE-1 in electrosynthetic systems is not new, with prior examples using Fe(II) as a mediator (Doud and Angenent, 2014; Rengasamy et al., 2018) or direct electron transfer (Bose et al., 2014; Guzman et al., 2019; Ranaivoarisoa et al., 2019; Rengasamy et al., 2018). However, there are some inconsistencies with these reports. The direct electron transfer observed by Bose et al. (2014) could be complicated by trace concentrations of iron used in the medium, as higher currents were reported with increased Fe(II) concentrations in the medium (Doud and Angenent, 2014). Even in the case of Fe(II) as a mediator, while one report suggests that soluble Fe(II) can be used as a mediator (Doud and Angenent, 2014), a different report suggests that the Fe(II) has to be immobilized before it may act as a mediator (Rengasamy et al., 2018). Given these contradictions in the literature, exploring alternative mediators like H<sub>2</sub> would be useful. Here, it was demonstrated that an electrosynthesis system comprising a photoautotrophic bacterium and an electrode to generate H<sub>2</sub> could be used to fix both CO<sub>2</sub> and N<sub>2</sub>. While there are a number of diazotrophs that can use H<sub>2</sub> as an electron source, only a very small subset exist that can also use light as a source of energy as well as CO<sub>2</sub> as a source of carbon. Since N<sub>2</sub> fixation by the bacteria is a very energy intensive process, the use of a phototroph provides a way to sustainably produce the ATP that is needed to drive N<sub>2</sub> fixation.

The growth rate (doubling time) was marginally different when grown with the 850 nm LED compared to the other wavelengths tested. However, when the growth was fit to the equation of exponential growth, the differences in growth rates were

negligible between the growth conditions. This suggests that the efficiency of photon capture and excitation of an electron by the photosystem is very similar between all wavelength conditions tested. It was interesting that the bacteria were able to grow just as well with 735 nm light (based on doubling times) given that they showed minimal absorbance in the 735 nm region of the spectrum (Figure 2.1). Thus it is possible that despite the lower absorbance in that region, the ability of the bacteria to capture and utilize the light for photophosphorylation is similar to other light conditions tested. The bacteria were also able to use 850 nm photons exclusively for growth under both photoheterotrophic, non-diazotrophic conditions as well as photoautotrophic, diazotrophic conditions with growth rates similar to standard light conditions (incandescent light). Given that the 850 nm photons would not be able to efficiently excite the bacteriochlorophyll that absorbs at 800 nm, it was interesting that no significant growth defects were observed.

The 850 nm LED was used successfully in cooperation with the electrochemical  $H_2$  generation to drive  $CO_2$ - and  $N_2$ -reduction by the bacteria with growth rates similar to what was observed non-electrochemically. The nitrogen and carbon were captured in the biomass, which can potentially be digested and used as fertilizer. Advantages of this hybrid approach over a non-electrochemical one are “*in situ*” generation of  $H_2$  from the electrocatalytic water splitting, which could be generated from renewable energy sources (e.g., light or wind). Additionally, other molecular mediators like neutral red and methyl viologen may be explored, which have been shown with other microorganisms as a replacement to the  $H_2$  evolution reaction (Liu et al., 2017). The use of these other mediators could reduce the applied potential and increase faradaic efficiencies, thereby resulting in a more energetically efficient system. Doubling time of the bacteria in the hybrid system was similar to what was observed under non-electrochemical conditions and no lag phase was observed in the hybrid

system. Under similar non-electrochemical conditions, a very significant lag phase of  $\sim 10$  days was observed. This could be due to the large quantity of  $H_2$  provided to the bacteria under non-electrochemical conditions ( $\sim 50\%$  of the headspace). In comparison, the  $H_2$  produced on average in the electrochemical system was probably sufficient to replenish dissolved  $H_2$  utilized by bacteria without affecting or inhibiting their growth during the initial period. This lack of a lag phase was also observed in a similar system with *Xanthobacter* (Liu et al., 2017).

By using a phototroph,  $CO_2$  and  $N_2$  reduction was achieved in a microbial electrosynthetic system at much lower currents than previously reported. Average current maintained in this system was less than 1 mA, while current of 10–12 mA was needed to drive  $N_2$  fixation in a system with *Xanthobacter* (Liu et al., 2017). Thus, the use of a phototroph provides a significant advantage over non-phototrophs by providing energy from light. This is evident from the calculated faradaic efficiencies of this system for  $N_2$  fixation ( $\sim 8.5\%$ ) as compared to a previously reported system that used the chemolithotroph *Xanthobacter* ( $\sim 4.5\%$ ) (Liu et al., 2017). Despite the large differences in currents between the two systems, the potential difference across the electrodes were the same in both studies. This could be optimized further by improving electrolyte concentrations, reducing distances between the electrodes or using a better  $H_2$ -evolution catalyst that is more resistant to passivation over longer durations. *R. palustris* has been found to tolerate relatively high amounts of salt, growing well with 200 mM NaCl concentration in this system. Further studies optimizing the bioelectrochemical system for salt concentration, electrode material, and reactor configuration could improve the energy efficiency of the system. Different configurations of light may also be used to improve bacterial growth and increase cell yields as has been found in another similar system (Doud and Angenent, 2014).

Another advantage in using *R. palustris* is that a genetic system for this bacteria

is well established and a complete genome sequence is available ([Larimer et al., 2003](#)). Thus genetic manipulation for extracellular ammonia production could be performed to obtain ammonia rather than organic nitrogen from biomass. In fact, a mutant of *R. palustris*, which constitutively expresses nitrogenase, has been shown to generate extracellular ammonium when grown under NF conditions ([Adessi et al., 2012](#)). Although the uptake hydrogenase is non-functional in the mutant and so cannot be applied to this system, this indicates that genetic manipulation of the bacteria could force them to release ammonia into the extracellular medium which would be readily accessible. This mutant would also be useful in systems that replace  $H_2$  with other non-gaseous mediators like neutral red or methyl viologen. The use of glutamine synthetase inhibitors would be another viable option, as was used in the study with *Xanthobacter*, to obtain extracellular ammonia ([Liu et al., 2017](#)). Apart from ammonia as the main product from  $N_2$  fixation, this system may also be potentially applied to drive  $CO_2$  reduction to valuable carbon compounds. Given the established genetic system in the bacteria, metabolic engineering of the bacteria can be performed to generate mutants that would be able to convert  $CO_2$  into precursors for bioplastics ([Ranaivoarisoa et al., 2019](#)), pharmaceuticals, or biofuels. For example, earlier studies showed that *R. palustris* can produce methane from  $CO_2$  in a single enzymatic step catalyzed by the nitrogenase enzyme ([Fixen et al., 2016](#)). When combined with the bacterial-electrode hybrid system, it is possible to develop an energy sustainable system for in situ production of both fixed C and N.

## 2.6 Acknowledgements

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## CHAPTER 3

### Laboratory Adaptation for Improved Growth with 2% N<sub>2</sub>

#### 3.1 Abstract

Understanding growth of diazotrophs under dilute N<sub>2</sub> condition is useful for improving yield and efficiencies of biohybrid N<sub>2</sub> capture technologies used in outer space. However, under low partial pressures of nitrogen, bacteria can enter a non-growing state and do not fix additional nitrogen. In this work, *R. palustris* TIE-1 was adapted to grow well with 2% N<sub>2</sub>, 20% CO<sub>2</sub> using H<sub>2</sub> or thiosulfate as an electron source. The strain grew to ODs of  $\sim 0.85$  (maximal ODs typically seen with 50% N<sub>2</sub>) while the wild type showed no growth over the same time period. There was no growth advantage observed with standard N<sub>2</sub> concentrations or with acetate as the electron and carbon source. When grown with acetate it was found that despite the lack of a growth advantage, the *in vivo* nitrogenase activity of the adapted strain was almost three times that of wild type. The adapted strain also displayed ammonia inhibition of nitrogenase activity, while this was abolished in wild type after extended nitrogen starvation. In combination, the data suggests that the strain has been adapted specifically for growth under 2% N<sub>2</sub>, under CO<sub>2</sub>-fixing conditions, using H<sub>2</sub> or thiosulfate as an electron source and that its nitrogen starvation response is different from the wild type strain. Apart from improving nitrogen capture in the biohybrid system under low N<sub>2</sub>, this strain can also guide targeted engineering approaches for improved biological nitrogen fixation for a variety of applications.

### 3.2 Introduction

Improving the efficiency of biological nitrogen fixation under different conditions is important for supporting life outside Earth, especially when the available nitrogen is very dilute. Although nitrogenase is an efficient enzyme, it still requires 8 ATP for the production of 1  $\text{NH}_3$  molecule under ideal conditions (Eady, 1996). With much lower concentrations of  $\text{N}_2$  (below the  $K_m$  for nitrogenase), the efficiencies decrease significantly, and limits the yield of fixed nitrogen and rates of biomass production. One approach to deal with this challenge would be to generate targeted mutants of nitrogenase (for a lower  $K_m$ ), or associated proteins to improve efficiency of N capture at low concentrations. However, this is not a trivial task.

Nitrogenase is a very complex enzyme. Despite being studied for decades, the exact site of  $\text{N}_2$  binding and a detailed mechanism of action is still elusive (Christiansen et al., 2001). Most known point mutants of the enzyme have either reduced or no  $\text{N}_2$  reduction activity (Christiansen et al., 2001; Seefeldt et al., 2009). So, generating targeted mutants of nitrogenase with lower  $K_m$  is extremely challenging. The  $K_m$  is also a function of other factors like temperature (Dilworth et al., 1993), ATP availability (Davis et al., 1975), the isozyme (Harris et al., 2019, 2018), etc. Furthermore, given the complex regulatory mechanisms involved in expression and activity, a combination of several mutations will likely be needed to achieve the same phenotype.

Engineering metabolism of the strains for improved nitrogen capture requires a detailed understanding of the cell under those conditions. Very little is known about the growth and metabolism under conditions of low  $\text{N}_2$ . Much of the research, particularly with purple non-sulfur bacteria, is related to biohydrogen production using nitrogenase under nitrogen starvation conditions (no  $\text{N}_2$  or fixed N sources). In these conditions, the bacteria enter a non-growing state and overexpress nitrogenase in an

attempt to capture any available nitrogen ([Arp and Zumft, 1983](#)). Since there is no  $N_2$ , the enzyme reduces protons and produces large amounts of hydrogen, most of which isn't reused by the cell. In most of the studies, the bacteria were grown heterotrophically (with an organic carbon source for electrons and carbon), and allowed to stay in this non-growing state. While these studies provide some insight into the metabolic changes under nitrogen starvation, they do not reveal any information about how they may efficiently capture nitrogen and grow.

Given the drawbacks of targeted genetic engineering techniques, laboratory adaptation of the bacteria is an ideal strategy. It requires the maintenance of a selective pressure for an extended period of time until a desired phenotype is obtained. This allows for mutations to occur across the genome, within both coding regions and regulatory regions which can be used to guide further targeted engineering strategies. Here we present the adaptation of a strain that can grow well with 2%  $N_2$  as the sole nitrogen source and its phenotypic and metabolic characterizations.

### 3.3 Materials and methods

#### 3.3.1 Media composition

The NF- $H_2$  medium is a defined mineral medium containing 12.5 mM of  $Na_2HPO_4$  and 12.5 mM of  $KH_2PO_4$  as the buffer components, 0.002 mg  $mL^{-1}$  of p-aminobenzoic acid and 0.1% (v/v) final concentration of the concentrated base solution [stock solution containing the following components (concentration in mM): nitrilotriacetic acid (105),  $MgSO_4$  (240),  $CaCl_2 \cdot 2H_2O$  (45),  $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$  (0.015),  $FeSO_4 \cdot 7H_2O$  (2.51), EDTA (0.855),  $ZnSO_4 \cdot H_2O$  (3.808),  $MnSO_4 \cdot H_2O$  (0.911),  $CuSO_4 \cdot 5H_2O$  (0.157),  $Co(NO_3)_2 \cdot 6H_2O$  (0.086),  $Na_2B_4O_7 \cdot 10H_2O$  (0.046)] as described before ([Kim and Harwood, 1991](#)). The media was supplemented with 1  $\mu M$   $NiSO_4$ , and 0.5% (v/v) fi-

nal concentration of Wolfe’s vitamins stock solution [stock solution containing (in g L<sup>-1</sup>): p-aminobenzoic acid (0.005), folic acid (0.002), lipoic acid (0.005), riboflavin (0.005), thiamine (0.005), nicotinic acid (0.005), pyridoxamine (0.01), pantothenic acid (0.005), cobalamin (0.0001), biotin (0.002)]. 30 mM NaHCO<sub>3</sub> was added as the carbon source after degassing.

