Electrochemical Ammonia and Hydrogen Production from Polymer-Immobilized Nitrogenase Enzymes

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12 October 2009

Why Ammonia?

- Renewable energy source
- Combustion does not produce greenhouse gases
  - $4NH_3 + 3O_2 \rightarrow 2N_2 + 6H_2O$
  - NO_x converted to H_2O and N_2
- Fuel cells
  - $4NH_3 + 5O_2 \rightarrow 4NO + 6H_2O$
- Infrastructure: Existing natural gas pipelines could be converted to ammonia transport

Exploring the Possibility

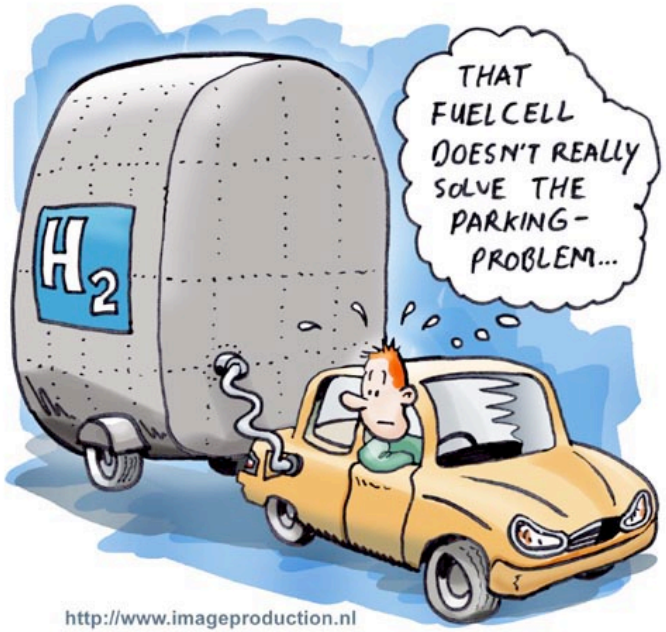
- $ cost and environmental tax
  - >1% of world's annual energy supply goes to synthesize NH_3
  - Modern synthesis (Haber-Bosch) requires natural gas as source of H_2 (3-5% of the world methane production)
  - High Temperatures (300-550 °C) and Pressures (15-25 MPa)
- An eco-friendly option possible?

Smith, B. E. *Science* **2002**, *297*, 1654-1655.

A Bit of Perspective

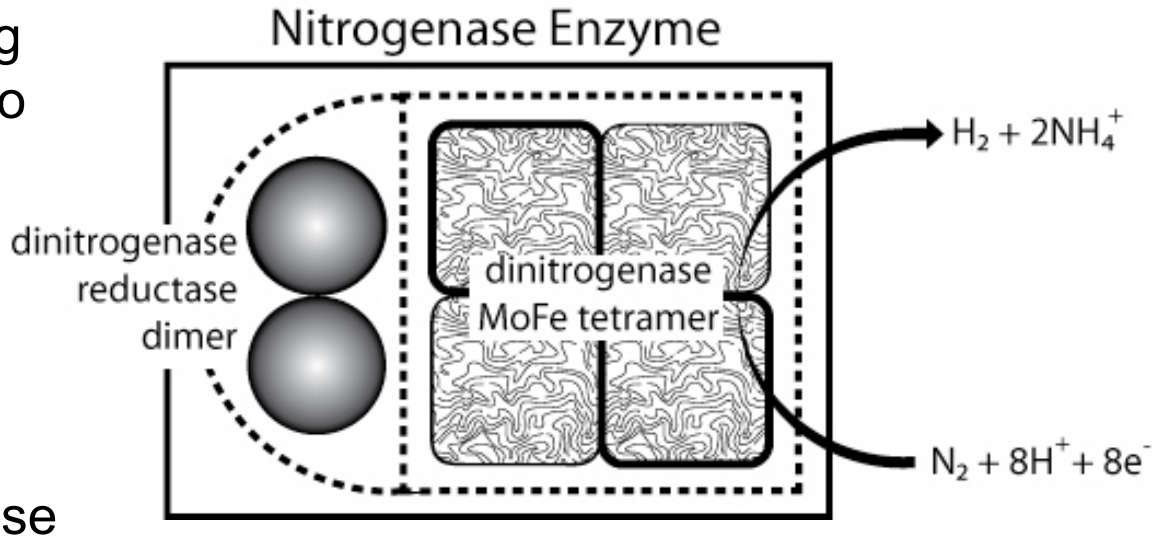
"Even though the huge and highly successful Haber-Bosch process is unlikely to be displaced readily by a new process, even if efficient and inexpensive, it must be kept in mind that >1% of the energy consumed by humans is consumed by the Haber-Bosch process."

Shrock, R. R. *Proc. Natl. Acad. Sci.* **103**, *17087*, 2006.



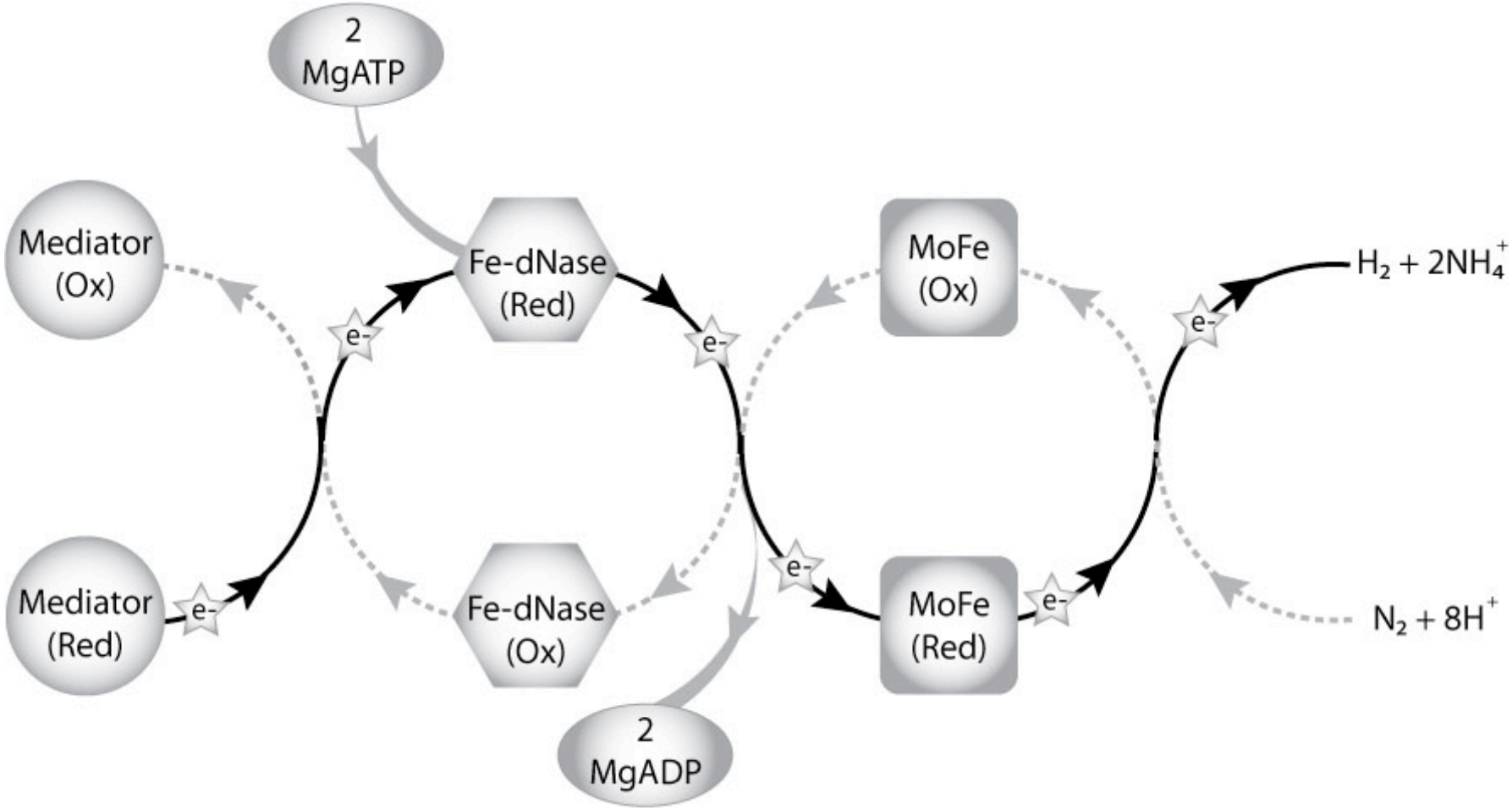
Biological Nitrogen Fixation

- Large scale NH_3 production: metalloenzymes in living organisms convert N_2 to NH_3 on a scale of ~10^8 tons/year.

