



PRINCETON



PENNSYLVANIA STATE UNIVERSITY



HAWAII



Air Force Office of Scientific Research
The Basic Research Manager of the Air Force

Renewable Bio-solar Hydrogen Production from Robust Oxygenic Phototrophs

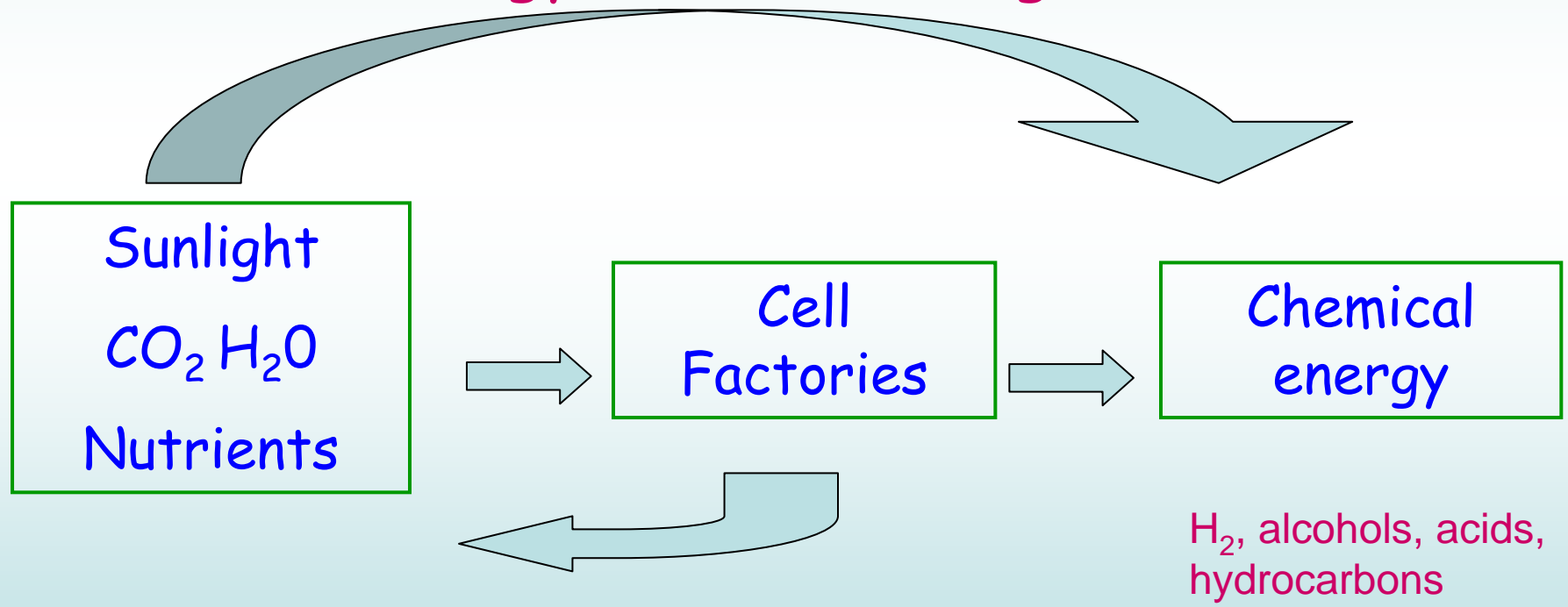
AFOSR MURI Progress Update: January 2007

-BioSolarH₂ → Team

- | | | |
|--------------------|------|--------------------------------------|
| Charles Dismukes | PU | photosyn. metabolism/chemistry |
| Donald A. Bryant | PSU | genetics/mol biology cyanos |
| Matthew Posewitz | CSM | genetics/mol biology algae |
| Eric Hegg | MiSU | enzymology/inorganic |
| Robert Bidigare UH | | microbial physiology/ecology |
| >2006 | | |
| John Peters | MoSU | crystallography & hydrogenases |
| Robert Austin | PU | microchemostats & cellular evolution |

The Approach:

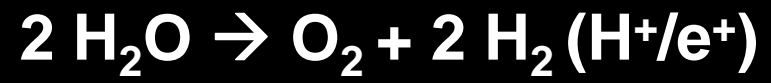
Algal & Cyanobacteria cell factories that produce chemical energy w/o sacrificing the microbe

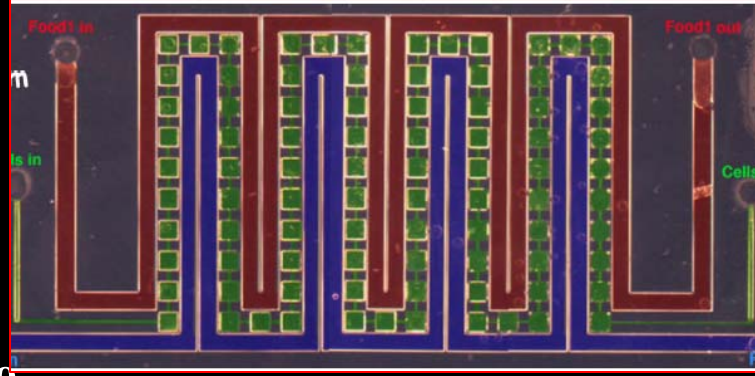
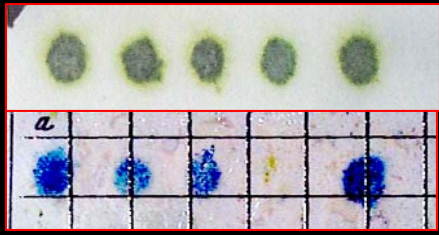


Native & GMOs that produce more energy



The Goal





**Rapid H₂ & Fluorescence
Screening**
CSM/NREL H₂ gas sensor

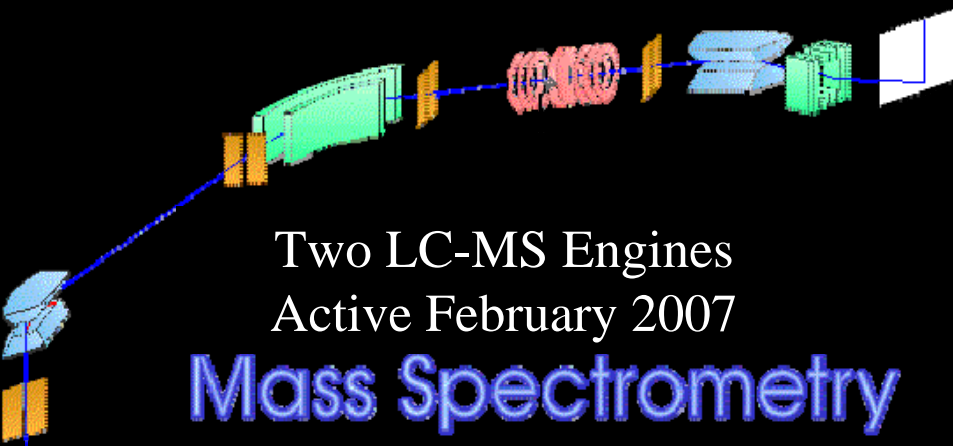
**Microarrayed chemostats
for directed evolution**