The NF-TS medium was the same as the NF-H<sub>2</sub> medium, with 10 mM thiosulfate and without the NiSO<sub>4</sub> or Wolfe’s vitamins. The photosynthetic medium (PM) was prepared similar to the NF-H<sub>2</sub> medium (without NiSO<sub>4</sub> or Wolfe’s vitamins), but with 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the nitrogen source. Photoheterotrophic (PH) growth medium was the same as PM medium with 15 mM or 30 mM sodium acetate as the carbon source. The pH of the medium was maintained at ~6.8–7.0, unless otherwise specified.

### 3.3.2 Growth conditions

*Rhodopseudomonas palustris* TIE-1 cells were used for all our experiments because it was the same strain used in the electrosynthetic system.

All growth trials were conducted in anaerobic vials (80 mL or 160 mL volume) sealed with blue butyl rubber stoppers and crimp seals to maintain anaerobicity. The vials were then filled with either 25 mL (80 mL total capacity) or 75 mL (160 mL total capacity) of the respective media. Then Ar gas was bubbled through the media for ~30 mins, and the headspace for ~15 mins to remove all traces of oxygen. The vials were then sealed, autoclaved and stored at room temperature till use.

The vials were degassed again with Ar immediately prior to use as described above and gases were added to obtain a defined composition and pressure (described below). The inoculum was centrifuged to separate the excess media, washed 2-3 times with NF-H<sub>2</sub> media and then resuspended in degassed media from the vial prior

to inoculation. All growths were conducted in duplicates at 20-25 °C with a 60 W incandescent or halogen bulb.

### 3.3.3 Setting up defined gas composition in headspace

The volume of headspace was determined by subtracting the volume of media in the vial from the total capacity. Since gas additions were calculated for a final pressure of 2 atm the calculated total volume of gases to be added was doubled (which in half the space provides twice the pressure based on ideal gas equation). The volumes of each individual gas were calculated as fractions of this total volume (see equation 3.1 for calculation).

$$\text{Gas volume} = 2 \times \text{Headspace volume} \times \text{Fraction of total composition} \quad (3.1)$$

Then the gas that contributed to 50% or more of the composition was identified. If this was Ar, the vials were degassed as described above. If it was a different gas, the headspace was exchanged with that gas by bubbling it through for at least 10 mins. The excess pressure in the headspace was then released, and calculated additional volumes of the gases were added individually using gas-tight syringes.

### 3.3.4 Laboratory adaptation

For the laboratory adaptation experiments, NF-TS media was used as a closely related strain (CGA009) showed faster growth under N<sub>2</sub>-fixing conditions with thio-sulfate than H<sub>2</sub>. The parent strain was cultured with decreasing concentrations of N<sub>2</sub> (in order - 80%, 50%, 20%, 10%, 2%). Once the bacteria reached a high visual density at a given N<sub>2</sub> concentration, a volume of this culture was used to inoculate at the subsequent lower concentration. The rest of the headspace was composed of 20% CO<sub>2</sub> and the balance (when applicable) was filled by Ar. The cultures were

not stirred or shaken and were left undisturbed during the growth period. Once the bacteria grew under 2%  $N_2$ , this condition was maintained for 2 more subcultures to establish the adaptation. A control experiment was set up with either the parent TIE-1 or the adapted strain under  $< 0.5\%$   $N_2$ , where growth was only observed with the adapted strain over a period of 2 months (data not shown). Freezer stocks were made of the strain with 20% glycerol which was revived with the PH media and then reinoculated into 2% or 0%  $N_2$  conditions. Only 1 of the revived cultures grew again under the low  $N_2$  conditions. The subpopulation that retained its adaptation after revival was used for the data presented.

### 3.3.5 Gas chromatography analysis of $N_2$ and $H_2$

The analysis of headspace gas compositions was made using a Shimadzu GC-8A gas chromatograph (Shimadzu Scientific Instruments, Inc.) with a thermal conductivity detector. The carrier gas used was argon (135 kPa), the injector/detector temperature was 100 °C and the column temperature was 60 °C. 200  $\mu$ L of the headspace gas was sampled using a gas tight syringe, and injected into the gas chromatograph.

To quantify the amount of  $N_2$ , a set of standards were prepared with the required amount of water, and known  $N_2$  concentrations in the headspace. The corresponding peak was integrated for their area using the LabSolutions software (Shimadzu Scientific Instruments, Inc.), and a standard curve was derived. This was used to quantify the amount of  $N_2$  in the culture headspace at different time points.

The same traces were also used to analyze and quantify hydrogen production with an appropriate standard.

### 3.3.6 *In vivo* characterization of nitrogenase

#### *In vivo* nitrogenase activity

14.4 mL vials were set up with 2% acetylene and 98% Argon as described for culture headspace previously. The excess pressure was released, for a final pressure of 1 atm. 1 mL of wild type or adapted strain culture grown with 15 mM acetate and 2% N<sub>2</sub> was injected into the vial to start the reaction. The vials were incubated with a 60 W halogen bulb at room temperature for 20 mins. At the endpoint, 40  $\mu$ L of the headspace was injected into a Shimadzu GC-8A gas chromatograph with a flame ionization detector (Shimadzu Scientific Instruments, Inc.). It was run with N<sub>2</sub> as the carrier gas (65 kPa), 110 °C column temperature, 180 °C injector/detector temperature, H<sub>2</sub>/Air detector gas. The quantification was performed with an appropriate standard curve. All assays were performed in duplicate.

#### Ammonium inhibition of *in vivo* nitrogenase activity

To test the ammonia inhibition of acetylene reduction activity, the vials were set up similar to the acetylene reduction assay and the headspace was sampled every 10 minutes. At the time indicated, 10  $\mu$ L of 10% ammonium chloride was added to the vials, and the ethylene production was monitored every 10 mins, for a total of 50-70 minutes. The assays were performed in duplicates.

#### Protein quantification of cell pellet

The acetylene reduction activities were standardized by the total amount of protein present in the culture. To measure this, 5 mL of the same culture was centrifuged at 3200 rpm for 15 mins. The supernatant was discarded and the pellet was resuspended in 600  $\mu$ L of cell lysis buffer from the Yeast/Bact DNA purification kit

(Qiagen Sciences) and incubated at 80 °C for 5 mins. Then, the DC assay (Bio-Rad Laboratories, Inc.) was used to quantify the amount of protein in the lysate using a BSA standard. The quantity of protein in 1 mL of culture was calculated from the identified concentrations using Equation 3.2.

$$\text{Amount of protein in assay} = \text{Concentration measured} \times \frac{0.6}{5} \quad (3.2)$$

### 3.4 Results

#### 3.4.1 Growth characteristics of the adapted strain

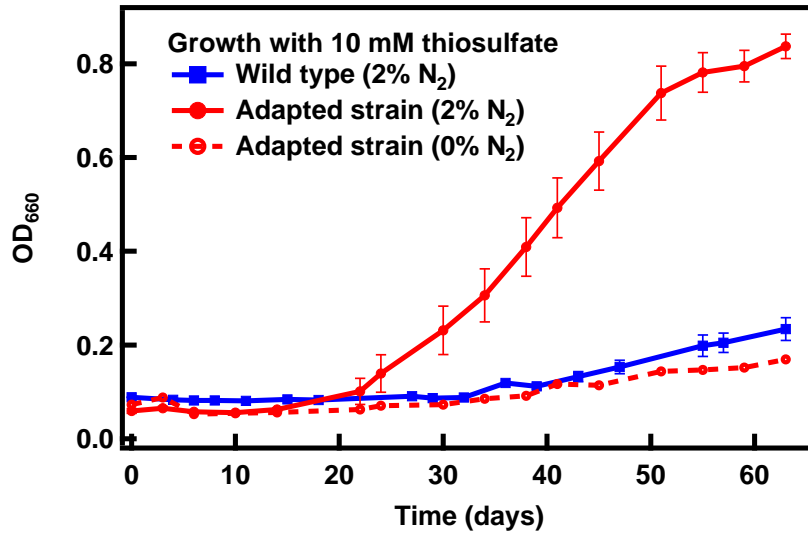


Fig. 3.1: Growth of WT and adapted *R. palustris* TIE-1 with thiosulfate as the electron source and 2% or 0% N<sub>2</sub> as the nitrogen source

After the laboratory adaptation was achieved, it was confirmed that the strain was adapted for low N<sub>2</sub>, with thiosulfate as the electron source (Figure 3.1). The adapted strain reached an OD of 0.83 in 63 days, while the wild type strain only reached a maximal OD of ~0.17. There was no growth seen when the adapted strain

was cultured with  $\sim 0\%$   $N_2$  in the headspace (Figure 3.1). However, unlike its closely related strain (CGA009) (Huang et al., 2010; Rolls and Lindstrom, 1967), wild type *R. palustris* TIE-1 did not grow well with thiosulfate under any of the conditions tested (Figure 3.2). So, further studies were conducted to elucidate if the adaptation was for growth with thiosulfate, or low  $N_2$ , or both.

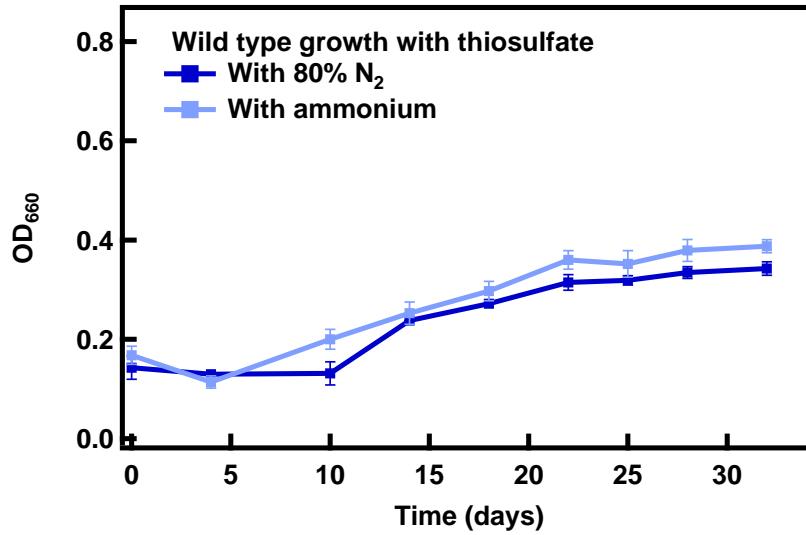


Fig. 3.2: Growth of the wild type TIE-1 strain with thiosulfate as the electron source and either 80%  $N_2$  or  $(NH_4)_2SO_4$  as the nitrogen source indicating that thiosulfate inhibits the growth irrespective of nitrogen availability.

It has been established previously that TIE-1 grows well under  $N_2$ -fixing conditions with  $H_2$  and could be used instead of thiosulfate to confirm the adaptation for low  $N_2$ . So, both the wild type and the adapted strains were grown with 50%  $H_2$  and either 50% or 2%  $N_2$ . A significant growth advantage was observed under 2%  $N_2$  compared to wild type (Figure 3.3). While the wild type strain did not grow at all over a period of  $\sim 50$  days, the adapted strain reached ODs of  $\sim 0.8$ . However, there was no obvious growth advantage when grown with 50%  $N_2$  (Figure 3.4).