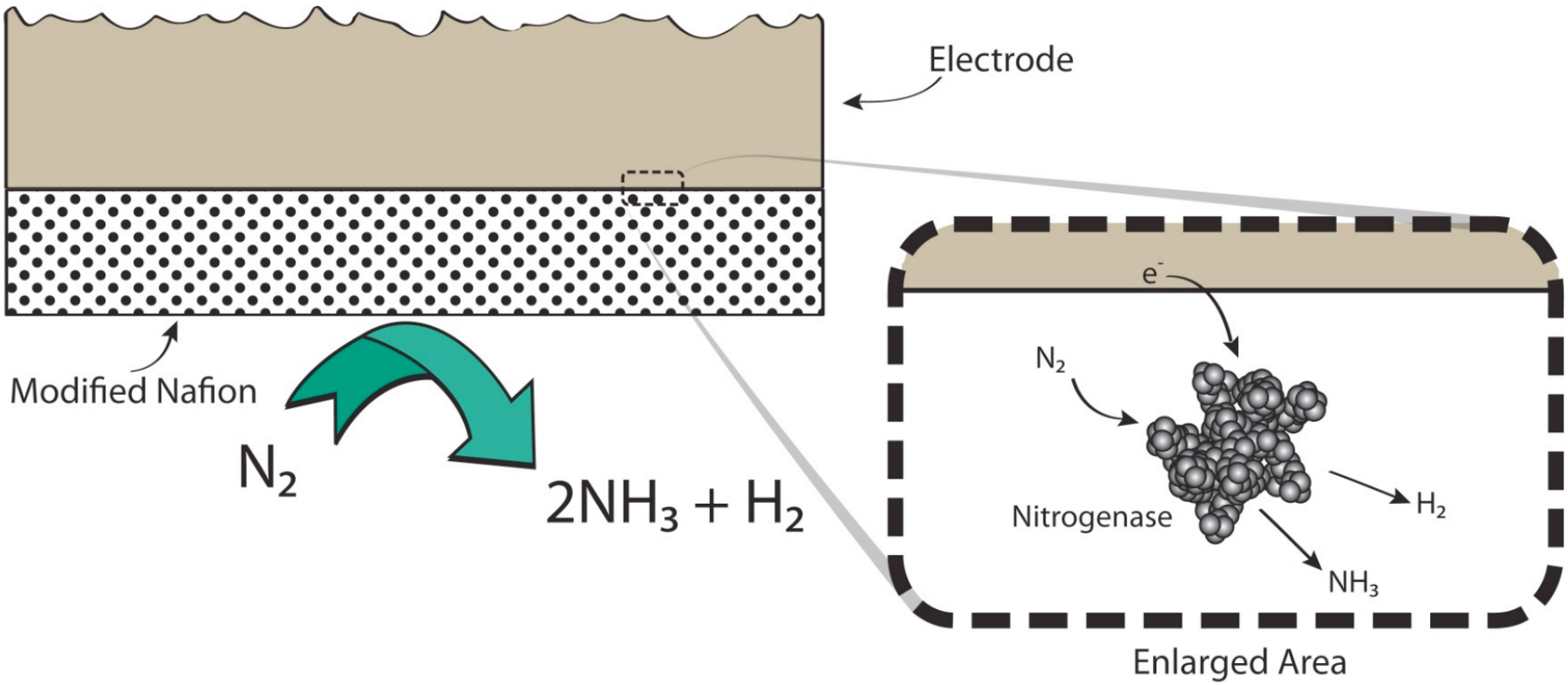


- Biologically fix N_2 to ammonia (via nitrogenase enzyme)
  - $N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16p_i$
  - Rate Determining Step: following et, Nase complex dissociation

Enzyme Cascade Reaction



The Big Picture



Energetic Inputs: sunlight (photosynthesis), reactant (atmospheric N_2), minimal electrical potential (1-3 V)

Outputs: NH_4^+ and H_2

Biological Enzyme Source

Cyanobacteria (Blue Green Algae) – *Anabaena variabilis*

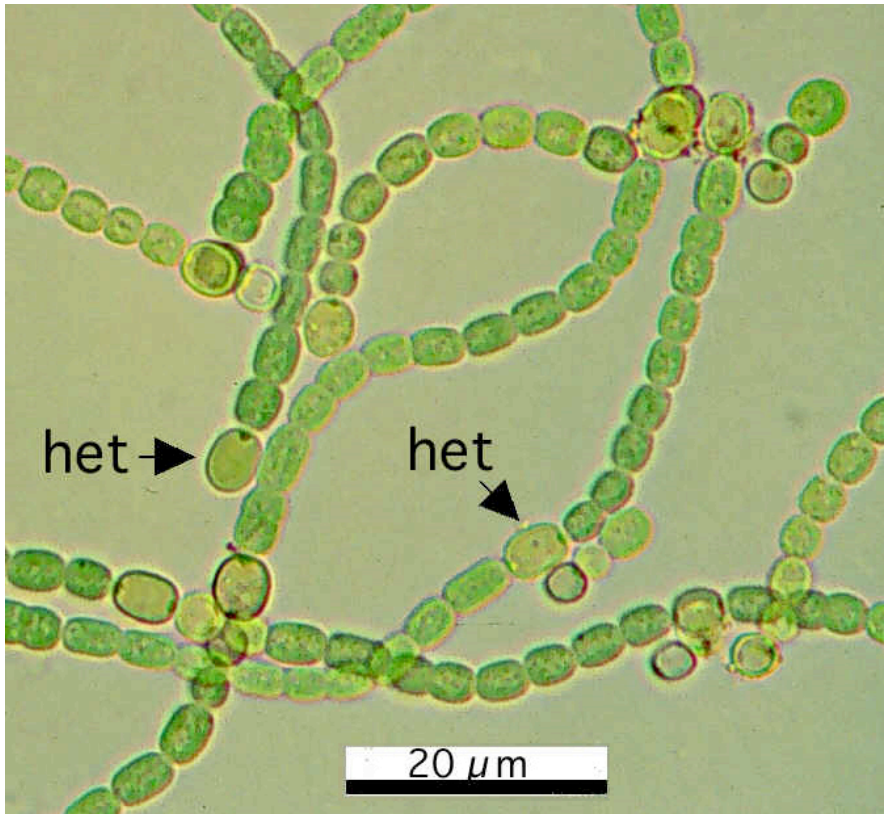
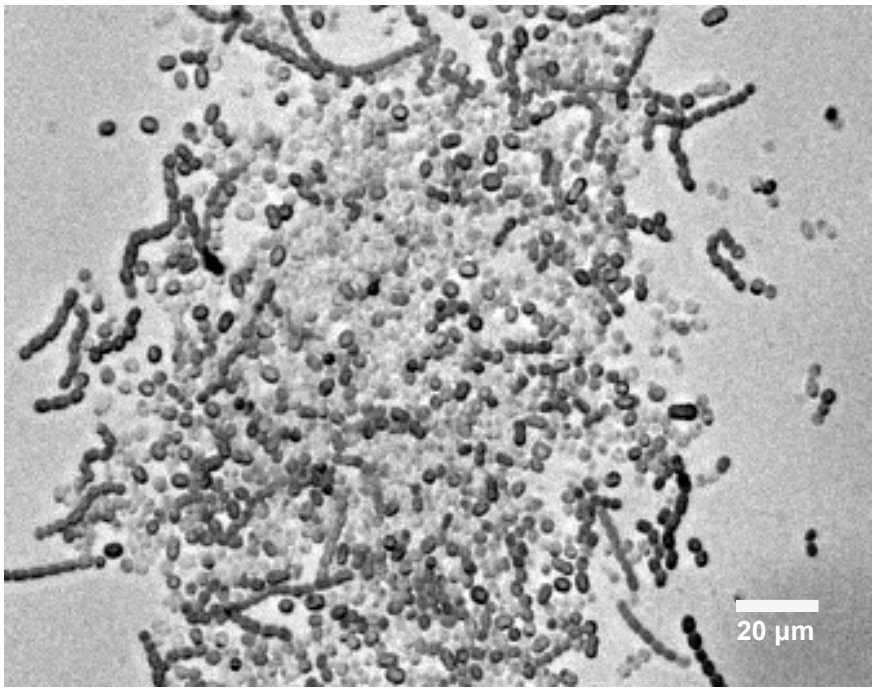
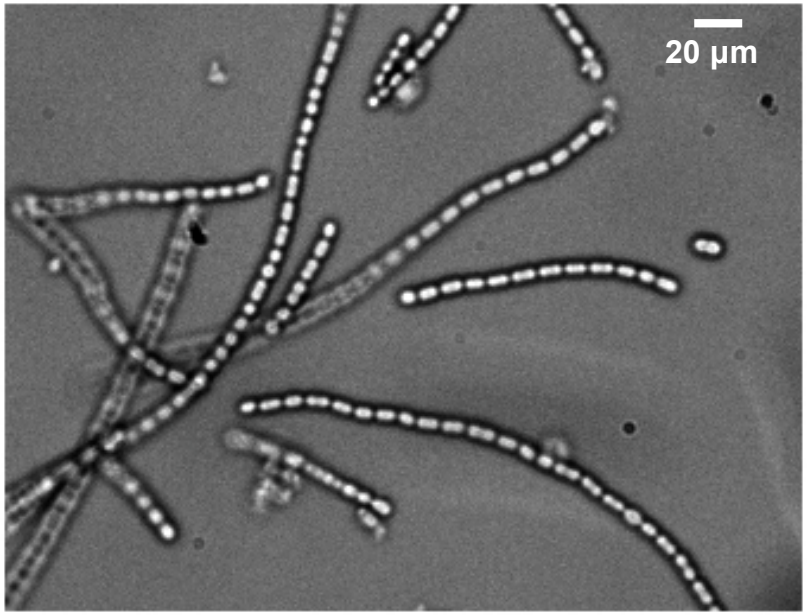


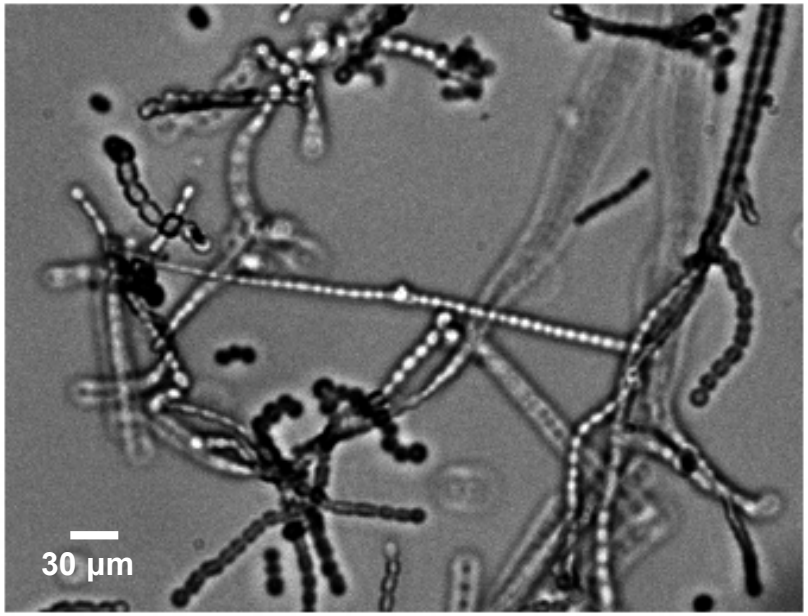
Photo courtesy of University of Seville, Institute of Plant Biochemistry and Photosynthesis
http://www.ibvf.cartuja.csic.es