**Fast repetition rate
fluorimeter**

Instrumentation Development

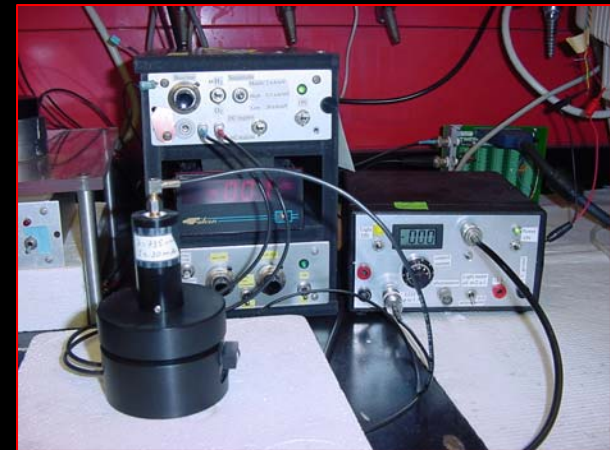
Metabolomics & Proteomics

**Dissolved H₂ & O₂
LED + Clark cells**

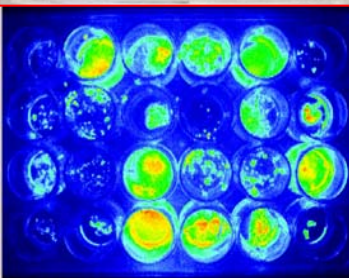
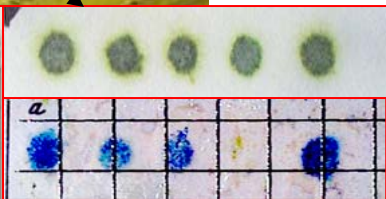
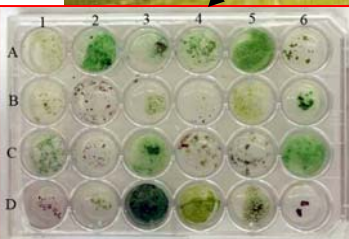
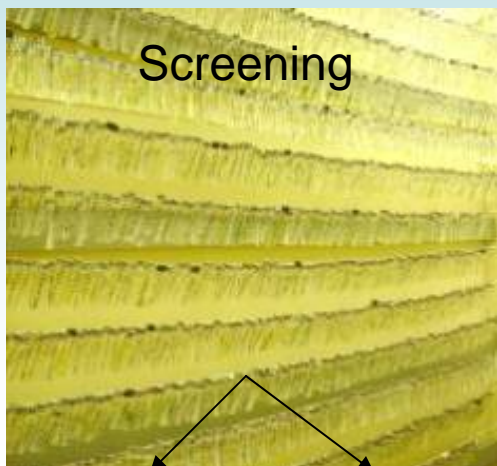


**Two LC-MS Engines
Active February 2007**

Mass Spectrometry



Sampling of Culture Collections in Progress



H₂ production

Photosynthetic
Quantum Efficiency

Marine Microbial Ecology and Diversity Center, University of Hawaii

- Patterson Collection.
 - Largest collection of cyanobacteria in the world
 - Approx. 1800 viable strains
 - Difficult to assemble a library of this scope due to sampling rights.
- NREL Collection.
 - 180 strains collected from the US southwest for NREL biodiesel program.
- Mitsui collection.
 - 165 strains marine cyanobacteria.
- Actively expanding collection
- Bioprospecting
 - Great Salt Lake & Yellowstone NP