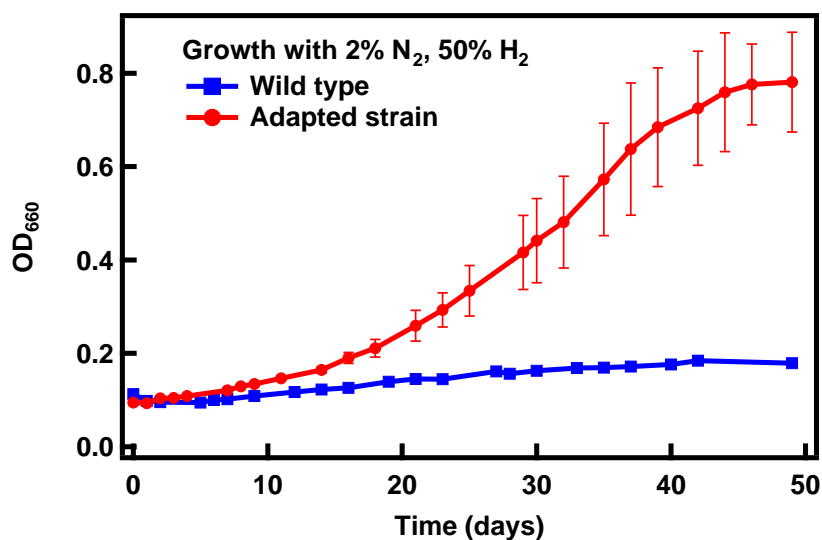


Fig. 3.3: Growth of the adapted strain with 2% N<sub>2</sub> and H<sub>2</sub> as the electron source confirming that the adaptation also supported growth under low N<sub>2</sub>

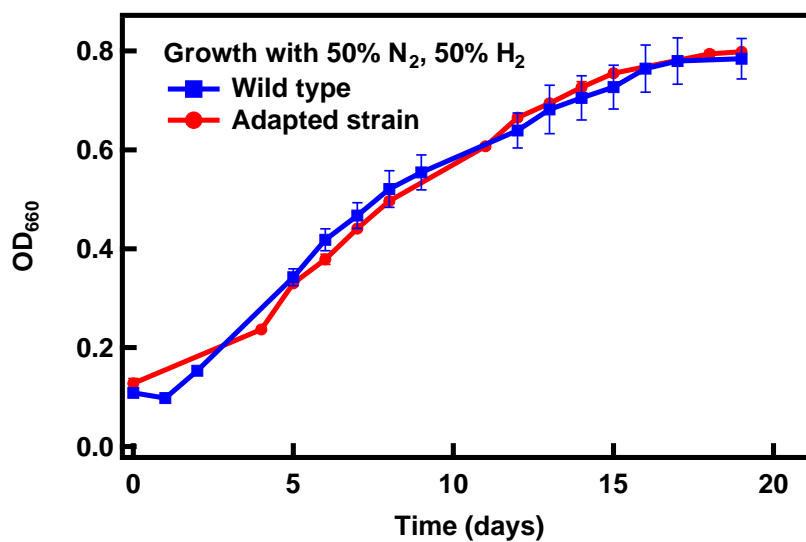


Fig. 3.4: Growth of the adapted strain with 50% N<sub>2</sub> compared to wild type TIE-1.

*R. palustris* has also been known to use organic carbon compounds like acetate, succinate, pyruvate, etc as a source of carbon and reducing equivalents. So the strains were also grown with 2% N<sub>2</sub> and 15 mM acetate to test if the adaptation extended to other sources of reducing equivalents. The growth rates for both wild type and

adapted strains were significantly increased compared to standard  $N_2$  concentrations with acetate. However, no growth advantage observed when acetate was provided as the carbon source even under 2%  $N_2$  (Figure 3.5).

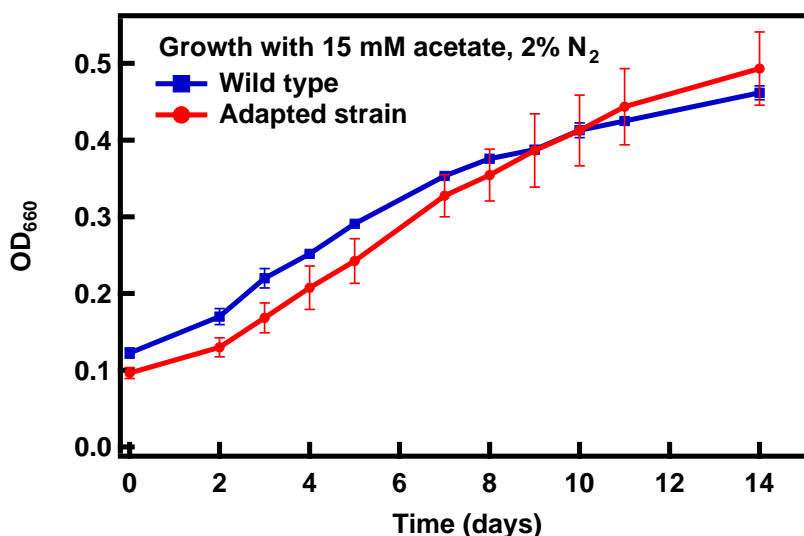


Fig. 3.5: Growth of the adapted strain with 15 mM acetate as the electron and carbon source and 2%  $N_2$  as the sole nitrogen source indicated no growth advantage compared to wild type.

### 3.4.2 *In vivo* nitrogenase activity

We hypothesized that the adaptation may have improved nitrogen capture by increasing baseline *in vivo* nitrogenase activities (either by overexpression or increased electron flux to the enzyme) which can be tested by the acetylene reduction assay. For these studies, cells grown with acetate were used since that was the only growth condition that provided sufficient cell mass for both wild type and adapted strains.

The *in vivo* nitrogenase activities were found to be 4.45 and 11.05 nmol ethylene (mg protein)<sup>-1</sup> min<sup>-1</sup> in the wild type and adapted strains respectively (Figure 3.6).

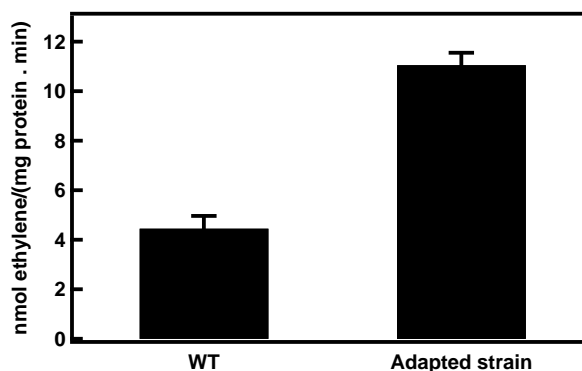


Fig. 3.6: *In vivo* nitrogenase activity characterization of the adapted strain. The cultures used for this analysis were grown with 15 mM acetate and 2%  $N_2$  for 14 days prior to the assay.

### 3.4.3 Nitrogen starvation response

Nitrogen starvation in this study is described as the condition with  $< 5\%$   $N_2$  and no fixed nitrogen source sufficient to support growth and biomass production. Hydrogen production is a well-studied nitrogen starvation response in purple non-sulfur bacteria. Significant hydrogen production was observed in both wild type and adapted strain cultures grown with acetate and 2%  $N_2$  (Figure 3.7A).

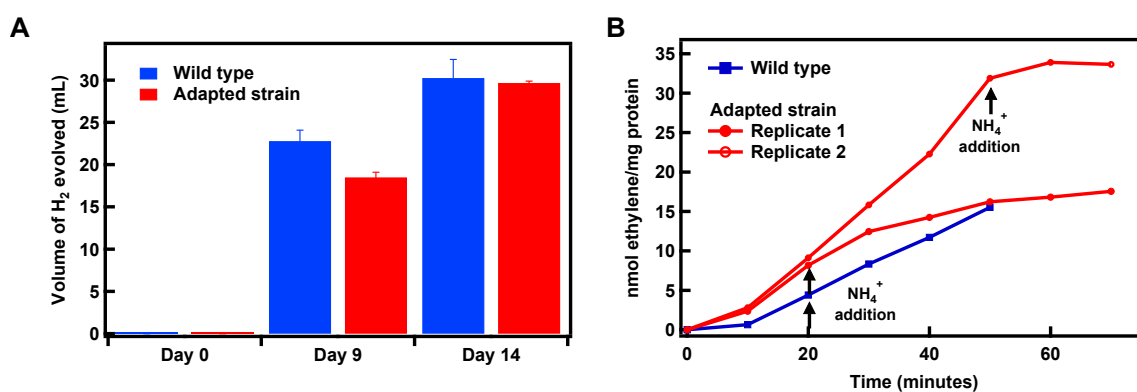


Fig. 3.7: Hydrogen production measured during the growth period with 15 mM acetate and 2%  $N_2$  (A) and loss of ammonium inhibition of nitrogenase activity observed in the wild type but not in the adapted strain (B).

Some studies have found that under short term nitrogen starvation conditions ( $\sim 24$  hours), there is a temporary loss of the ability of  $\text{NH}_4^+$  to inhibit *in vivo* nitrogenase activity (Alef et al., 1981; Cejudo and Paneque, 1988). It is unclear from these studies why this occurs, but the loss is temporary. The authors observed that after transferring the bacteria to an  $\text{N}_2$ -rich condition the bacteria regained the ability to respond to  $\text{NH}_4^+$ . After growing the wild type and adapted strain for 14 days with 2%  $\text{N}_2$ , this loss of inhibition was observed in the wild type strain, but not in the adapted strain (Figure 3.7B).

### 3.5 Discussion

Biohybrid technologies for  $\text{N}_2$  reduction are the most logical choice for extraterrestrial applications. But they require bacteria that have evolved under the high  $\text{N}_2$  concentrations on Earth to function in extremely nitrogen limited conditions. Although we did not model the Martian atmosphere exactly, by successfully adapting the bacteria to grow with 2%  $\text{N}_2$ , this study lays the foundation to further improve such systems for application in similar nutrient-limited conditions.

The laboratory adaptation was performed with thiosulfate since a closely related strain (CGA009) has been shown to grow very well with thiosulfate under  $\text{N}_2$ -fixing conditions (Huang et al., 2010; Rolls and Lindstrom, 1967). However, thiosulfate was found to inhibit growth of wild type TIE-1 under all tested conditions. This was surprising given the high level of similarity between the genomes of the two strains (almost 98% identity) (Simmons et al., 2011). Further experiments confirmed that the strain had been adapted for growth with 2%  $\text{N}_2$  with  $\text{H}_2$  as the electron source, but this observation did not extend to acetate. Although this was surprising, given the lack of background literature supporting these observations, it is unclear whether it is unexpected. No growth advantage was identified with 50%  $\text{N}_2$ .

One possible mechanism could be that under low  $N_2$  the adapted strain is able to increase electron flux to nitrogenase (either by overexpression of nitrogenase itself, or electron donors to the enzyme) and so improve nitrogen capture under those conditions. This would result in higher baseline *in vivo* nitrogenase activity compared to wild type which may be tested by the acetylene reduction assay. Even though the nitrogenase activity of the adapted strain was almost three times that of the wild type, this did not result in improved growth phenotype under the same conditions. This could be due to limitations in ammonium assimilation pathways that limit biomass production under those conditions.

The DraT/DraG system is a post-translational mechanism to regulate nitrogenase activity *in vivo* through the ADP-ribosylation or deribosylation of the Fe protein ([Nordlund and Ludden, 2005](#)). When grown with sufficient  $N_2$ , the addition of ammonia triggers a cascade that activates Dinitrogenase Reductase ADP-ribosyl Transferase (DraT). This results in the transfer of an ADP ribosyl group to the Fe protein and prevents electron delivery to the *M*Fe protein. Once all the ammonia is consumed, the Dinitrogenase Reductase ADP-ribosyl Glycohydrolase (DraG) protein hydrolyzes the group and restores activity. However, studies have shown that when cells undergo short term nitrogen starvation ( $\sim 24$  hours), there is a temporary loss of this ability of ammonia to inhibit *in vivo* nitrogenase activity ([Alef et al., 1981](#); [Cejudo and Paneque, 1988](#)). In our experiments, it was found that ammonia was still able to inhibit acetylene reduction by the adapted strain but not the wild type under similar conditions. This indicates that the adapted strain likely senses and/or responds to nitrogen starvation differently compared to wild type.

Another hallmark of nitrogen starvation stress is hydrogen production by purple non-sulfur bacteria. In the absence of  $N_2$ , the bacteria overexpress nitrogenase which reduces protons instead to produce large amounts of hydrogen ([Arp and Zumft, 1983](#)).

Interestingly, significant quantities of hydrogen were produced by both the wild type and adapted strains when grown with acetate or thiosulfate. This suggests that despite sensing/responding to nitrogen starvation differently, N<sub>2</sub> may still be limiting the reaction rate at the enzymatic level. Due to the constraints of the growth conditions, hydrogen production in growth with H<sub>2</sub> as the electron source could not be studied.

Whole genome sequencing of the adapted strain is currently underway to identify SNPs that could contribute to the adaptation. Based on the experimental observations, it is likely there are mutations in regions of the genome involved in nitrogenase regulation (particularly *draT*, *draG*, the *ntrBC* operon), uptake hydrogenase, genes involved in thiosulfate oxidation and possibly even in the genes encoding the nitrogenase isozymes (*nifHDK*, *vnfHDK*, *anfHDK*). It will be particularly interesting to see if there are mutations within the nitrogenase proteins that result in amino acid substitutions that could affect the kinetic parameters of the protein. Other mutations could include genes involved in carbon fixation pathways. RuBisCO has been found to be essential for maintenance of the redox status of the cell under both autotrophic and heterotrophic conditions ([McKinlay and Harwood, 2010](#)). Since nitrogen fixation requires a large amount of electrons, it is possible that there are mutations in carbon fixation genes that could allow for improved electron flow to nitrogen fixation under starvation conditions. However, since this is the first study with growth reported under such low N<sub>2</sub> concentrations, it is difficult to predict exactly what may be found.