A. var. cluster, 40x

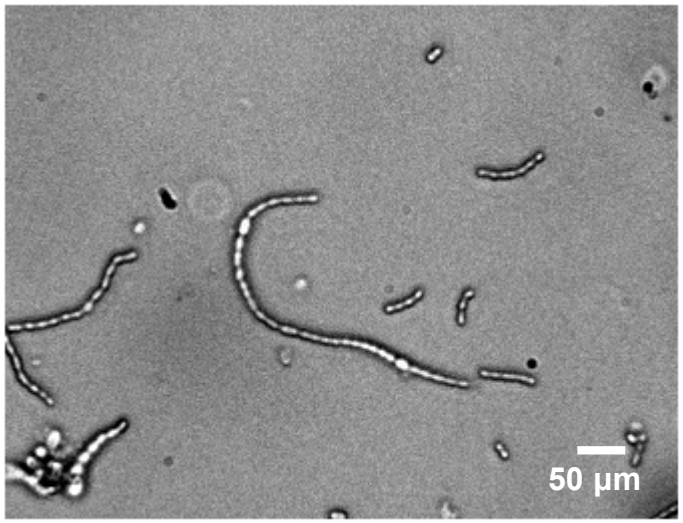
Anabaena variabilis

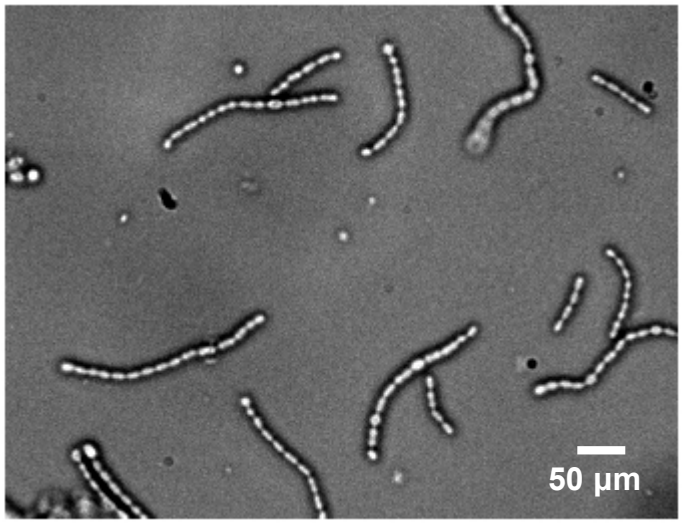


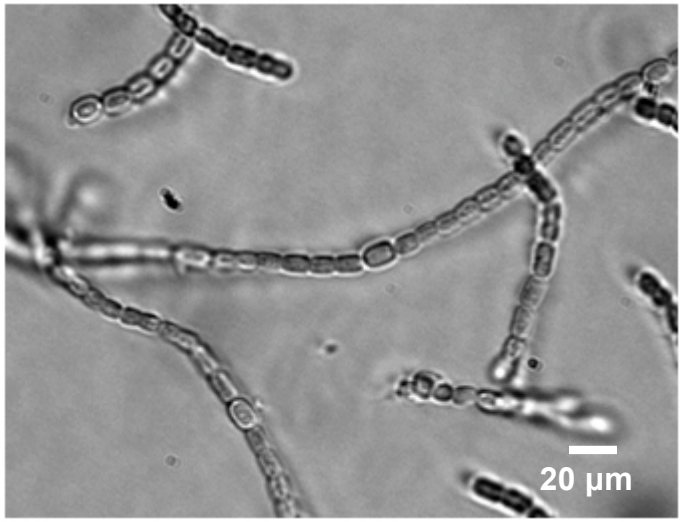


Filamentous, fresh water, archaebacteria. Classic "N_2 Fixers."

Anabaena variabilis SA1





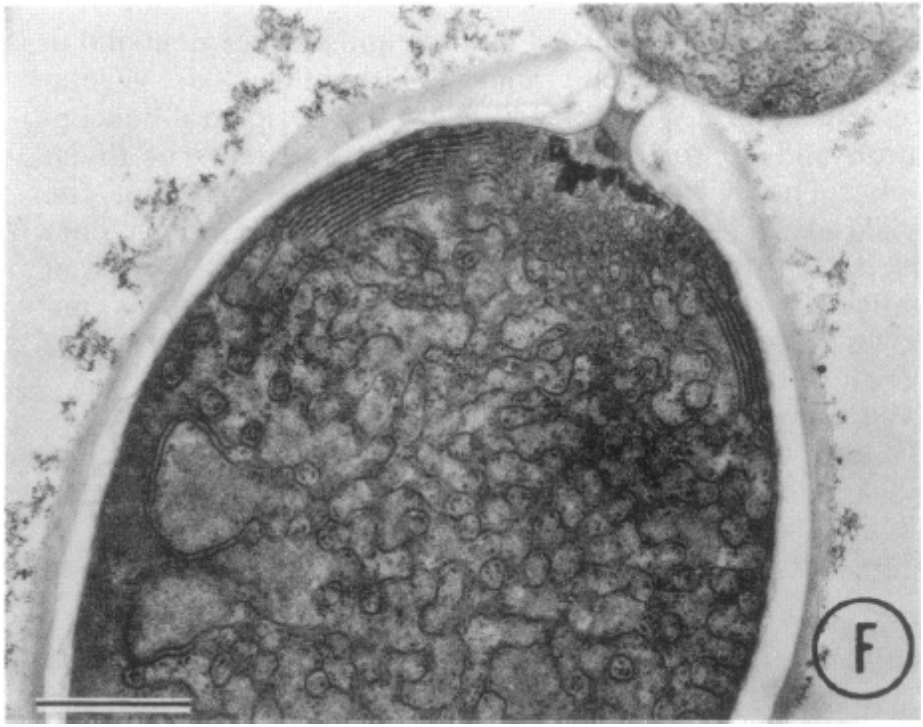


Genetic Mutant Nitrogenase Derepressed strain of *A. var.*

Spiller, H.; Latorre, C.; Hassan, M.E.; Shanmugam; K.T. *J. Bacteriol.* **1986**, *165*, 412-419.

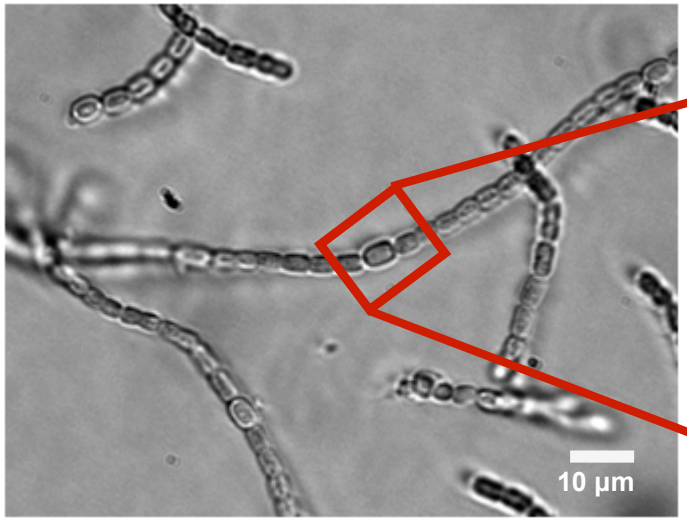
Why *Anabaena variabilis*?

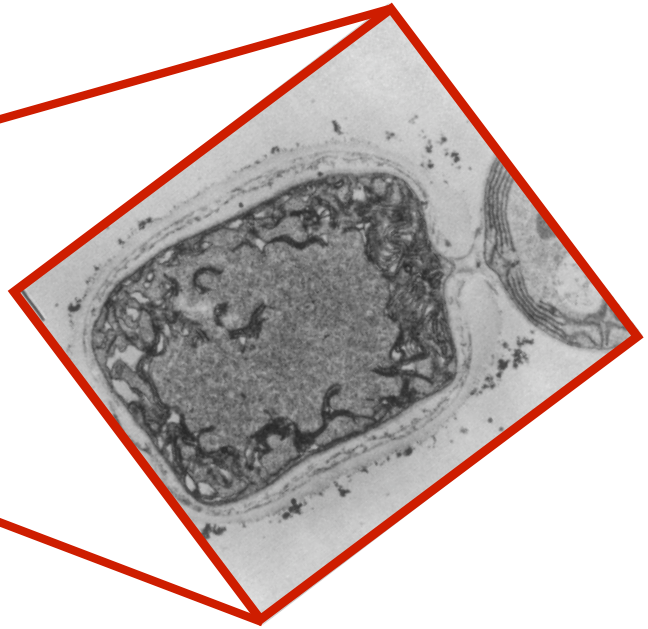
- O_2 is a competitive inhibitor
  - Evolution of heterocysts – thickened and relatively oxygen impermeable membranes
- *A. var.* are not able to reuptake of H_2
- Well-studied organism
- Low mutation over time
- Easy to culture and maintain

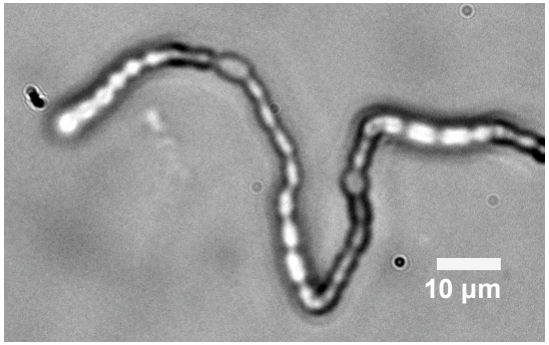


Lang, N. J., Krupp, J. M. & Koller, A. L.
J. Bacteriol. **169,** *920-923* (1987)