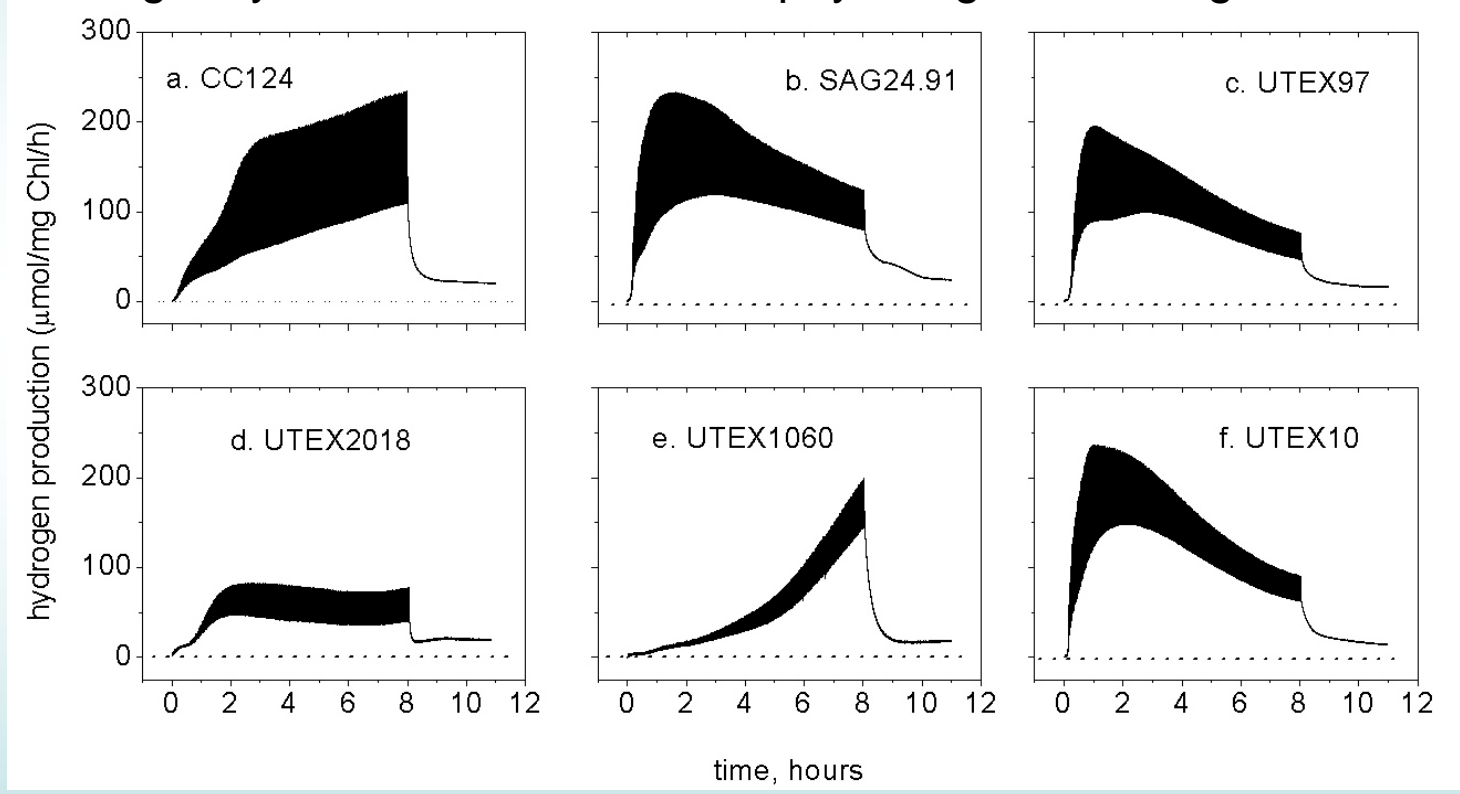
Why Screen?

Jonathan Meuser & Gennady Ananyev

Screening has revealed major differences in photo-H₂ production and rates of anaerobic induction between ecologically distinct strains of monophyletic genera of algae



Microalga
Chlamydomonas



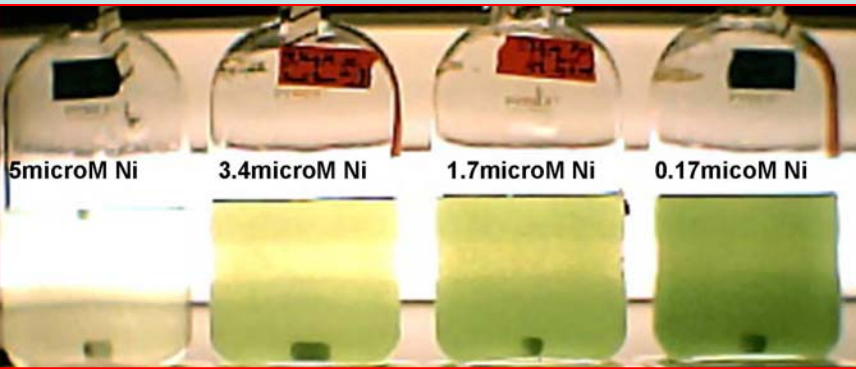
The time profiles reveal the kinetics of induction of photo-H₂ production capacity that occurs following initiation of anaerobiosis at time zero of cultures grown on light photosynthetically. Six strains of *Chlamydomonas*