Such genomic and transcriptomic studies are essential to understand how the bacteria evolved under prolonged nitrogen starvation. They can further inform metabolic engineering targets to increase rates and efficiencies of nitrogen fixation under more diverse growth conditions. One of the biggest challenges in developing biological technologies for nitrogen fixation is that the yield and rates of production are slower than

the current industrial method for ammonia production. By understanding how the bacteria have improved nitrogen capture under low  $N_2$  partial pressures, we might be able to understand how to increase rates of nitrogen fixation even under high  $N_2$  as on Earth, especially if mutations are identified within the nitrogenase protein.

### 3.6 Acknowledgements

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## CHAPTER 4

### Summary and Future Directions

#### 4.1 Introduction

Nitrogen fixation is the process of conversion of dinitrogen ( $N_2$ ) into a biologically available form like ammonia. It is a critical part of the nitrogen cycle that supports all life as we know it. In order to sustain manned missions to deep space, *in situ* nitrogen fixation is essential for the functioning of all other biological processes like food or pharmaceutical production. The research presented in this thesis provides the groundwork for developing biological nitrogen fixation technologies for nitrogen capture under highly minimalistic conditions.

#### 4.2 Microbial electrosynthesis for sustainable $N_2$ fixation

In chapter 2, a sustainable approach for nitrogen capture was presented using a phototrophic bacterium, *R. palustris* TIE-1. The bacteria were grown in an electrochemical cell fixing  $N_2$  and  $CO_2$  simultaneously, using *in situ* produced hydrogen as the sole electron source and infra-red light as the sole energy source. Simply by our choice of organism, we were able to demonstrate a faradaic efficiency twice that of a similar system with *Xanthobacter* (Liu et al., 2017). This work also established that low energy infra-red photons could be used to grow these bacteria without a significant effect on growth rates (and hence nitrogen yield) compared to standard light conditions. To our knowledge, this is the first study that established the effect of different wavelengths on *R. palustris* growth after standardizing for photon flux. While this is the first step in developing sustainable  $N_2$ -reduction technologies,

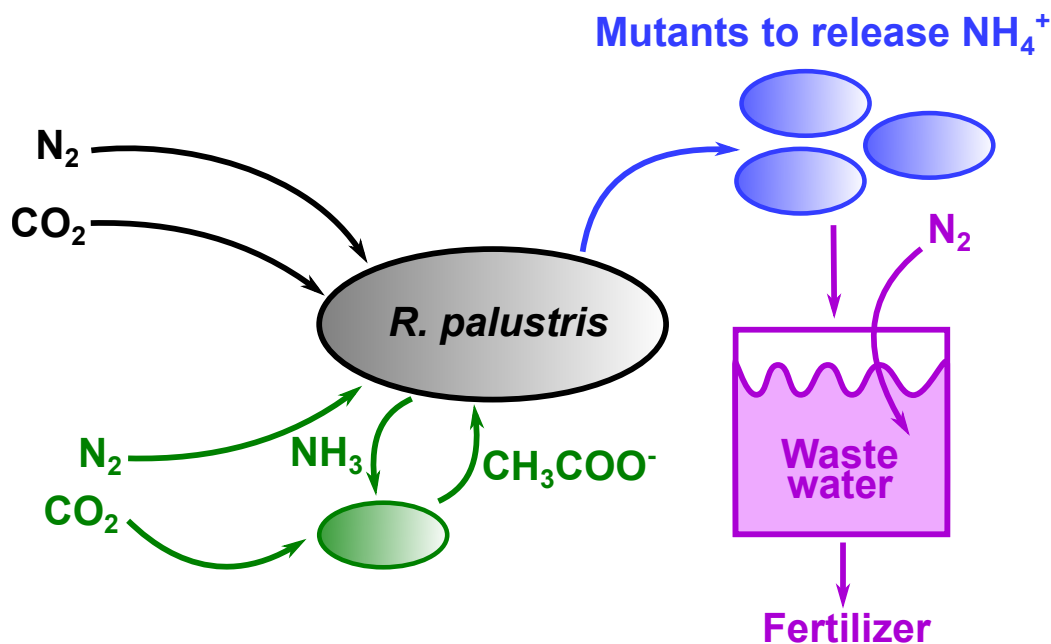


Fig. 4.1: Possible future directions to improve  $N_2$  yields. One direction (outlined in blue) would be to generate mutants of the bacteria designed to release  $NH_4^+$  which would reduce the amount of fixed nitrogen sequestered in the biomass. Furthermore, it would be available more readily for downstream use. Other directions (outlined in green and magenta) could include finding sustainable organic sources of electrons to support nitrogen fixation like acetate or organics present in waste streams like sewage or plant waste.

more research is needed to optimize the system and improve nitrogen yields. Specific research projects in this direction are outlined below:

#### 4.2.1 Generating ammonium excretion mutants

The strain used in this work was a wild type strain that captured all of the fixed nitrogen as proteins and other biomolecules in its biomass. When considering application as a fertilizer, inorganic nitrogen sources like ammonium or nitrates are preferable as they are more easily taken up by plants. Mutants of free-living diazotrophs that can produce ammonium are not new. Most common mutations involve the genes *nifA* (Adessi et al., 2012), *nifL* (Barney et al., 2017) and *amtB* (McCully et al., 2018). NifA and NifL are known transcriptional regulators of the nitrogenase

system ([Martinez-Argudo et al., 2004](#); [Masepohl, 2017](#)). NifA is a transcriptional activator of the *nif* operon while NifL binds to NifA and represses its activity. This binding is regulated by the presence of ammonium and energy status of the cell. Thus they have opposite functions, where NifA activates transcription of nitrogenase, and NifL represses transcription in response to environmental signals. Other mutants generated have involved knockouts of the ammonium transporters like AmtB ([McCully et al., 2018](#)). Ammonium transporters have been found to be useful in recapturing any ammonia that escapes the cell as well as in ammonium sensing and nitrogenase regulation under specific conditions ([Moure et al., 2019](#); [Yakunin and Hallenbeck, 2002](#); [Zhang et al., 2012](#)).

Mutants of *nifL* have been generated in *A. vinelandii* to promote extracellular ammonium production by knocking out the *nifL* gene ([Barney et al., 2017](#)). A NifA\* mutant of *R. palustris* CGA009 contains a modified NifA protein that cannot bind NifL ([Adessi et al., 2012](#)). In both these mutants the nitrogenase is constitutively expressed at high levels, which drastically increases intracellular ammonia concentrations. The excess ammonia that isn't incorporated into the biomass leaks out of the cell, producing micromolar quantities of ammonium in the media. One study found that double mutants of NifA\* and  $\Delta$ amtB produced significantly more extracellular ammonium compared to the single NifA\* mutation ([McCully et al., 2018](#)). The reproduction of similar mutants in the TIE-1 strain will be essential for improving the performance of the  $N_2$ -fixing electrochemical system since the strain used in these other studies does not have a functional uptake hydrogenase.

In addition to generating mutants of known targets, it might be useful to perform flux balance analysis and *in silico* knockout studies to identify other metabolic targets that can be used to improve ammonium production. Nitrogenase regulation is highly complicated, with several genes involved in transcriptional and post-

translational regulation of expression and activity. Since nitrogen fixation is a central metabolic pathway, dramatic mutations that produce increases in ammonium excretion may significantly impair the survivability of the mutant. So, identifying multiple small targets, that can have a large overall effect will allow us to achieve the same effect without affecting the viability of the strain.

#### 4.2.2 Alternate systems for generation of electron sources

The research described here demonstrates the ability to fix  $N_2$  under highly nutrient limited conditions, where the bacteria would have to fix their own  $N_2$  and  $CO_2$ . In the cell, these two energy and electron demanding pathways compete with each other, and a balance is achieved to support growth. When  $CO_2$ -fixation is reduced or inhibited, there is an increase in electron flow towards nitrogenase in order to maintain a redox balance. This was observed when RuBisCO was knocked out in *R. rubrum* leading to increased biohydrogen production (Wang et al., 2010). However, similar knockouts in *R. palustris* result in non-viable cells since it is a central component in redox cycling and maintenance of redox status.

It is also possible to reduce the flux of electrons through RuBisCO by growing the bacteria under heterotrophic conditions with an organic carbon source like acetate or succinate. These can be produced by hybrid bio-inorganic systems (Liu et al., 2015), or abiotic inorganic systems (Ripatti et al., 2019) that produce large quantities of acetate. Preliminary results indicate that the yields of fixed nitrogen are significantly improved under such conditions (Unpublished data). Further studies need to be done to characterize the system in terms of volumetric productivities, as well as efficiency of energy and electron usage.

Purple non-sulfur bacteria also have a high metabolic versatility and are capable of metabolising a number of different organic carbon compounds like those found in

waste streams like compost and sewage ([Larimer et al., 2003](#); [McKinlay and Harwood, 2010](#)). They have been studied extensively for waste-water remediation in conjunction with biohydrogen production (using nitrogenase). It would be interesting to study how waste streams (with a high organic carbon and nitrogen content) may be used to support nitrogen capture, particularly by using the above mentioned mutants that reduce sensitivity to ammonium. This approach would combine waste-water remediation and fertilizer production and reduce the footprint of the system needed on Mars.

### 4.3 Laboratory evolution for improved growth under low $N_2$

Chapter 3 describes the successful laboratory adaptation of a strain for growth under partial pressures of  $N_2$ . To our knowledge, this is the first study that has demonstrated growth of bacteria under such low  $N_2$  concentrations. The growth characteristics under different metabolic conditions were also characterized and it was identified that the adaptation is specific for  $N_2$ - and  $CO_2$ -fixing growth with 2%  $N_2$ , and  $H_2$  or thiosulfate as an electron source. Further research in this project is needed to understand the mechanism of adaptation and the metabolic modifications that have occurred to support growth under these conditions.

Although the growth characteristics and *in vivo*  $N_2$  fixation activities have been studied, there is little to no understanding of what genomic changes may have resulted in the modified growth characteristics. Given the lack of background literature that could inform specific hypotheses about the mechanisms of adaptation, most of the directions forward require an -omics approach.

Efforts are ongoing to sequence the genome of the adapted strain and identify SNPs that could play a role. But this is only the first step in understanding the mechanism of adaptation. Transcriptomic studies are needed to further elucidate how the

identified SNPs affect expression and regulation patterns. Identifying genes that are up- or downregulated can also provide a blueprint to recreate similar effects for more diverse growth conditions.

Expression of alternative nitrogenases in *R. palustris* seems to be regulated by nitrogen starvation rather than metal availability (Oda et al., 2005). Alternative nitrogenases (V- and Fe-nitrogenase) have different affinities and activities for reduction of alternative substrates like CO<sub>2</sub> and CO to useful C-1 and C-2 products. So understanding mechanisms of induction of these isozymes will be useful in producing valuable carbon compounds in the biohybrid system. This strain is ideal to understand how nitrogen starvation conditions are sensed in these bacteria as it was found to respond to nitrogen starvation differently compared to wild type. Proteomic and transcriptomic data generated for both strains under conditions of high and low nitrogen can reveal which proteins are essential for this process. It would also help us identify genes involved in nitrogen starvation responses and which could further inform metabolic engineering strategies for improved growth under limited nitrogen conditions.

#### 4.4 Acknowledgements

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## APPENDICES

## APPENDIX A

### Future Directions: Microbial Electrochemistry

#### A.1 Introduction

Microbial electrochemistry is the study and application of interactions of microbes with an electrode either directly or through mediators. This is a fast growing field that can be applied for wastewater remediation, or electrosynthesis based on the direction of electron transfer (from microbe to electrode or vice versa) ([Rabaey and Rozendal, 2010](#); [Schröder et al., 2015](#)). With most microorganisms, the first trials couple it to an electrode use  $H_2$  as the mediator (as demonstrated in chapter 2 of this thesis) ([Liu et al., 2018](#)). This is because a lot of microbes have hydrogenase enzymes which can oxidize the dihydrogen to produce reduced NADH or NADPH needed for reductive biosynthesis. There are also many efficient inorganic catalysts for production of hydrogen by electrolysis of water, which allows for constant regeneration of the mediator *in situ*. However, there are issues associated with using  $H_2$  as the mediator.

1. The mechanism of  $H_2$  production is by reduction of protons at the cathode coupled with the generation of oxygen at the anode. This is often accompanied by formation of hydroxyl ions, which dramatically shift the pH during operation and requires constant monitoring.
2. The potential required for water electrolysis to be supported is quite high, and may not be energy-efficient for practical applications.

3. Since  $\text{H}_2$  is not very soluble in water ( $K_H=7.9 \times 10^{-6}$ ), a significant amount of it is lost to the atmosphere and results in non-productive  $\text{H}_2$  generation. Using a soluble mediator or direct electron transfer would prevent this loss and should result in higher faradaic efficiencies.