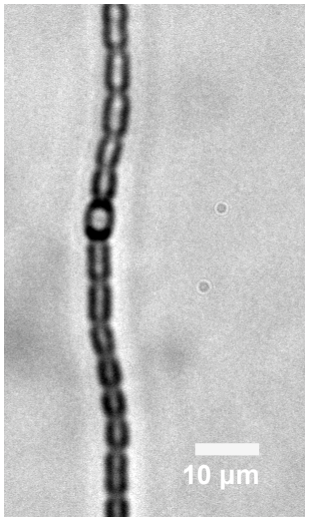
Heterocysts – Enzyme Site







- O_2-impermeable
- Photosystem I, not II.
- ~5-9% of total cells



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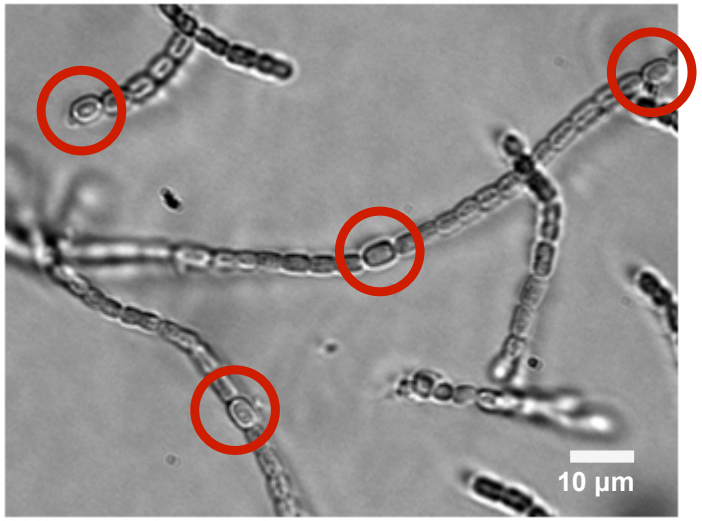

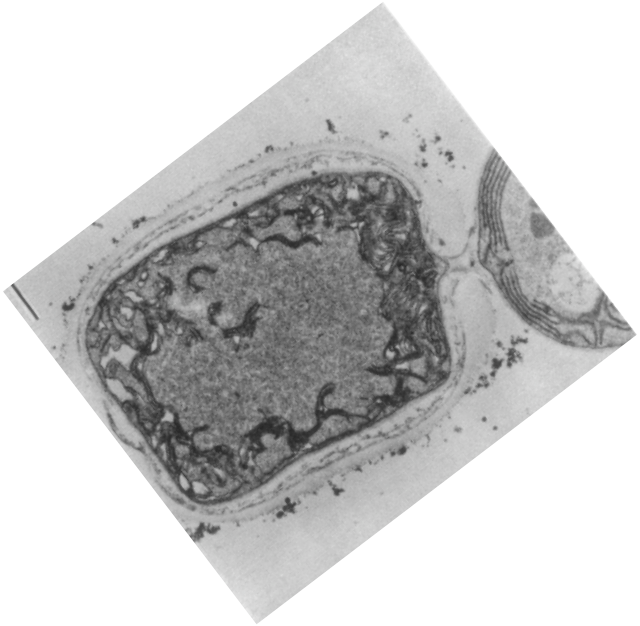

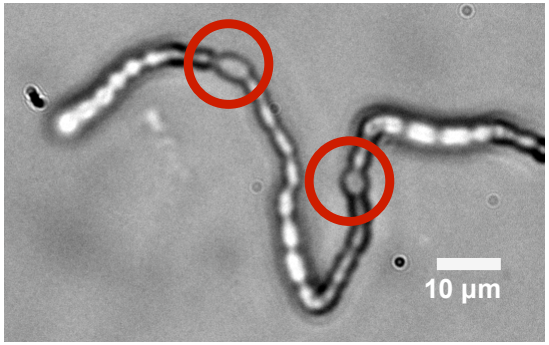

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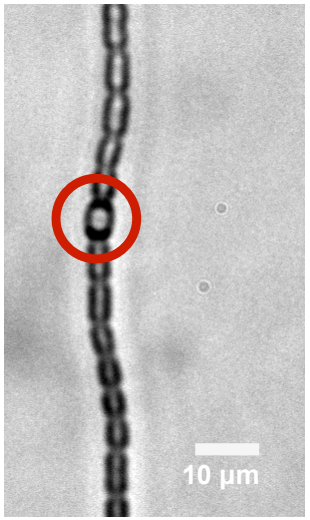
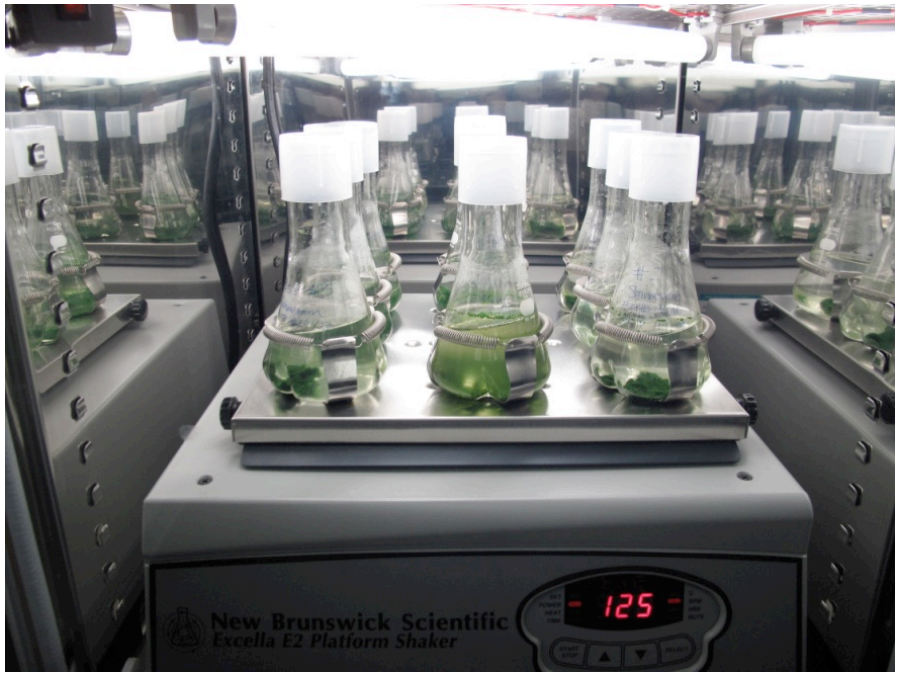


Culture Conditions

- Temperature: 32 °C (lab), ambient *in vivo*
- Light: full spectrum fluorescent bulbs, 32 µmol/m^2·s
  - 24-hour illumination
  - PAR – Photosynthetically Active Radiation
- Atmosphere: ambient
- Shake: 125 orbital oscillations/minute
- Media: BG-11_0 metal salt broth, no serum required
- Media Change: bi-weekly
- Volume: 125-mL media in 250-mL baffled flasks, covered

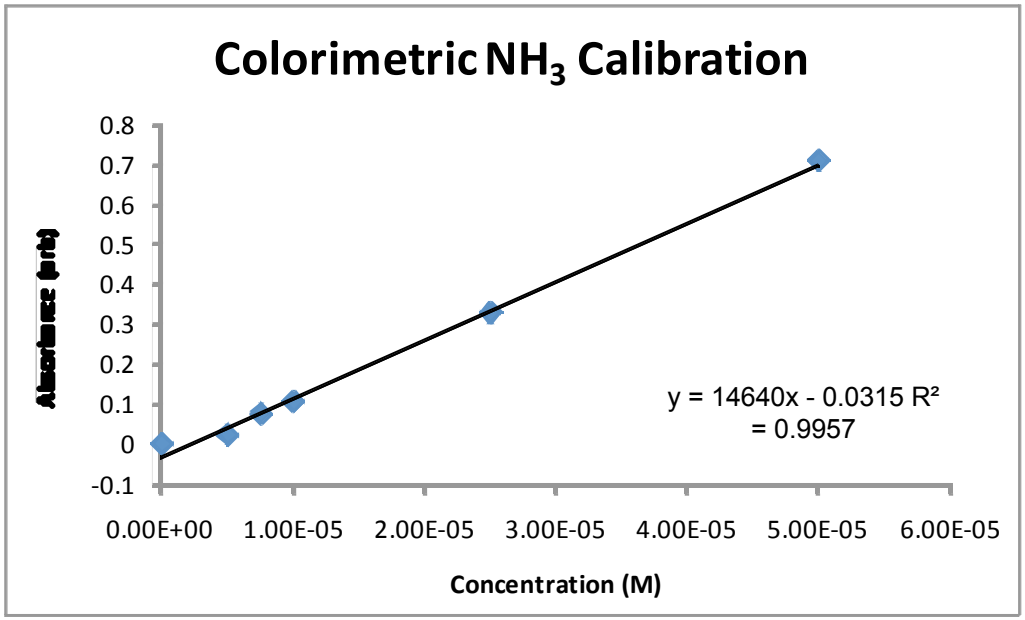


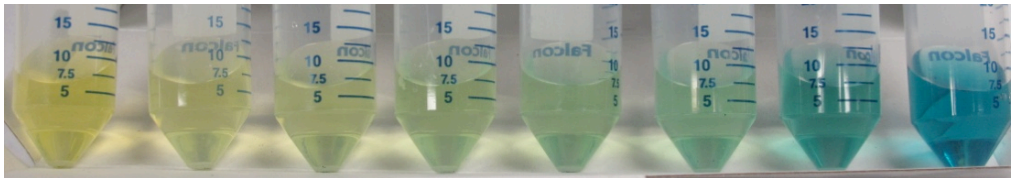
Culture Measurements

- Turbidity – A_{750}
- Chlorophyll a (Chl a)
- Dry Mass
- Imaging
- Enzyme Activity – C_2H_2 to C_2H_4 conversion
- Ammonia Production/Detection

Ammonia Detection

- Colorimetric – catalytic oxidation of phenol in the presence of NH_4^+ to yield indophenol blue. Measure absorbance at 650 nm.
  Lower LOD: 5 x 10^{-5} M (Solorzano, L. *Limnol. Oceanogr.* **1969**, *14*, 799-801).