Micronutrients, growth & bioreactor optimization has produced large improvements in H₂ production

1. Sufficiency of Nickel during growth **17X H₂ increase**
2. Duration of prior photoautotrophic growth. **oldies but goodies**
3. Anaerobicity & darkness during fermentation. **strong respiring strains**
4. Selection of salt-tolerant strain. **synthesis of fermentable sugars**
5. Mechanical agitation used for photoautotrophic growth. **minimize shearing**
6. Higher fermentation temperature. **2x (23→30 C)**
7. Lower light intensity 3x after attaining steady-state. **Fermentation adaptation**

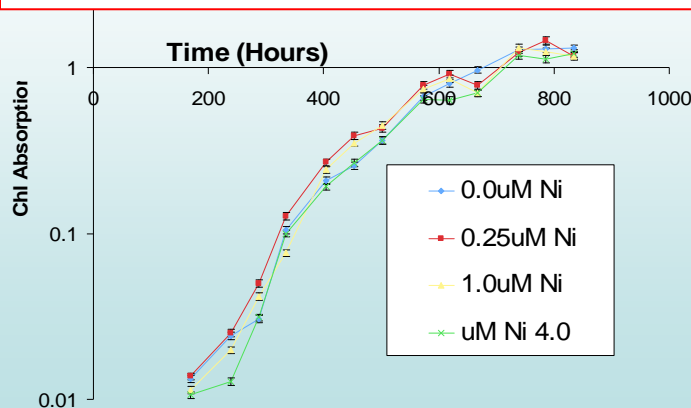
Ni²⁺ Supplementation on Growth & H₂ Production by *A. Maxima*