## A.2 Methods

All the electrochemical studies were performed using the MultiEmStat potentiostat, with Ag/AgCl reference electrodes and Pt counter electrodes.

Cyclic (CV) and square wave (SWV) voltammetric studies were used for preliminary studies of feasibility for added mediators as well as direct electrochemical studies. Glassy carbon or graphite rod electrodes were used, and the voltage was scanned from -1 V to +1 V at a scan rate of 20 mV/s. The respective media without the mediator or bacterial strain was used as the blank scan. Between CV studies of different samples, the electrode was washed thoroughly with water and polished to remove traces of adsorbed compounds that may interfere with the analysis.

For all of the chronoamperometry experiments either graphite rod electrodes or carbon foam working electrodes were used. The electrodes were poised at -0.8 V or at an appropriate voltage depending on the midpoint potential of the redox peaks observed and the resulting current response was recorded. During the course of the chronoamperometry experiment, cyclic voltammetric studies were performed.

## A.3 Results and Discussion

### Exploring mediators for microbial electrosynthesis

A number of external mediators have been used in microbial electrochemistry systems, including neutral red, methylene blue, methyl viologen, formates, humic

Table A.1: Mediators tested for redox activity

Mediator	Presence or absence of redox shoulders
FeCl <sub>3</sub>	No
Fe-NTA	No
Hydroquinone	Yes
Methylene blue	Yes
Neutral Red	Yes
Methyl Viologen	No

acids, etc (Liu et al., 2018). Several mediators reported in the literature were tested for feasibility in electrochemical systems with purple non-sulfur bacteria (listed in Table A.1). Of the mediators tested, neutral red, methylene blue and hydroquinone displayed observable redox behavior (Figure A.1 in the growth medium as the electrolyte and graphite rods as the working electrode (cathode). However, the redox potentials of methylene blue and hydroquinone were too high to support reductive biosynthesis (based on the standard potential of the NADP<sup>+</sup>/NADPH couple) and further experiments were conducted with neutral red.

Most mediators used in electrochemistry experiments are toxic to cells under high concentrations. So neutral red was tested qualitatively for their effects on photoheterotrophic growth of *R. palustris* TIE-1 cells. Growth was observed with 200  $\mu$ M neutral red (data not shown), suggesting that it did not significantly inhibit the growth under the tested conditions. The bacteria did not grow with neutral red as the sole carbon source, indicating that they couldn't metabolize and break down the mediator (data not shown).

Chronoamperometry experiments were performed with *R. rubrum*, *R. capsulatus* and *R. palustris* TIE-1 as well as the NifA\* mutant of *R. palustris* CGA009 using neutral red. There was no evidence of electron transfer from the electrode to the bacteria under CO<sub>2</sub>-fixing conditions with any of the strains (data not shown).

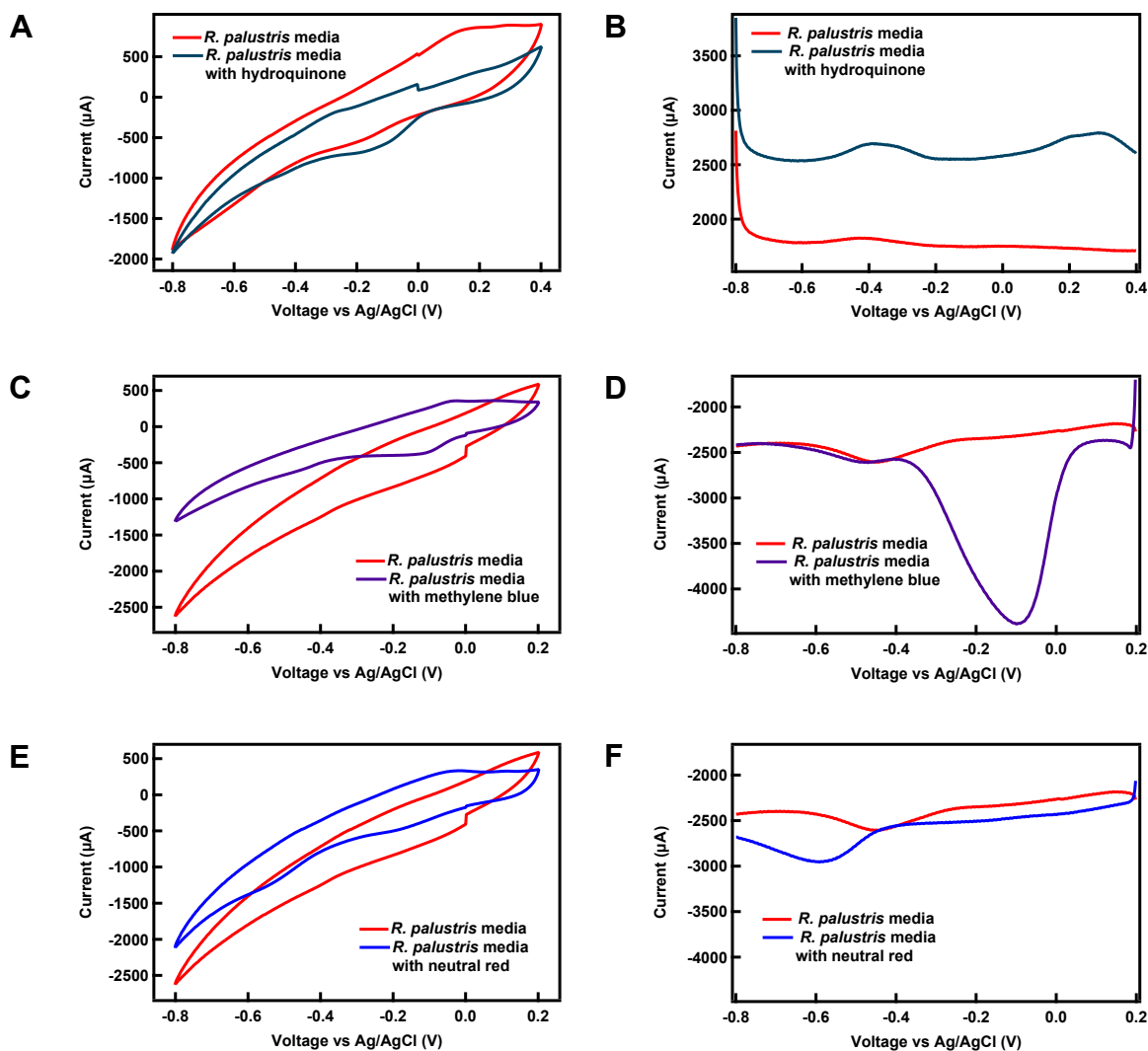


Fig. A.1: CV and SWV studies of hydroquinone (A, B), methylene blue (C, D) and neutral red (E, F) in the *R. palustris* growth media with graphite rod as the working electrode. The observed redox shoulders for hydroquinone and methylene blue were not in the right potential range for electrosynthesis, but the midpoint potential of neutral red was ideal for electrosynthesis studies.

## Exploration of direct electron transfer in purple non-sulfur bacteria

Purple non-sulfur bacteria are known for their metabolic versatility and have been studied in electrochemical systems. *R. capsulatus* was used as a single inoculum in a microbial fuel cell and was found to oxidize organic compounds coupled with electricity generation using mediators like osmium redox polymers (Hasan et al., 2015). There are even some reports of direct electron transfer to *R. palustris* in the literature. In one study, the strain TIE-1 was grown under CO<sub>2</sub>-fixing conditions (Bose et al., 2014), consuming electrons from the electrode and were also shown to produce small amounts of biopolymers (poly-hydroxybutyrates) under similar conditions (Ranaivoarisoa et al., 2019). Another strain of the same organism, DX-1, was reported to be electroactive in a microbial fuel cell, driving electricity production by oxidation of organic carbon in waste streams (Xing et al., 2008). So we wanted to test if strains of purple non-sulfur bacteria that would be capable of consuming electrons directly from an electrode to support N<sub>2</sub>- and CO<sub>2</sub>-fixation could be identified.

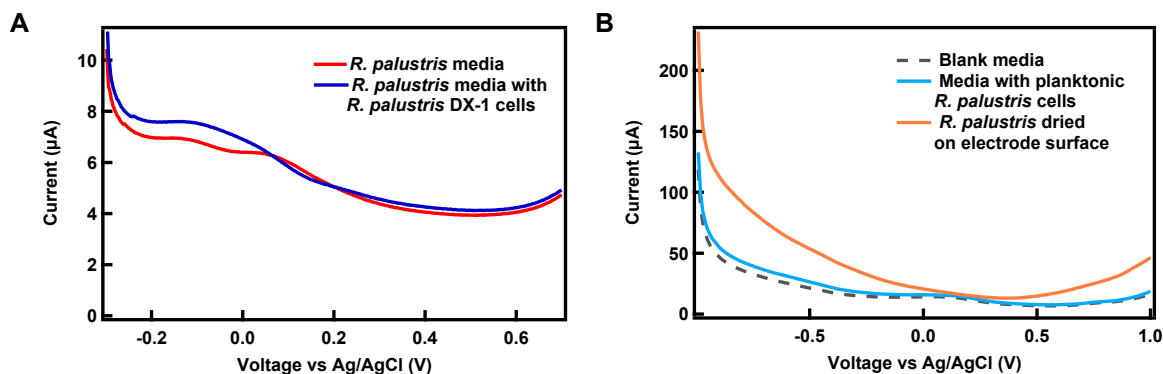


Fig. A.2: SWV studies of *R. palustris* DX-1 (A) and TIE-1 (B) with a glassy carbon electrode indicating the absence of redox shoulders representative of possible redox active proteins on the cell surface capable of electron transfer from the electrode to the cells.

In our experiments, *R. palustris* TIE-1, *R. palustris* DX-1, *R. capsulatus* UR-1

and *R. rubrum* B10 were tested for electroactivity and the possibility of direct electron transfer. Despite several trials of chronoamperometry, in our hands, no evidence of direct electron transfer with *R. palustris* TIE-1 was found (data not shown). This was further supported by the lack of distinct redox shoulders with either planktonic or cells adsorbed onto the graphite electrode (Figure A.2B). This was also true for the strain DX-1 which showed no discernable shoulders that would suggest interaction with the electrode (Figure A.2A).

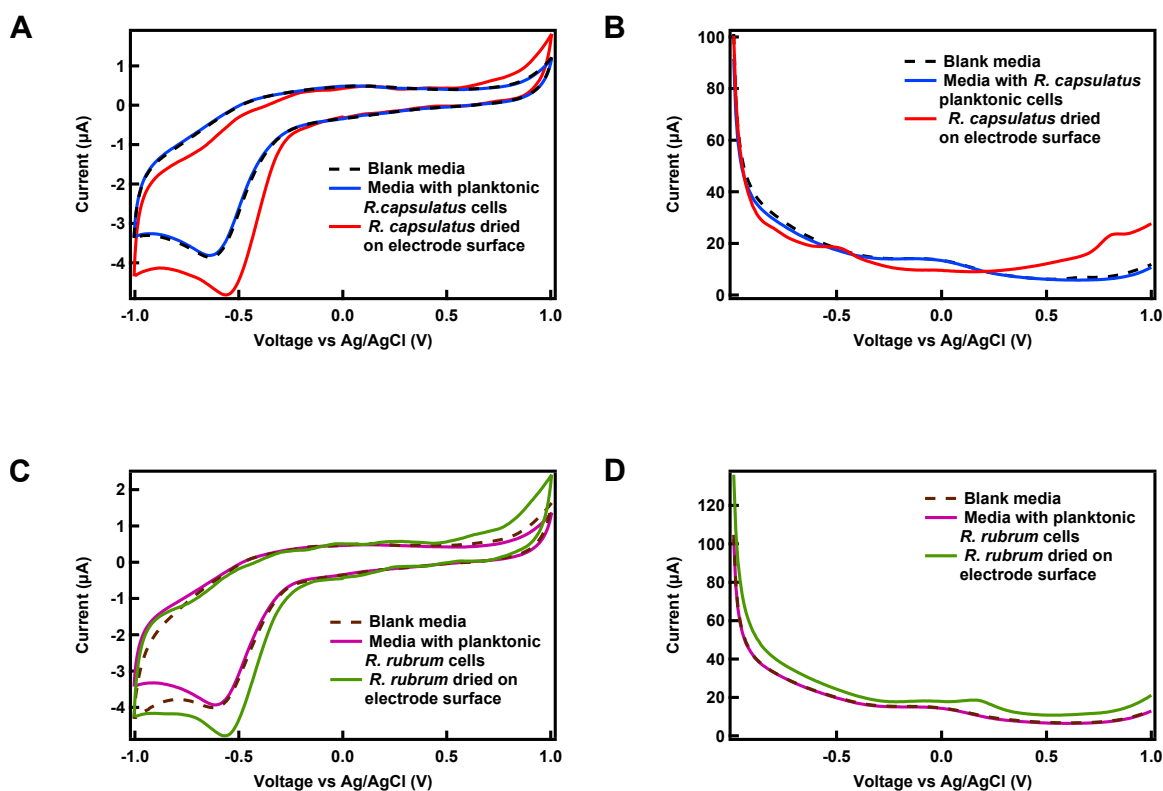


Fig. A.3: CV and SWV studies of *R. capsulatus* (A, B) and *R. rubrum* (C, D) indicate that redox shoulders that weren't observed with planktonic cells were apparent with adsorbed cells. This could indicate that the redox active proteins are in very low concentration on the cell surface.