- Labor intensive
- Time consuming
- Waste generating

- Less subjective
- Non-technical

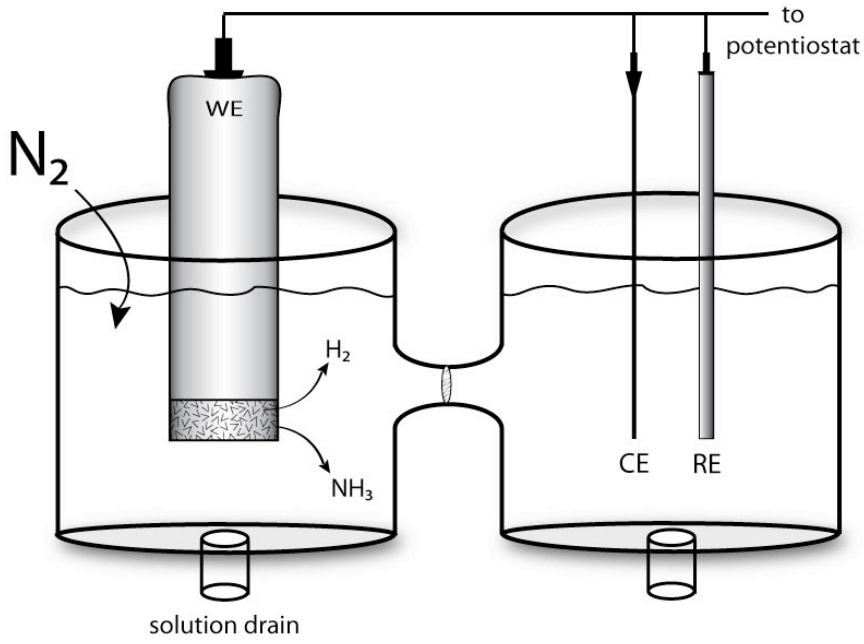
Ammonia Detection

Cell Type	$[NH_3]$ (μM)	μmol NH_3/μg Chl a
A. var. (Shanmugam)	1.3 ± 0.3	0.0124
A. var. SA1 (Shanmugam)	2.09	0.0198
A. var. (ATCC)	1.5 ± 0.3	0.0118

- Ammonia Ion Selective Electrode (gas sensing) – spike with strong base, drive conversion from ion to NH_3 gas. Lower LOD: 5×10^{-6} M
  - Drift near lower LOD
  - Quick, robust, minimal sample preparation
- Initial Quantification (preliminary data, small cultures)

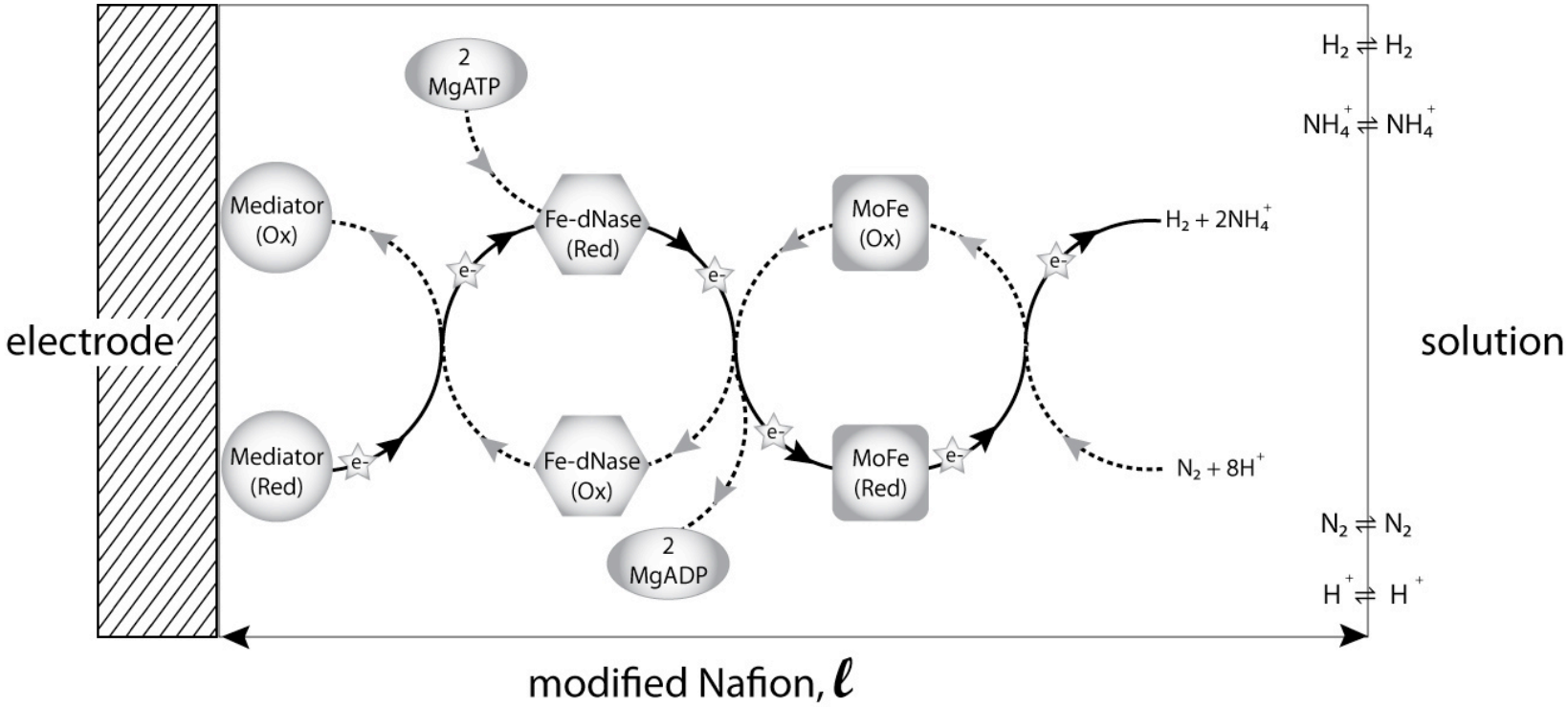
Increase Production

- Scale up of culture/cell counts
- Chemical additives to prevent reuptake of NH_4^+
  - Nitrite – via nitrite reductase
  - MSX – methionine D,L-sulfoximine
- Removal of NH_4^+ from external media – constant refresh – towards the device construction.



Jeanfils, J.; Loudeche, R. *Biotechnol. Lett.* **1986**, *8*, 265-270.

Proposed "Bioelectrocatalysis"



- Electrochemistry of Nitrogenase – not a walk in the park
  - $NAD^+ + ne^- \rightarrow NADH$ ($E° = -0.32$ V for NADH)
  - Dithinoite: Na_2S_2O4 ($E° = -0.66$ V at pH 7)
  - Ferredoxin: Fe_2S_2 ($E° = -0.495$ mV)
  - ATP recycling?

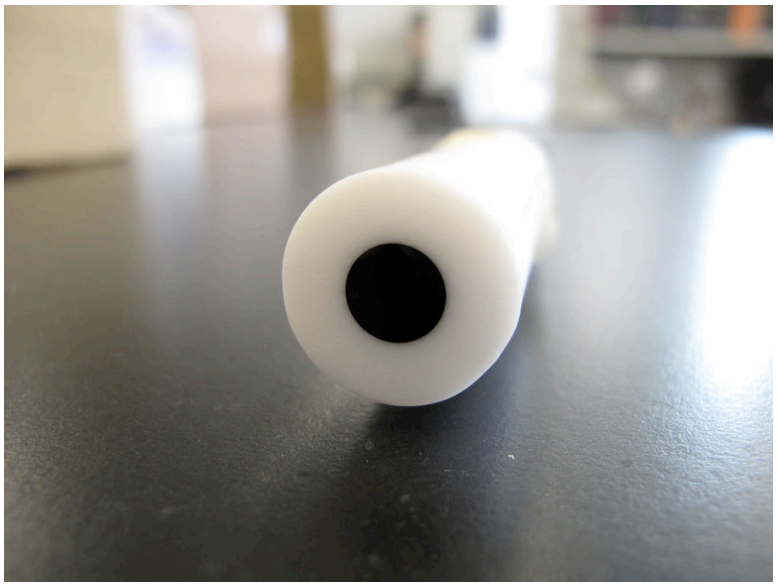
Immobilized Enzymes

- Enzyme lifetimes can be extended > 1 year by entrapment in within hydrophobically modified Nafion or chitosan.
  - Increased biocatalytic capacity due to increased cell densities
  - stabilization of enzyme activities
  - avoid wash out of cells at high dilution rates
  - the possibility for continuous operation
  - lower cost of isolation of products

Musgrave, S. C.; Kerby, N. W.; Codd, G. A.; Stewart, W. D. P. *Biotechnol. Lett.* **1982**, *4*, 647-652.
Brouers, M.; Hall, D. O. *J. Biotechnol.* **1986**, *3*, 307-321.
Atanassov, P.; Minteer, S. D. *Interface* **2007**, *Summer*, 28-31.