Damian Carrieri & Gennady Ananyev

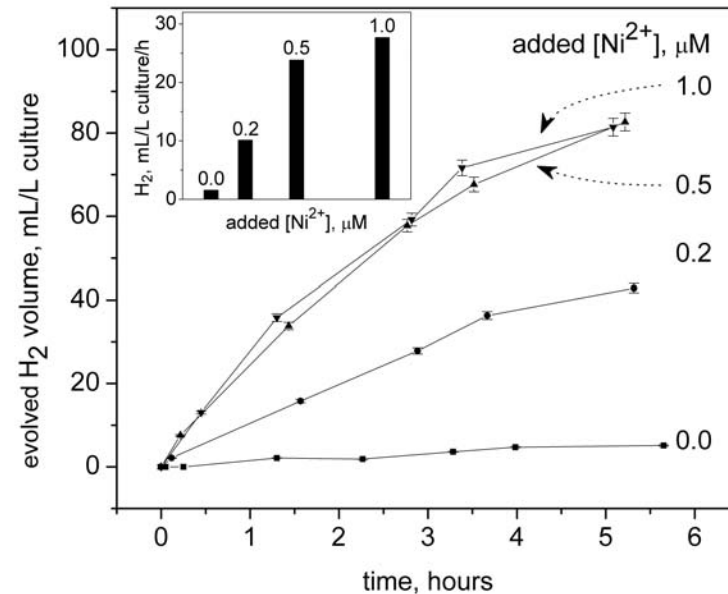


A. [Ni²⁺] causes chlorosis during initial stage of photoautotrophic growth (130 μE/m²sec).

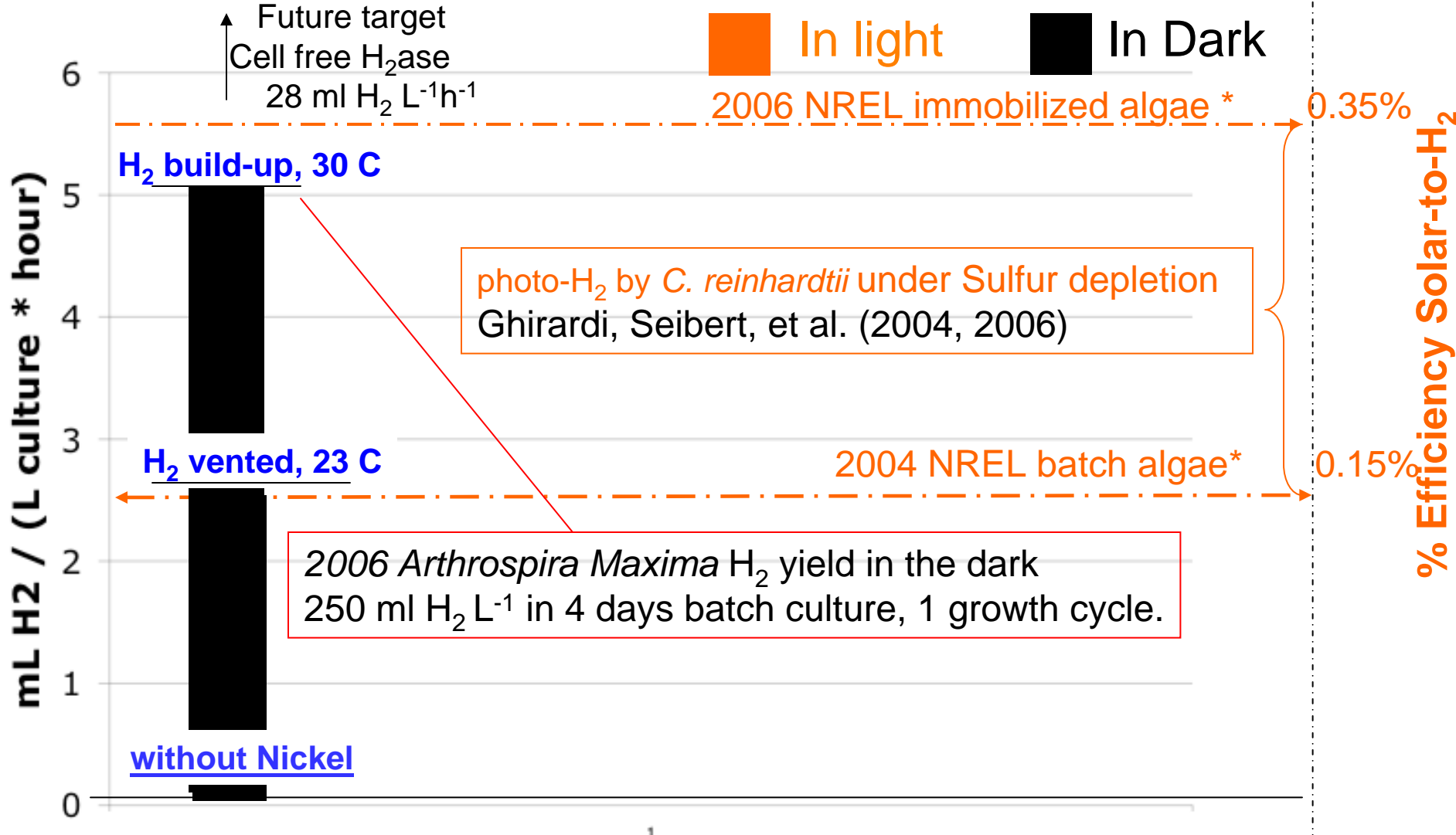
B. After chlorosis induced lag, cells recover & grow photoautotrophically to normal cell density. NO Effect.