In contrast, when *R. capsulatus* and *R. rubrum* were adsorbed onto the electrode surface, there were distinct electroactive shoulders in square wave voltammetry studies

that were not observed in the cyclic voltammetry signals (Figure A.3). However, chronoamperometry studies with both strains did not show any evidence of reductive biosynthesis in the absence of added mediators (data not shown). This is consistent with a previous report about *R. capsulatus* that found that direct electron transfer was not observed in a microbial fuel cell (Hasan et al., 2015).

### Identification of self-produced mediators for electrosynthesis

In most cases of direct electrochemistry, the bacterial cells are directly attached to the electrode, often forming a biofilm on the electrode surface. However, in the experiments with different purple non-sulfur bacteria, no evidence of biofilm formation was found on the electrode. We hypothesized that induction of biofilm formation could promote transfer of electrons to the bacteria without added mediators. p-coumarate is a common plant-derived compound that has been used to induce biofilm formation in *R. palustris* to adhere to walls of a vial for biohydrogen production (Harwood, 2009; Schaefer et al., 2008).

So, chronoamperometry experiments were conducted with 0.5 mM p-coumarate in the growth media as the catholyte and grew the bacteria under CO<sub>2</sub>-fixing conditions. A significant bacteria-dependent increase in current was observed (Figure A.4) compared to control experiments when bacteria or p-coumarate were left out of the system.

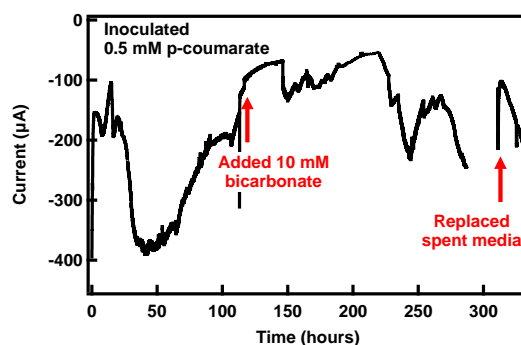


Fig. A.4: Chronoamperometry trace of *R. palustris* TIE-1 under CO<sub>2</sub>-fixing conditions with 0.5 mM p-coumarate.

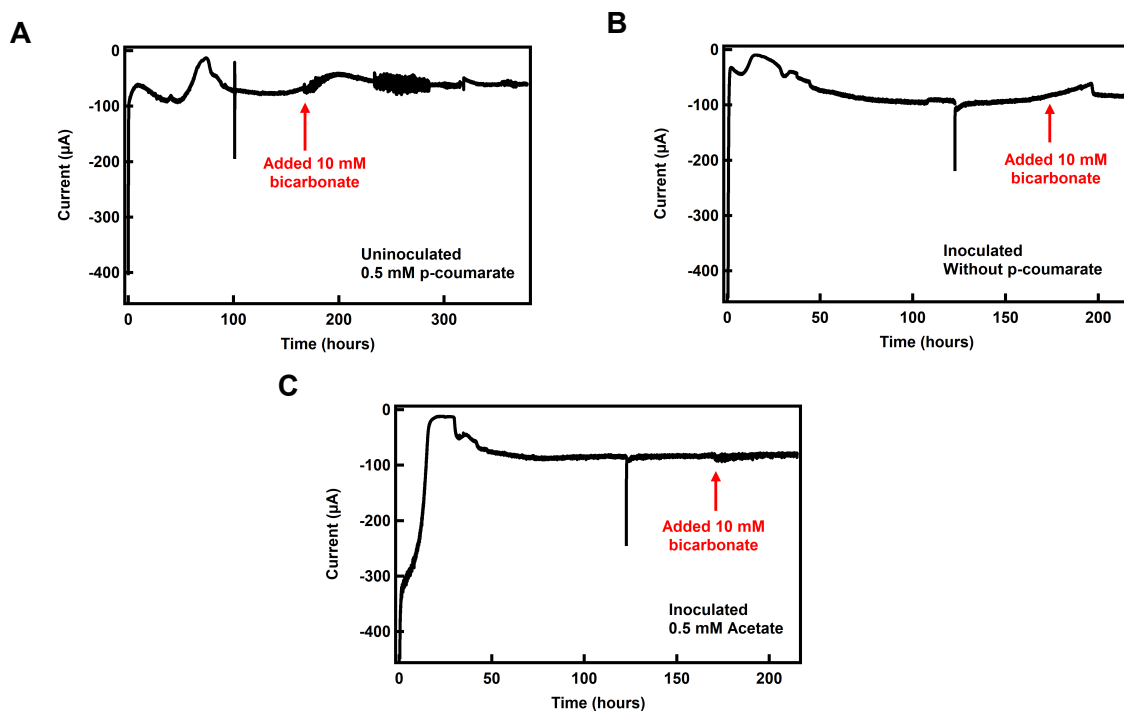


Fig. A.5: Chronoamperometry traces in the electrochemical cell without bacteria (A), p-coumarate (B), or with 0.5 mM acetate instead of p-coumarate (C).

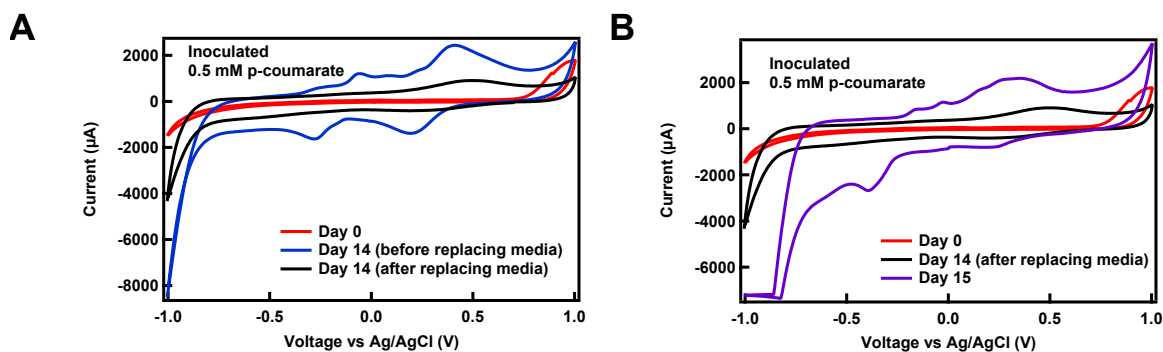


Fig. A.6: CV traces of the electrochemical cell set up with 0.5 mM p-coumarate and inoculated with *R. palustris* TIE-1 indicate that a diffusible mediator is produced in the presence of both the components.

Cyclic voltammetry performed during the experiment indicated that novel redox peaks had developed since day 0, suggesting a possible electrochemical connection

between the bacteria and the electrode (Figure A.6). The media was replaced on day 14, to probe whether the electron transfer was direct or mediated. If the electron transfer was direct, the distinct redox shoulders would still remain after the media was replaced (since the biofilm would not be washed away by replacing the media). But if it were mediated by a diffusible compound, the redox peaks would be lost. Cyclic voltammetry studies performed before and after replacement of the media indicated that the electron transfer components were diffusible (Figure A.6).

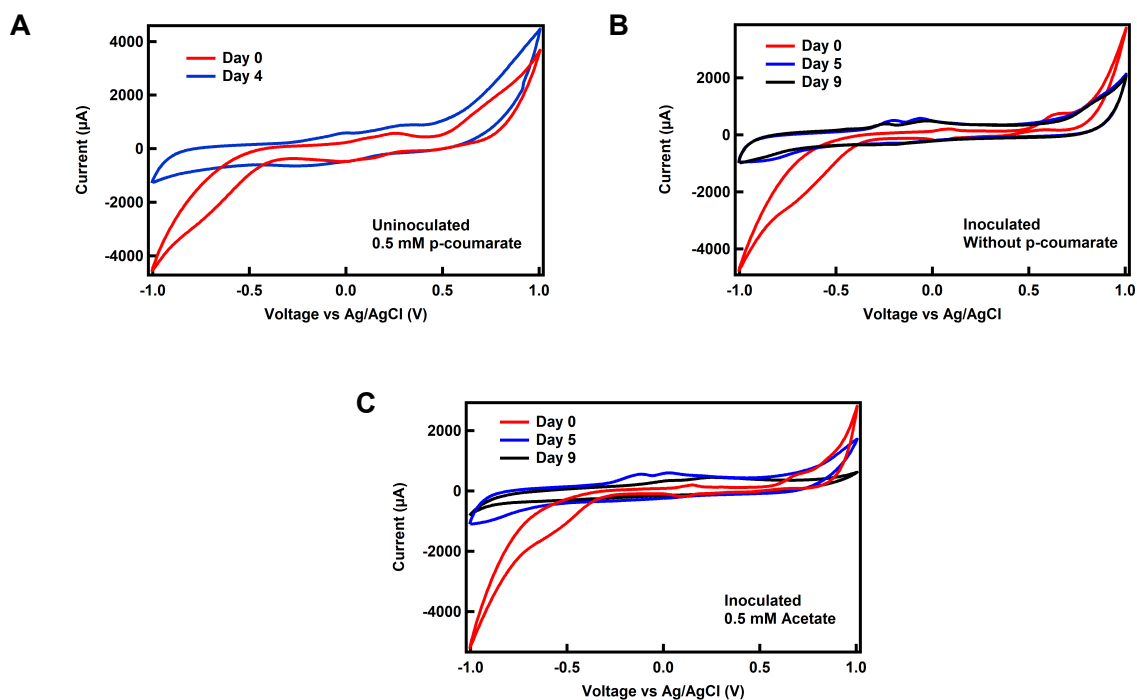


Fig. A.7: CV traces from electrochemical cells in Figure A.5 indicate that the redox peaks observed in Figure A.6 are dependant on the presence of bacteria as well as p-coumarate

Based on the cyclic voltammetry studies performed before inoculation, this redox activity could not be due to the addition of p-coumarate to the medium (Figure A.6A). This suggests that when p-coumarate was added in the medium, the bacteria produce a mediator to consume electrons from the electrode under  $\text{CO}_2$ -fixing conditions. This

was not observed when there was no bacteria, no p-coumarate or 0.5 mM acetate instead of 0.5 mM p-coumarate in the media (Figure A.7).

p-coumarate has also been found to be a precursor of a quorum sensing signal in *R. palustris*. It is be metabolized to form p-coumaroyl homoserine lactone that, among others, induces biofilm formation(Schaefer et al., 2008). It is possible that in the presence of an electrode potential, with p-coumarate, the bacteria were induced to produced a diffusible compound that could mediate electrons between the electrode and the bacteria. Intriguingly, further experiments showed that the application of a poised potential inhibited growth of the bacteria with 0.5 mM p-coumarate (data not shown).

#### A.4 Summary and future directions

The observation of a self-produced mediator is promising as it eliminates the need for mediators like neutral red or methylene blue which break down over time and are toxic in higher concentrations. Although the experiments performed here demonstrate initial feasibility of the system, there are still a lot of conditions to optimize. Some of the immediate next steps is to repeat the experiments under N<sub>2</sub>-fixing conditions to confirm that the mediator supports growth under N<sub>2</sub>- and CO<sub>2</sub>-fixing conditions, as well as to further characterize the effect of the electrode potential on bacterial growth under these conditions.

A key piece of the project is analyzing the mediator produced by the bacteria. Techniques like LC-MS will be useful in identifying compounds that are produced only in the electrochemical cell with p-coumarate. The identified compound can be added into the electrolyte to confirm it's use as a mediator.

Another interesting direction of study is understanding how p-coumarate promotes mediator production. *R. palustris* can consume p-coumarate as a carbon source

in addition to using it to produce a quorum sensing signal. Using the synthetic quorum sensing compound (p-coumaroyl homoserine lactone) instead of p-coumarate could help illustrate if the mediator is a metabolite of p-coumarate or if it induces the production of redox compounds. These studies can help optimize the electrosynthesis system and improve efficiencies and yield for biofertilizer production.