Polymer Modified Electrodes

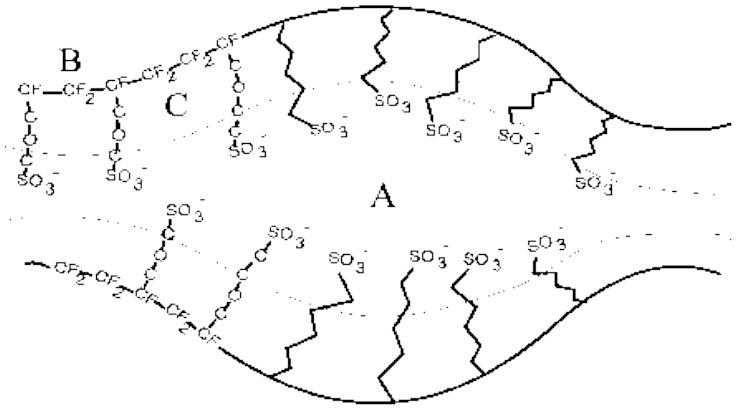
- Preparation
  - Apply liquid Nafion suspension to electrode surface.
  - Dry in vacuum desiccators for 1 hour
- Voltammetric characterization
- Modified Nafion
  - Increased flux
  - Larger, but fewer, pores
  - Lower proton-selectivity (more neutral and buffered pH environment)

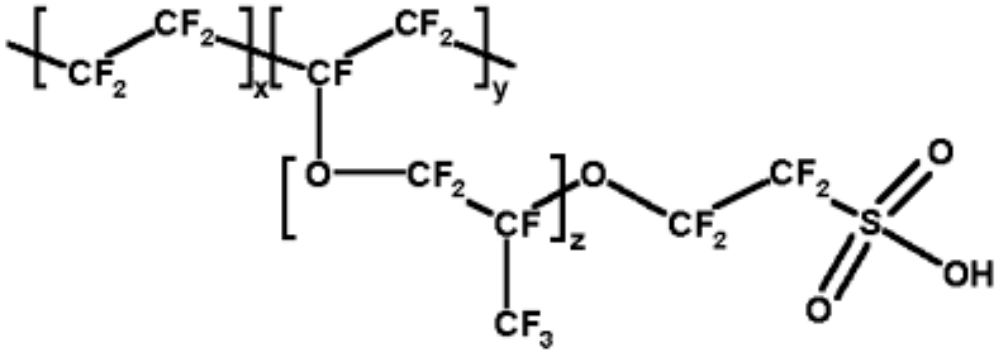


Klotzbach, T. L.; Watt, M.; Ansari, Y.; Minteer, S. D. *J. Membr. Sci.* **2008**, *311*, 81.

Polymer Details

- Nafion – structural support
- Micellar structure – not large enough for heterocysts
- Modify with organic modifiers – long alkyl chains to increase pore size
- Ion-selectivity of Nafion significantly reduced when modified
- Modified Nafion and the hydrophobic environment actually increases the enzyme kinetics.

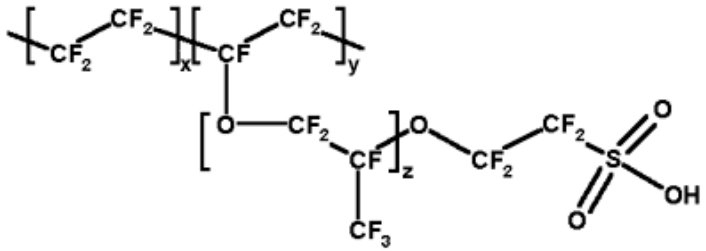


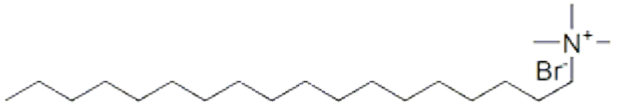


Moore, C. M.; Akers, N. L.; Hill, A. D.; Johnson, Z. C.; Minteer, S. D. *Biomacromol.* **2004**, *5*, 1241-1247
Klotzbach, T.; Watt, M.; Ansari, Y.; Minteer, S. D. *J. Membr. Sci.* **2006**, *282*, 276-283.

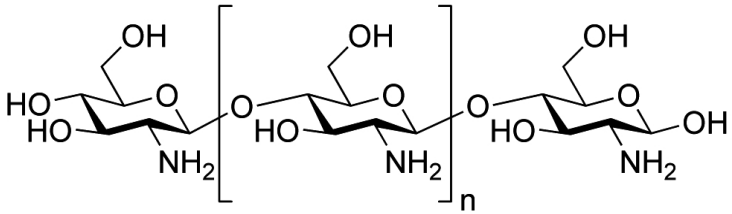
Immobilization Polymers

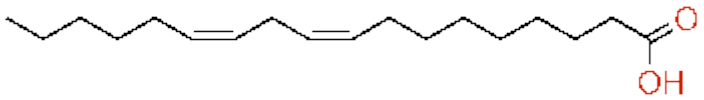
- Modified Nafion
  - octadecyltrimethylammonium bromide (TMODA) Nafion in propanol – pore sizes: ca. 10 μm





- Modified Chitosan
  - linoleic acid chitosan in acetate buffer (hydrophobic)
  - naturally abundant - exoskeletons



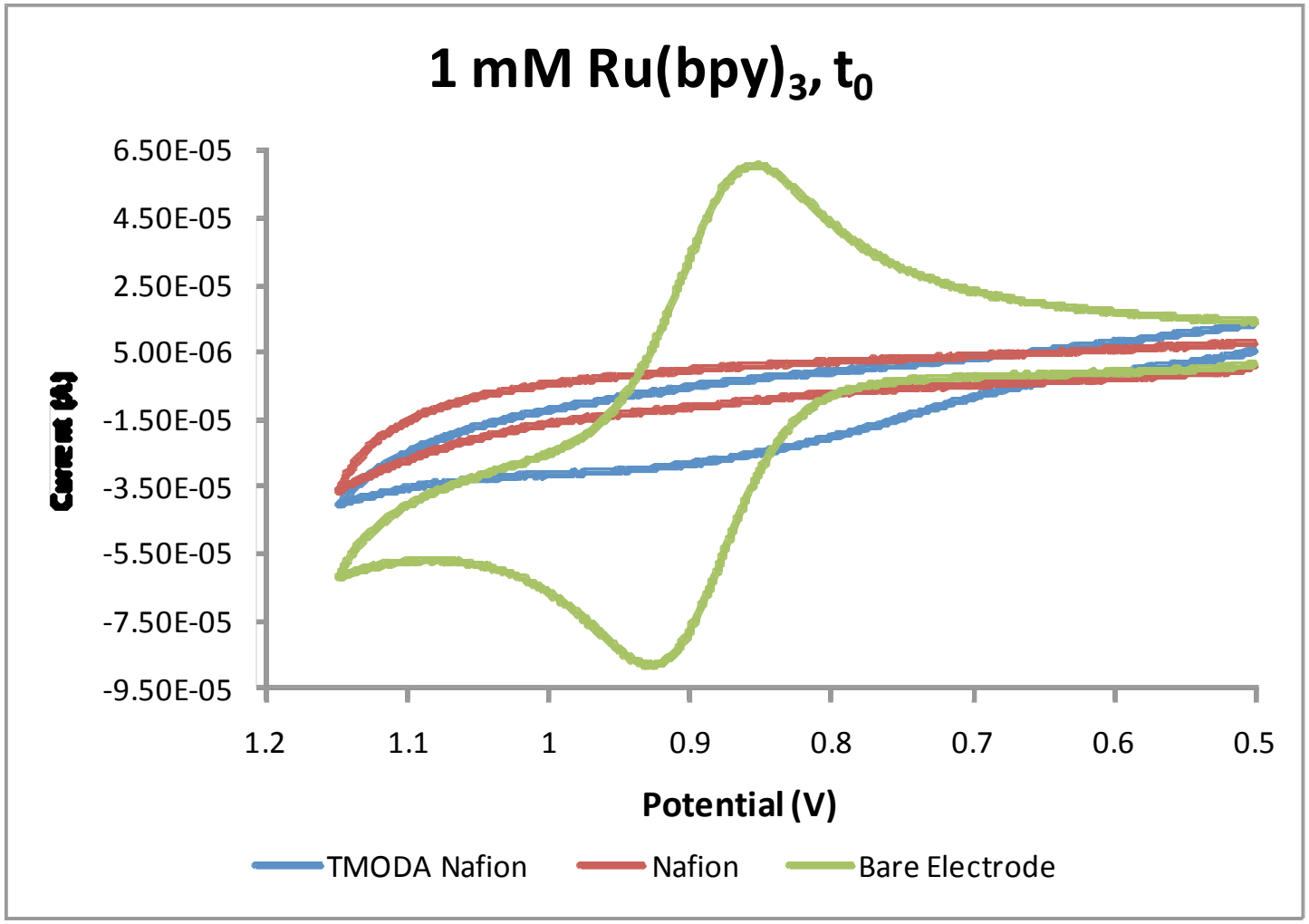


Minteer, et. Al.

Polymer Characterization

- Use CV to measure the flux of the redox species through the polymer membrane at the electrode surface
- Cyclic Voltammetry Conditions
  - Scan Rate: 100 mV/s
  - 25 °C
  - 100 mM HNO_3 electrolyte
  - Silver/Silver Oxide Reference
  - pH = 6.5