C. Ni²⁺ stimulates cell's capacity to evolve hydrogen by **20 fold!**



2006 rates & yields of H₂ by cyanobacteria & algal strains

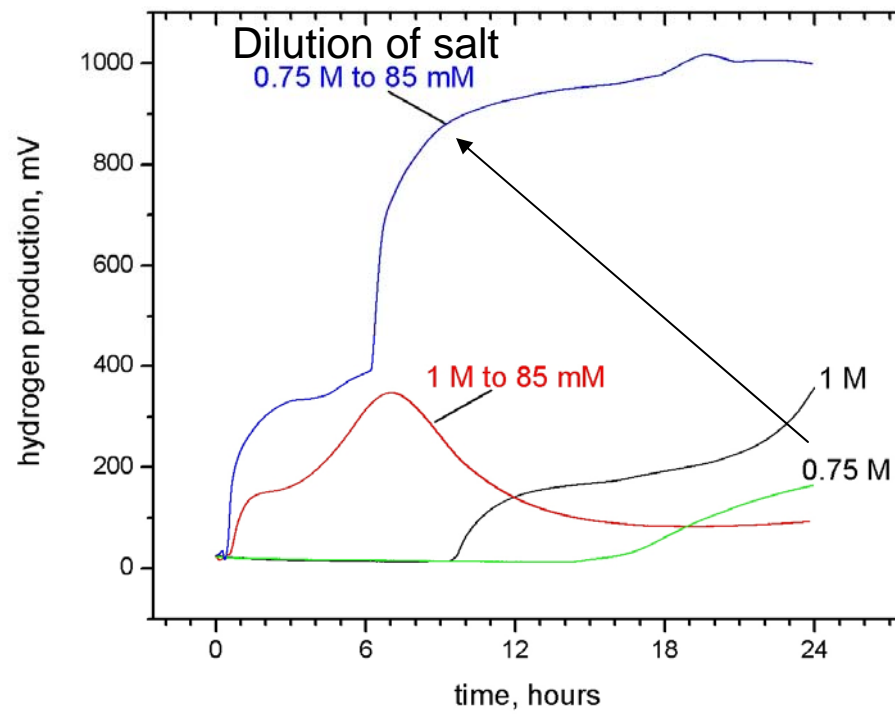


Osmotic Stress by Dilution of Growth Medium Boosts H₂ Yield by 18 X!

Arthrospira maxima acclimated to NaCl



250 mM Carbonate medium + 250 mM NaCl +500 mM NaCl +750 mM NaCl



Arthrospira exhibits large increases in fermentative H₂ production by application of stresses
or
by carbon supplementation :

1. Osmotic stress of salt-tolerant strains. **18X increase**
2. Nitrate removal during fermentation (if sole N source). > **5-20X**
3. Add fermentable carbon substrates. > **10X with glucose**

These results with the Princeton microbioreactor indicate promising Applications for larger scale fermentation trials in progress

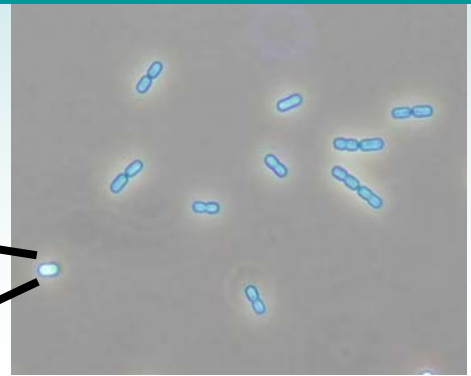
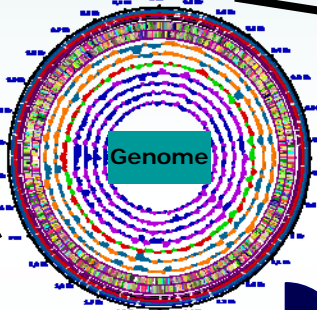
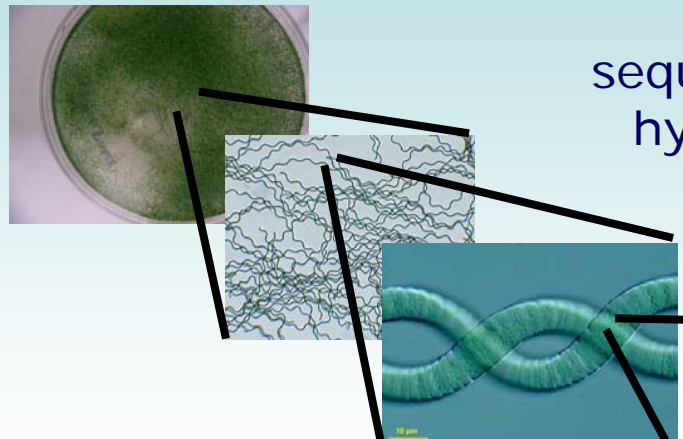
Genetic Opportunities Ahead