## A.5 Acknowledgements

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## APPENDIX B

### Future Directions: Characterizing the Use of Bacterial Biomass as Fertilizer

#### B.1 Introduction

The use of bacterial biomass as fertilizer has been studied for decades, primarily for their growth promoting effects on plants (Naik et al., 2019). In the case of bacteria that are commonly used in our experiments, like *A. vinelandii* and *R. palustris* and other purple non-sulfur bacteria, there are several studies characterizing the effects on plant growth as well as the potential mechanisms of interactions (Das, 2019; Sakarika et al., 2019). However, in most studies, the bacteria are added as an inoculum along with fertilizer, designed to grow and survive in the soil or substrate, and are not considered as the primary source of nitrogen. Due to the material and weight constraints on Mars, it is unlikely that all the nutrients for plants can be transported from Earth. So, a good understanding of how the bacterial biomass can perform as the sole fertilizer as well as the nitrogen flux through the system is needed for a complete design of the mission.

#### B.2 Methods

##### Media composition and growth conditions

*A. vinelandii* trans strain was grown using the established 100 L fermenter protocol and media recipes used commonly in the laboratory, with some slight modifications. While the scale up from plates to six 500 mL culture volumes was performed with urea, no urea was added to the media in the 100 L fermenter. So the bacteria

grew under nitrogen-fixing conditions from the time of inoculation into the 100 L fermenter to the time of harvest. All 6 flasks of inoculum were added to 100 L of the medium.

### **Plant growth trials**

Wheat plants (cv, Apogee) were grown in calcined clay with Utah hydroponic recipe in four treatments – with nitrates, with biomass (+N+B); with nitrates, without biomass (+N-B); without nitrates, with biomass (-N+B); without nitrates, without biomass (-N-B) – with 4 plants in each treatment group. For the no nitrates treatment, the nitrate salts were replaced with their corresponding sulfate salts.

First, the calcined clay was washed to remove smaller particles and split into two tubes. Each tub was saturated with 2 L of the respective nutrient solution (+/-N) for  $\sim 1$  day. The solutions were further split into 2 more pots and the nitrogen-fixing biomass produced was evenly distributed between the 2 +B treatments ( $\sim 126.05$  g wet biomass/treatment group). The nutrient solution with or without biomass was then added to 4 pots each for plant growth trials.

The plants were watered everyday and monitored during growth. At 26 days, the plants were harvested and their fresh and dry weights were measured for comparison.

### **B.3 Results and Discussion**

Pictures were taken on the day of harvest for visual comparison of the effects of the treatments (Figure [C.1](#)), and the fresh and dry weights were recorded (Table [B.1](#)).

Table B.1: Average plant weight (g) at harvest in the different treatment groups

Treatment	Average total weight (g)		Percent increase	
	Fresh weight	Dry weight	Fresh weight	Dry weight
<b>+N+B</b>	33.69	4.74	129.01	102.02
<b>+N-B</b>	14.71	2.35	N.A.	N.A.
<b>-N+B</b>	21.96	3.82	637	356.89
<b>-N-B</b>	2.98	0.84	N.A.	N.A.



Fig. B.1: Representative figures for Apogee wheat plants grown in four treatment groups (+N+B, +N-B, -N+B, -N-B). The presence of *A. vinelandii* significantly improved yield irrespective of presence of nitrates in the nutrient solution and decreased observed chlorosis.

In both conditions tested, the addition of bacterial biomass significantly improved the plant yield, irrespective of the presence of nitrate in the nutrient solution (Figure B.1). Unsurprisingly, this effect was more prominent in the -N treatment groups. Chlorosis was observed in the absence of biomass, indicating that addition of biomass alleviated the nitrogen deficiency in its absence (Figure B.1). The mild chlorosis observed in the +N-B treatment is likely because nitric acid, that is typically added to maintain the pH, was not added in our experiments.

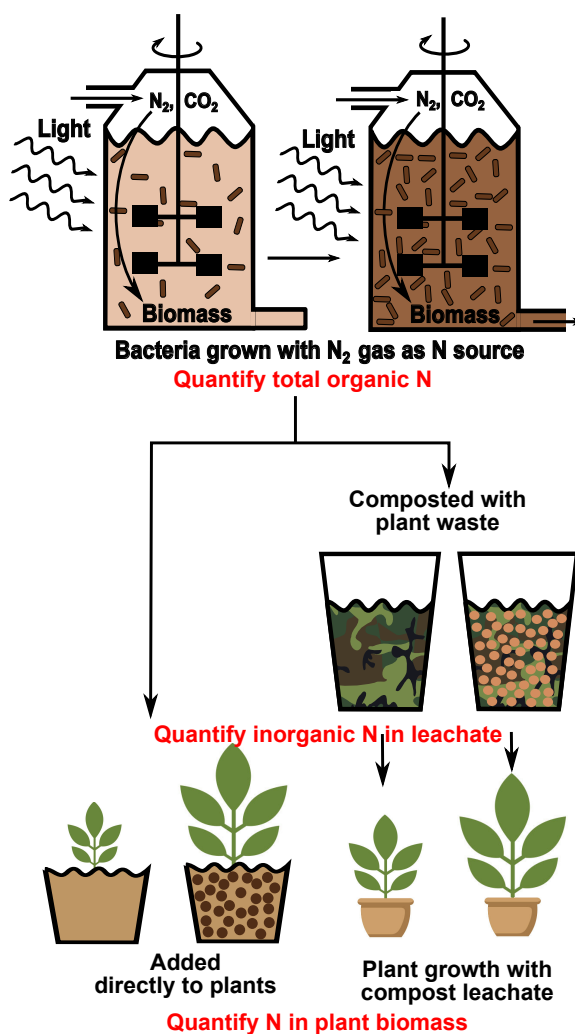


Fig. B.2: A proposed approach to quantify the nitrogen flux from bacteria to plants when the bacterial biomass is used as the sole nitrogen source for plant growth.

While this preliminary result is promising, more experiments are needed to establish the nitrogen flux through the system. One of the biggest concerns in this preliminary experiment is that growth in the 100 L fermenter is not axenic, and so there are likely to be other bacteria that either sped up the degradation of the biomass, or provided beneficial plant-microbe interactions that further enhanced the effects on growth. Experiments are currently underway to grow large amounts of the biomass under more sterile conditions for both *A. vinelandii* and *R. palustris*.

The organic nitrogen in the biomass is likely to be mineralized by microbial action in the soil, and so the nutrients are released slowly compared to chemical fertilizers. Slow release fertilizers have been shown to be beneficial for plant growth ([Geng et al., 2015](#)), but if the rate of release is slower than the rate of utilization, that will inhibit plant growth and yield. So experiments are necessary to identify the rate of breakdown of the organic N in the same system. These experiments could also be used to define the flux of N through the system and measure what percentage of the fixed N is utilized directly by plants for plant growth. A plan for the study is proposed in figure [B.2](#).

Other experiments further down the line could look into how processing the biomass prior to addition to plants affects the bioavailability and growth promoting effects observed. Extending these studies to purple non-sulfur bacteria, is also important to understand how the mission-relevant bacterial strains perform as biofertilizers.

#### **B.4 Acknowledgements**

I would like to thank Paul Kusuma for collecting the data related to plant growth as well as his inputs for experimental design.

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## APPENDIX C

### Supplementary Material for Chapter 2

#### C.1 Calculations of faradaic efficiencies

$$\text{Faradaic efficiency} = \frac{\text{Amount of N or C fixed} \times \text{Number of electrons needed}}{\text{Total number of electrons delivered at cathode}} \times 100 \quad (\text{C.1})$$

#### **N<sub>2</sub>-, CO<sub>2</sub>-fixing bioelectrochemical cell**

At OD  $\sim 0.9$ , cell dry weight = 300 mg/L.

Therefore, at an OD of  $\sim 0.12$  (OD at the start of the experiment),

Cell dry weight = 40 mg/L

At OD  $\sim 0.5$  (OD at the end of 8 days in 100 mL bioelectrochemical cell),

Cell dry weight = 170 mg/L

Cell dry weight fixed in 8 days = 130 mg/L

#### **Fixed N:**

Assuming 14% of cell dry weight is N,

Weight of fixed N after 8 days = 1.82 mg/100 mL

Average rate of fixed N production = 0.23 mg/100 mL/day or 16.25  $\mu\text{mol N}/100$  mL/day

Number of electrons delivered at cathode = 576  $\mu\text{mol}/100$  mL/day

Number of electrons needed to fix one N atom (Assuming 0% recycling of  $H_2$  produced by nitrogenase) = 4

Faradaic efficiency of N fixation = 11.3%

Number of electrons needed to fix one N atom (assuming 100% recycling of  $H_2$  produced by nitrogenase) = 3

Faradaic efficiency of N fixation = 8.5%

### **Fixed C:**

Assuming 50% of cell dry weight is C, weight of C fixed after 8 days = 6.5 mg/100 mL

Average rate of fixed C production = 0.8 mg/100 mL/day = 67.7  $\mu$ mol C/100 mL/day

Number of electrons delivered at cathode = 576  $\mu$ mol/100 mL/day

Number of electrons needed to fix one C atom = 4 (2 NADPH, and hence 4 electrons per  $CO_2$  needed for the Calvin cycle only; not considering electrons needed for further reduction to sugars or fatty acids)

Faradaic efficiency of C fixation = 47%

### **$CO_2$ -fixing bioelectrochemical cell**

At OD  $\sim 0.9$ , cell dry weight = 300 mg/L

Therefore, at OD  $\sim 0.095$  (OD at the start of the experiment),

Cell dry weight = 31.7 mg/L

At OD  $\sim 0.41$  (OD at the end of 8 days in 100 mL bioelectrochemical cell),

Cell dry weight =  $\sim 137$  mg/L

Cell dry weight fixed after 8 days = 105 mg/L

**Fixed C:**

Assuming 50% of cell dry weight is C, weight of C fixed after 8 days = 5.26 mg/100 mL

Average rate of fixed C production = 0.66 mg/100 mL/day = 54.8  $\mu$ mol C/100 mL/day

Number of electrons delivered at cathode = 577  $\mu$ mol/100 mL/day

Number of electrons needed to fix one C atom = 4 (2 NADPH, and hence 4 electrons per CO<sub>2</sub> needed for the Calvin cycle only; not considering electrons needed for further reduction to sugars or fatty acids)

Faradaic efficiency of C fixation = 38%

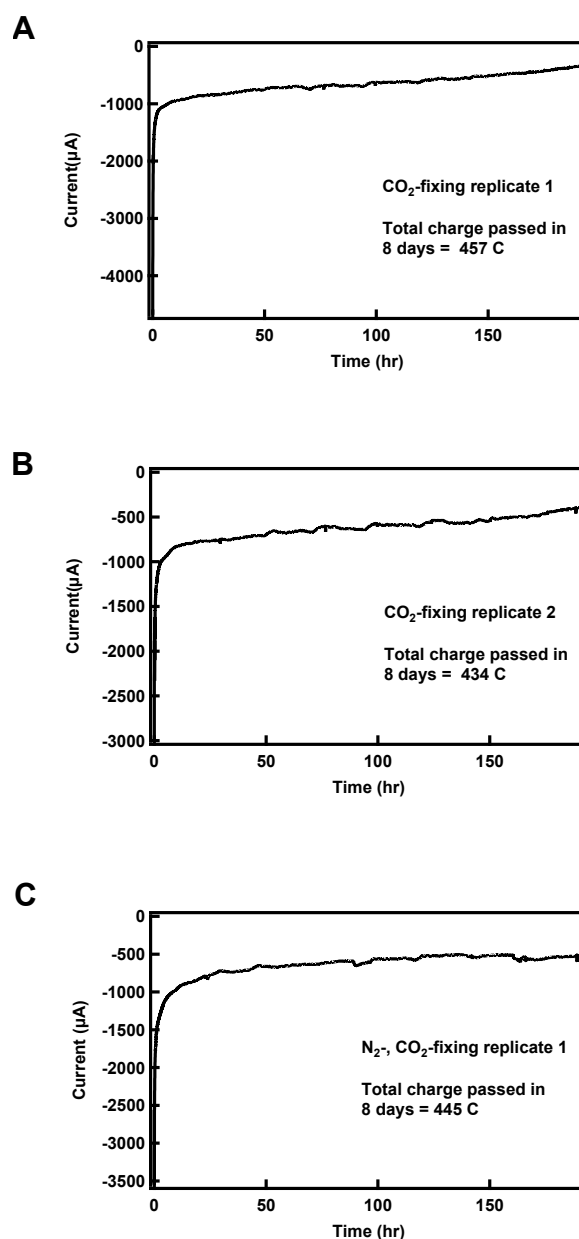


Fig. C.1: Current passed through the bioelectrochemical system under different growth conditions. All the bioelectrochemical growths were performed at a potential difference of -3 V with respect to the counter electrode ( $\sim -1.4$  V vs Ag/AgCl reference) with 16-gauge platinum wires as the cathode and anode. The electrolytes were not stirred under CO<sub>2</sub>-fixing conditions, but were stirred under N<sub>2</sub>- and CO<sub>2</sub>-fixing conditions to allow for higher currents that were necessary to support bacterial growth under N<sub>2</sub>-fixing conditions