Cyclic Voltammograms



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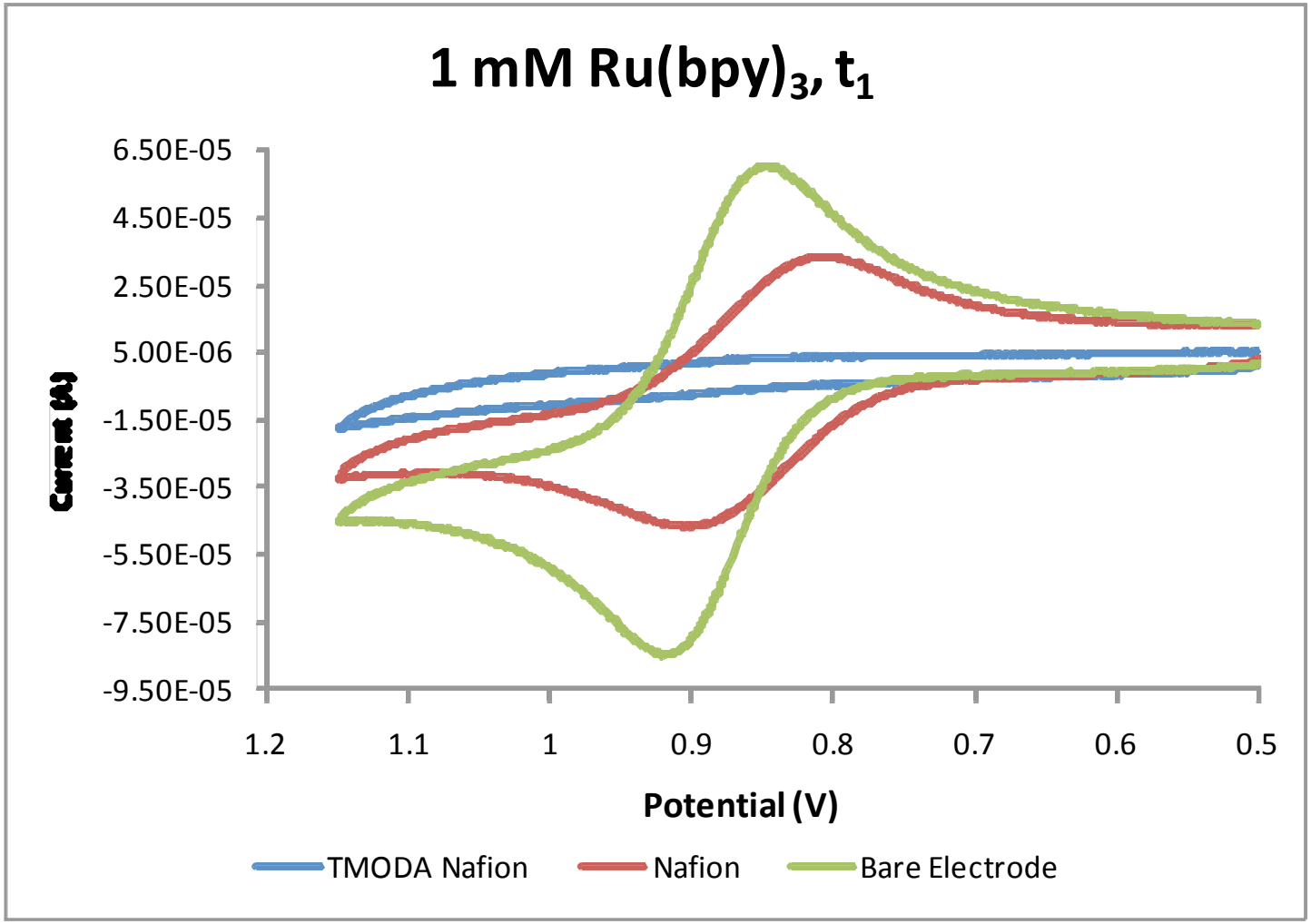
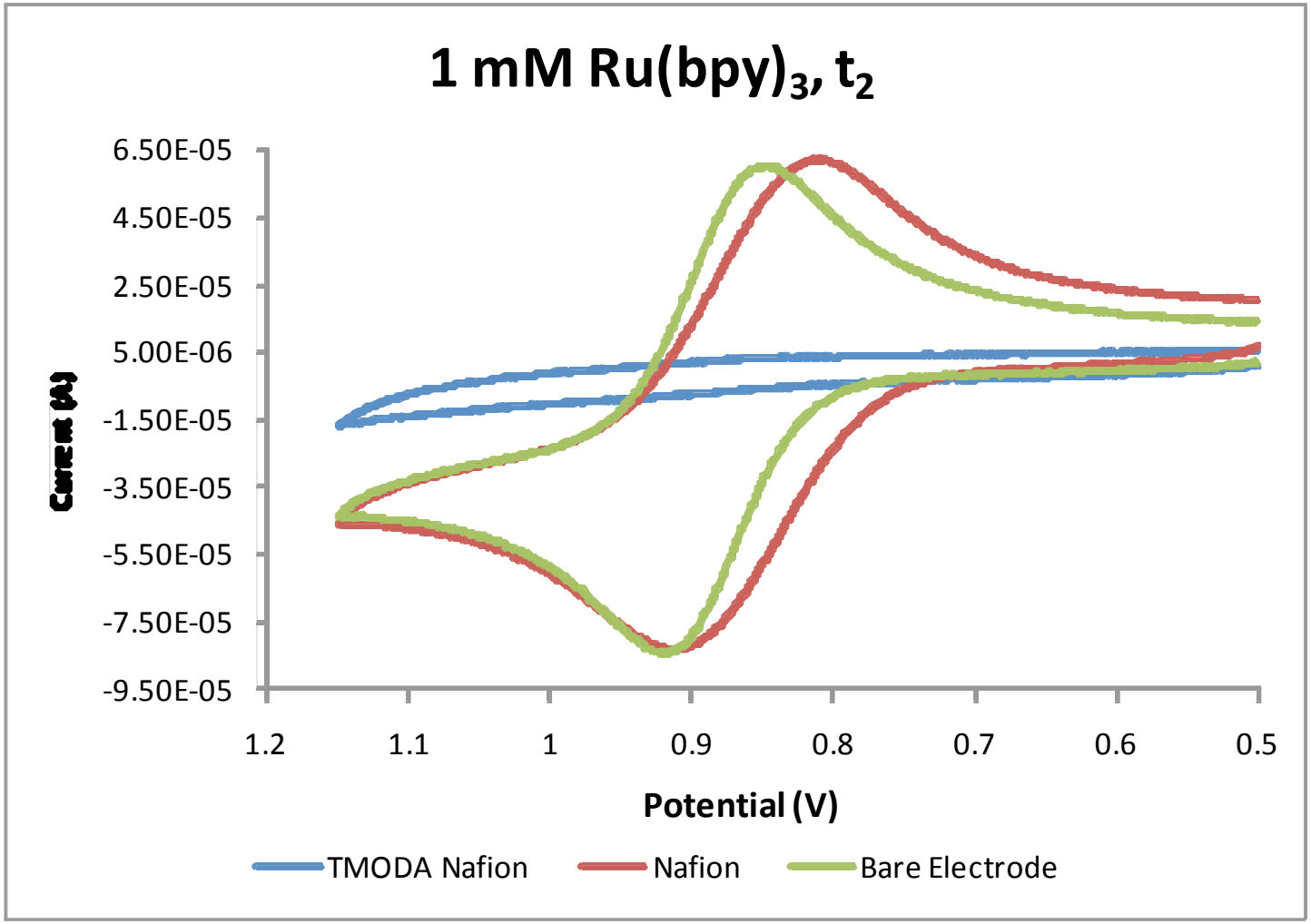
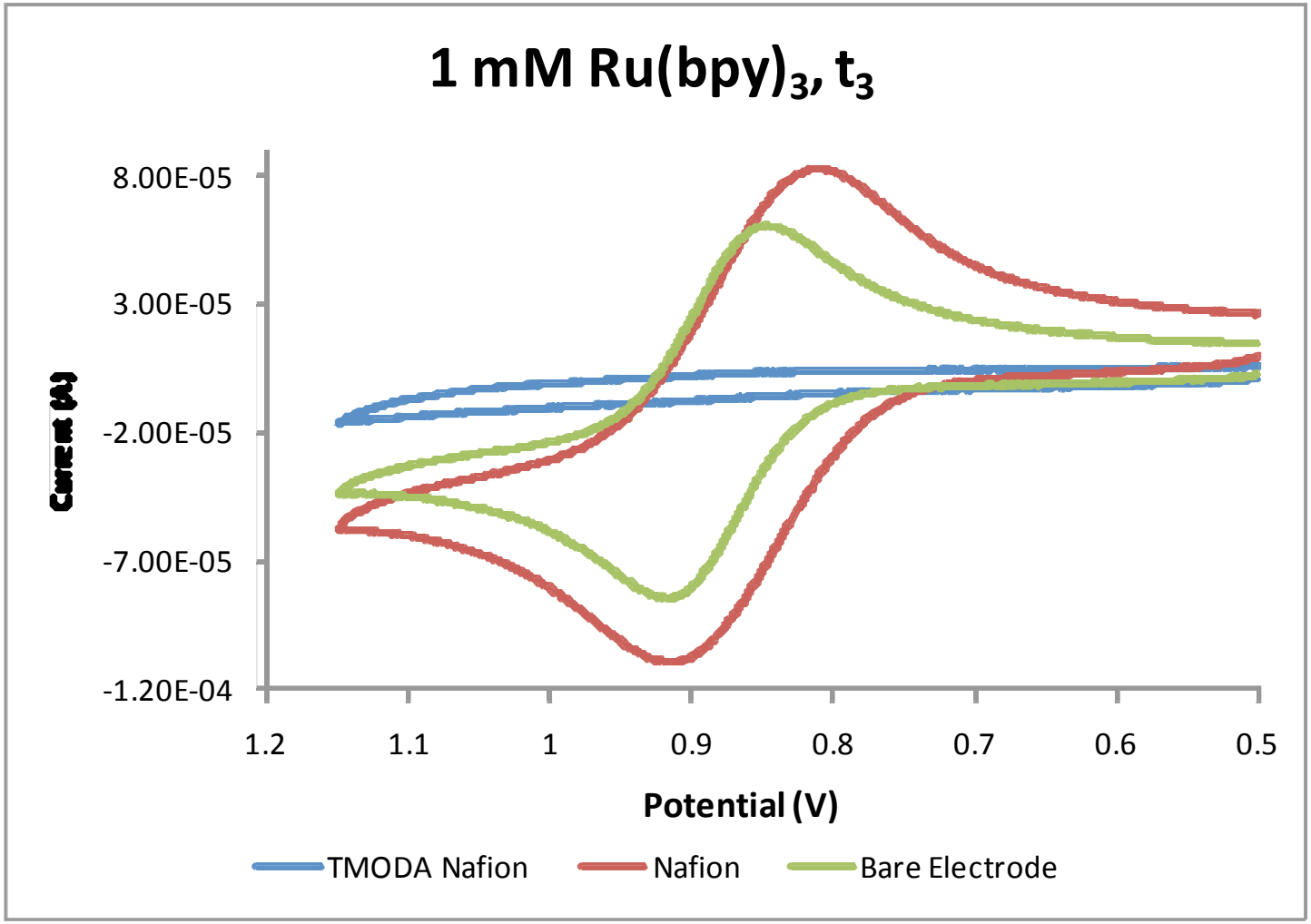