## C.2 Whole cell absorbance spectra of bacteria grown under different light conditions

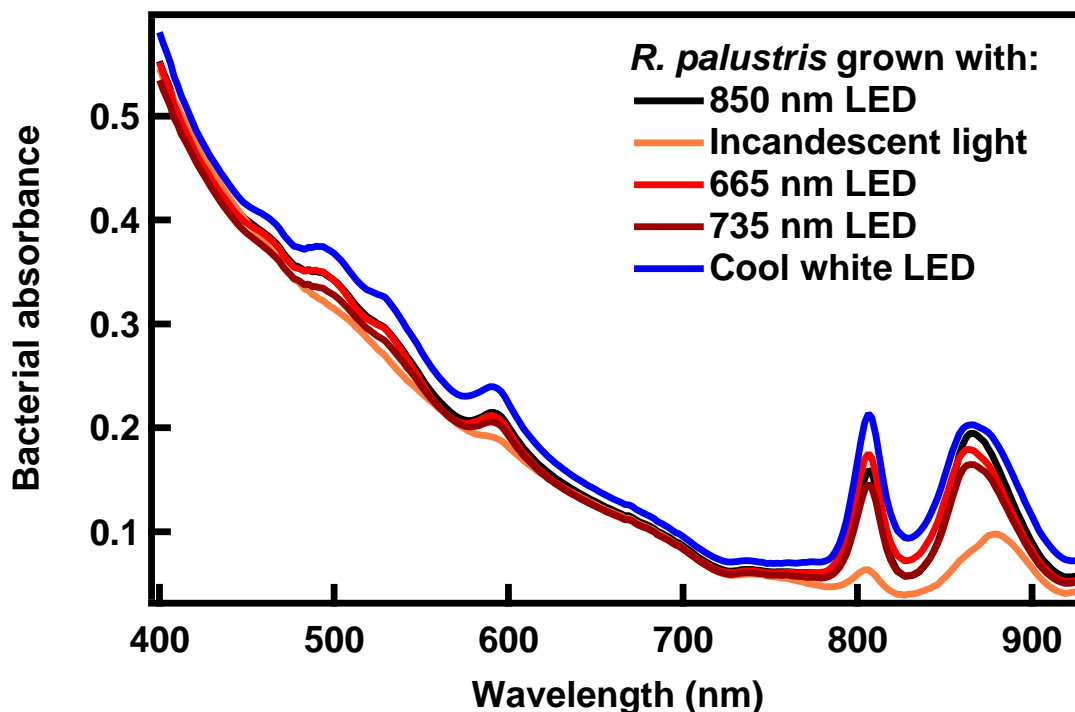


Fig. C.2: Whole cell absorbance spectra of *R. palustris* TIE-1 grown under different light conditions. The cell densities were normalised by the OD<sub>660</sub>

## C.3 Calculation of doubling time

Doubling times were calculated from the average OD measurements. The data was plotted in Microsoft Excel and the linear region was identified by fitting different time ranges to a linear trendline that would give an  $R^2$  value of 0.95 or greater (Supplementary figure C.3). Within the identified linear phase, the time taken for one doubling of the bacteria was identified and reported as the doubling time. The doubling times are summarized in supplementary table C.1.

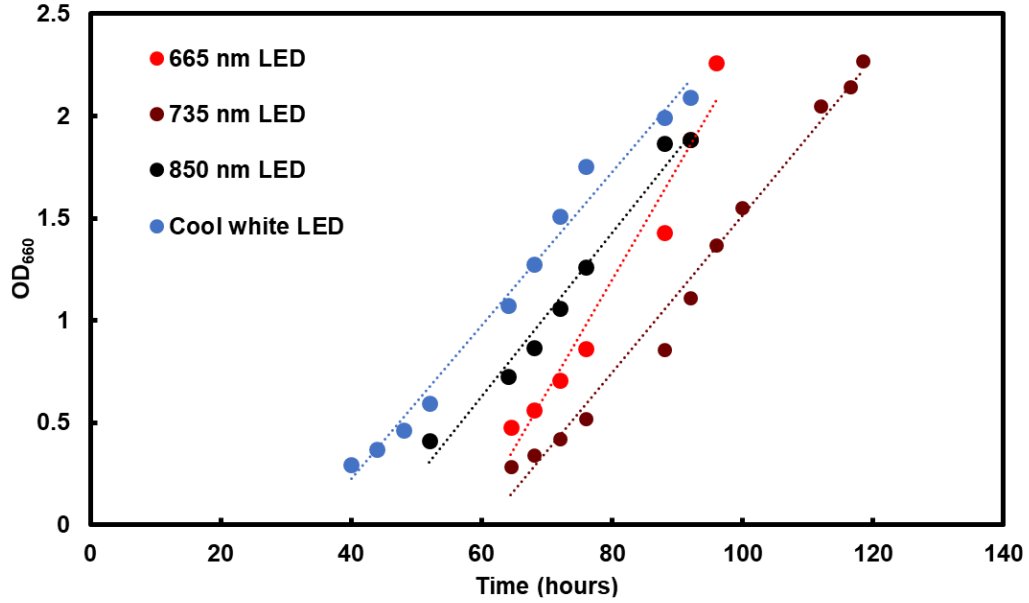


Fig. C.3: Regions of linear growth identified for the four different light conditions identified by fitting the data to a linear trendline on Microsoft Excel 2016 and selecting the range that had the highest  $R^2$  value. All  $R^2$  values for the given range were above 0.95.

#### C.4 Calculation of specific growth rate

$$\text{Specific growth rate} = \frac{\ln \frac{A_1}{A_0}}{t_1 - t_0} \quad (\text{C.2})$$

Where  $A_0$ ,  $A_1$  are absorbances at times  $t_0$  and  $t_1$  respectively.

The region of exponential growth was identified by fitting the data to an exponential curve on Microsoft Excel 2016 and identifying the data range that provided the highest  $R^2$  value. (Supplementary figure C.4). The first 40 hours was ignored for all wavelength conditions as this most likely represented the lag phase of bacterial growth. The specific growth rate identified was the average of the growth rates during the identified intervals for each growth curve. The identified growth rates are summarized in supplementary table C.1.

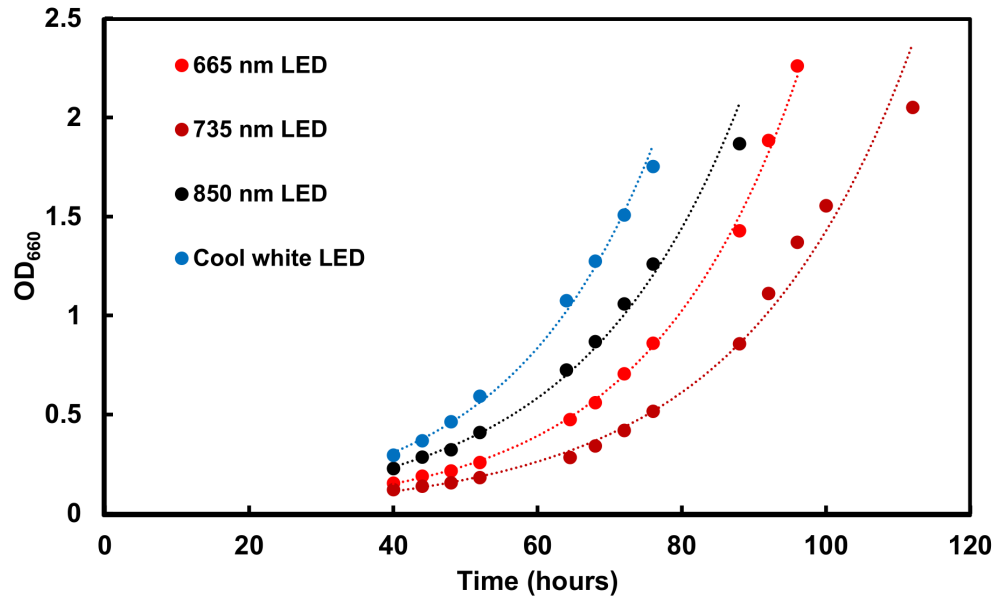


Fig. C.4: Regions of exponential growth identified for the four different light conditions identified by fitting the data to an exponential curve on Microsoft Excel 2016 and selecting the range that had the highest  $R^2$  value. All  $R^2$  values for the given range were above 0.99.

Table C.1: Summary of growth rates calculated for growth with different wavelengths of light

Wavelength Condition	Cool white LED	850 nm LED	665 nm LED	735 nm LED
Doubling time (h)	16	20	16	16
Specific growth rate ( $\text{h}^{-1}$ )	0.05	0.05	0.05	0.04

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## EDUCATION

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### Utah State University

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- Microbial electrosynthesis of special nitrogen and carbon compounds for applications on deep-space missions.
- Characterization of bacterial metabolism under nutrient limiting growth conditions.
- Characterization of bacteria as biofertilizer

### Graduate Teaching Fellow, Utah State University

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- Study of genetic susceptibility to colorectal cancer in people with Type 2 Diabetes.
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## JOURNAL PUBLICATIONS

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1. M. Soundararajan, R. Ledbetter, P. Kusuma, S. Zhen, P. Ludden, B. Bugbee, S. Ensign, L. Seefeldt. **Phototrophic N<sub>2</sub> and CO<sub>2</sub> Fixation using a *Rhodopseudomonas palustris*-H<sub>2</sub> Mediated Electrochemical System with Infrared Photons**. *Frontiers in Microbiology*. (2019). doi: 10.3389/fmicb.2019.01817.
2. M. Soundararajan, S. Kannan. **Fibroblasts and Mesenchymal Stem Cells - Two sides of the same coin?**. *Journal of Cellular Physiology*. (2018). doi:10.1002/jcp.26860.

## RESEARCH PRESENTATIONS

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1. M. Soundararajan, R. Ledbetter, P. Kusuma, S. Zhen, P. Ludden, B. Bugbee, S. Ensign, L. Seefeldt. **Microbial Electrosynthetic N<sub>2</sub> and CO<sub>2</sub> Reduction at Ambient Conditions for Application in Deep-Space Missions**. Astrobiology Science Conference, Seattle, WA, June 2019.
2. M. Soundararajan, J. Hilzinger, K. Wetmore. **Directed Evolution of *R. palustris* TIE-1 for Improved Growth Under 2% N<sub>2</sub>**. Centre for Utilization of Biological Engineering in Space Semiannual Meet, Davis, CA, May 2019.
3. M Soundararajan, R. Ledbetter, P. Kusuma, S. Zhen, P. Ludden, B. Bugbee, S. Ensign, L. Seefeldt. **N<sub>2</sub>- and CO<sub>2</sub>-Fixation Using Electrochemistry – Capturing Resources on Mars**. University of Minnesota, Minneapolis, MN, January 2019.
4. M Soundararajan, R. Ledbetter, P. Kusuma, S. Zhen, P. Ludden, B. Bugbee, S. Ensign, L. Seefeldt. **Whole Cell N<sub>2</sub>- and CO<sub>2</sub>-Fixation Using Electrochemistry – Capturing Resources on Mars**. European Nitrogen Fixation Conference, Satellite meeting on Molecular aspects of Regulation and Metabolism in Nitrogen Fixation, Stockholm, Sweden, 2018.
5. M. Soundararajan, R. Ledbetter, P. Kusuma, S. Zhen, P. Ludden, B. Bugbee, S. Ensign, L. Seefeldt. **N<sub>2</sub> Reduction to NH<sub>3</sub> Using a Microbial Electrosynthesis System**. Centre for Utilization of Biological Engineering in Space Semiannual Meet, Berkeley, CA October 2018.
6. M. Soundararajan. **Capturing Resources on Mars – Our First Steps**. Centre for Utilization of Biological Engineering in Space Monthly Virtual Seminar, May 2018.
7. M. Soundararajan, R. Ledbetter, P. Kusuma, S. Zhen, P. Ludden, B. Bugbee, S. Ensign, L. Seefeldt. **Microbial Electrocatalysis for Simultaneous N<sub>2</sub> and CO<sub>2</sub> Reduction**. Centre for Utilization of Biological Engineering in Space Semiannual Meet, Berkeley, CA April 2018.

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