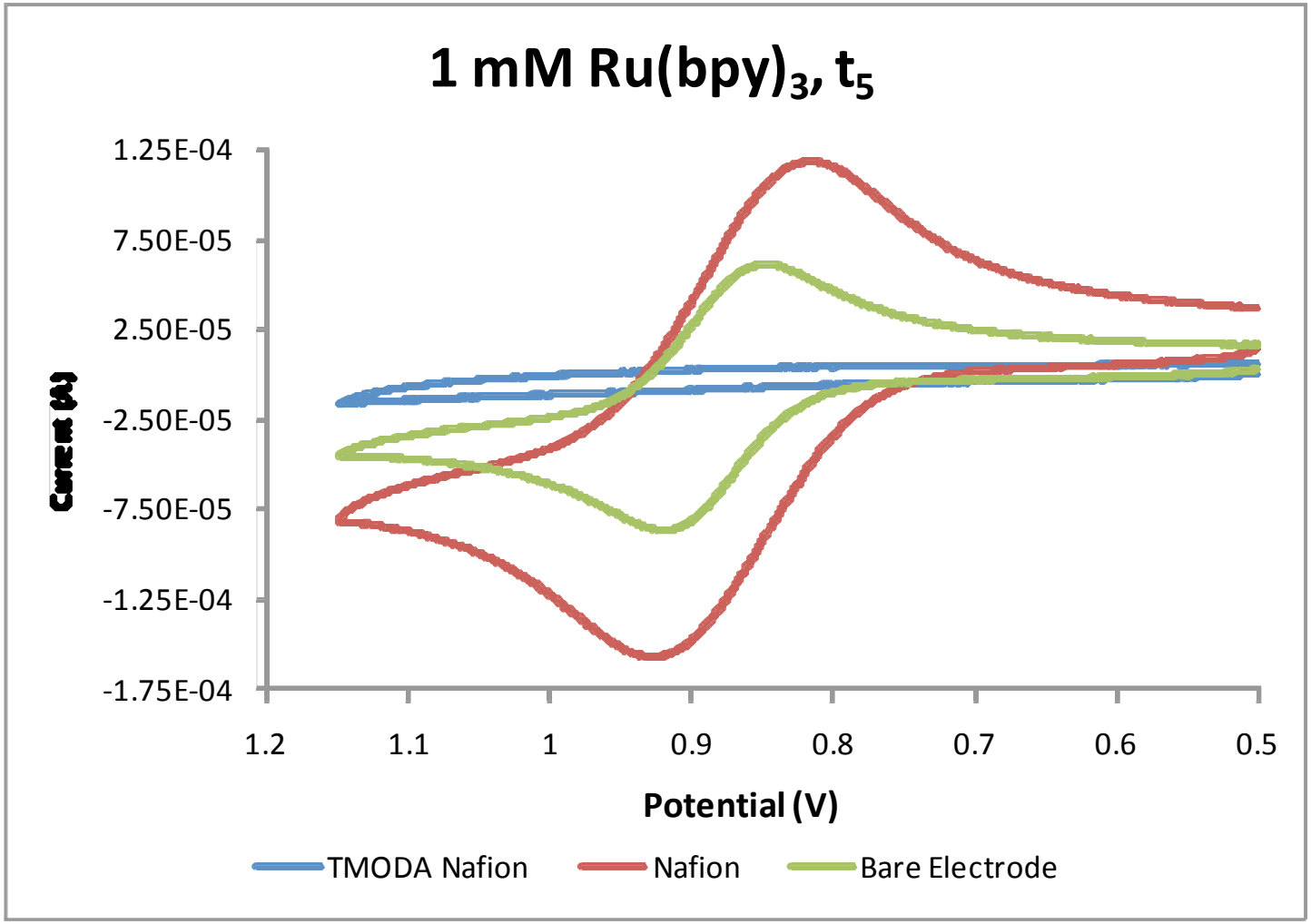
Cyclic Voltammograms



Cyclic Voltammograms



Cyclic Voltammograms



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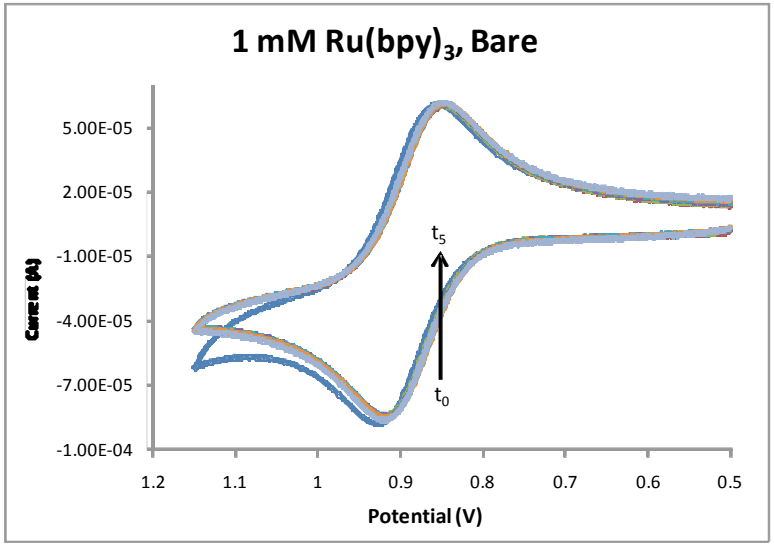

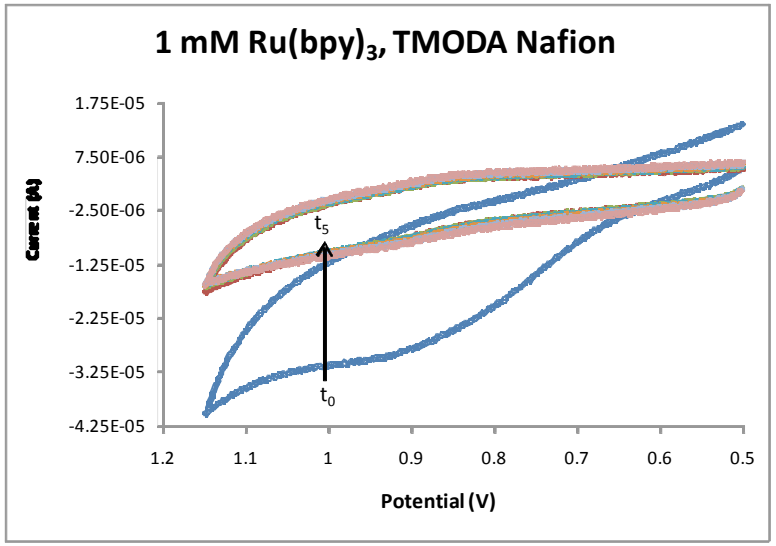

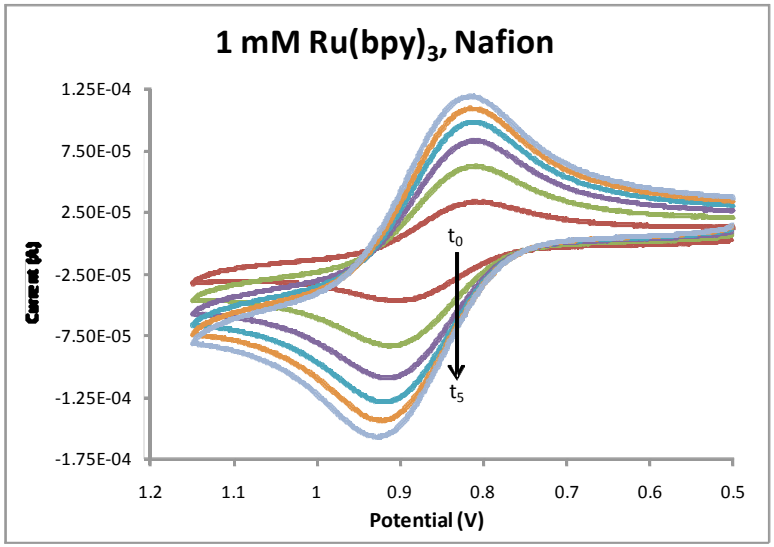

Summary

- Commercial production of NH_3 is energetically costly and environmentally taxing
- Nature has a process that produces significant amounts of NH_3 – nitrogen fixation
- *Anabaena variabilis* is a cyanobacteria capable of fixing N_2 in oxygenic environments – releasing H_2 as a byproduct
- We have cultured these cells, detected ammonia, and are optimizing parameters to build a bioelectrocatalytic device.

Future Directions

- Increase NH_3 production into the media
- Enzyme activity assay
- Heterocyst isolation
- Further polymer characterization
- Integration of heterocysts into polymer

Acknowledgments

- The project described is supported by Grant Number 08F-04 from the Iowa Energy Center
- K.T. Shanmugam (University of Florida) for providing an initial cultures of *Anabaena variabilis* and *Anabaena variabilis* SA1 variant strain
- T. Thiel (University of Missouri – St. Louis) for helpful conversations
- The Leddy Research Group
- Anna Riessen and Kaylee Lanz – Undergraduate Chemistry Majors – The University of Iowa

Options to Consider

- Biological Source
  - Cells - Different strains of cyanobacteria: *A. cylindrica*, *A. sp* – marine strain
  - Plants – symbiotic inoculation - Rhizobius legumes (clover, alfalfa, soybeans)
- Polymers
  - Different types (biocompatible interfaces), different Nafion/Chitosan modifications (chain length on modifiers, pH tolerance, solvent systems)
- Redox Mediators
  - Type, function, solution based or immobilized, concentration
  - ATP recycling system – use NAD+/NADP for active phosphorylation/dephosphorylation
  - Other coenzymes to enhance electron transfer to nitrogenase FeMoCo