- Develop a genetic system for *Arthrospira* sp.
- Determine the optimal conditions for H₂ase expression in cyanobacteria
- Over-express H₂ase(s) in cyanobacteria
- Introduce foreign H₂ase(s) into cyanobacteria
- [NiFe]-hydrogenase engineering of NAD vs NADP selectivity.
- [NiFe]-hydrogenase gene shuffling
- Engineer cyanobacterial strains with reduced amounts of light-harvesting antenna
- Use comparative genomics, molecular genetics, and strain engineering to optimize H₂ production



Obtain genome sequences from robust, hydrogen-producing oxyphototrophs

Synechococcus sp. PCC 7002



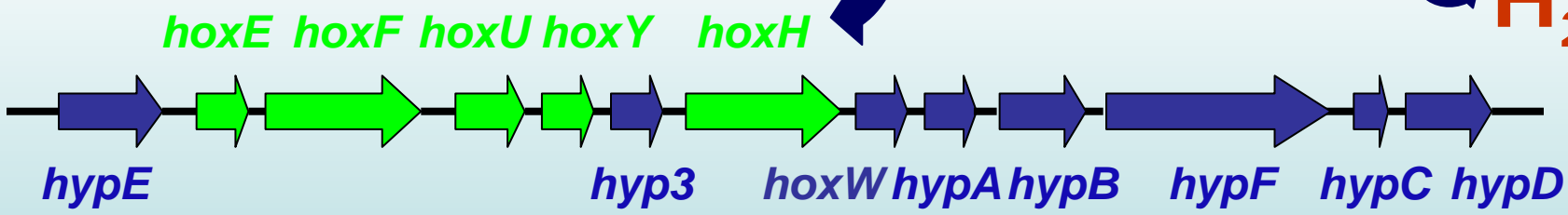
Arthrospira maxima

Develop genetics in this excellent H₂-producer; genome will be sequenced in 2007 by JGI-DOE

Apply "-omics"
Transcriptomics
Proteomics
Metabolomics

Metabolic Engineering

H₂

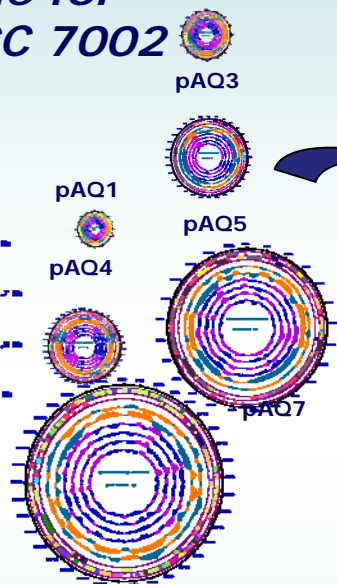
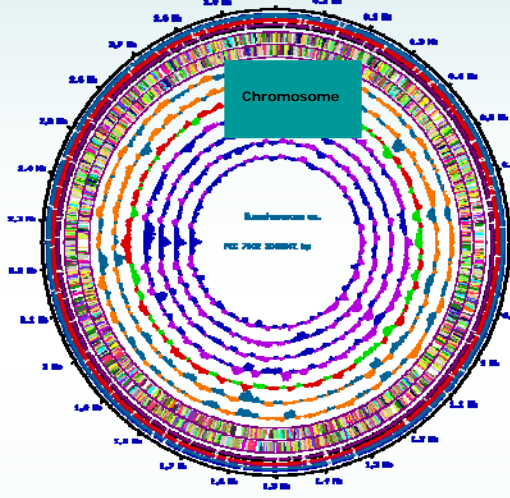


In *Synechococcus* sp. PCC 7002, the genes encoding the Ni-Fe hydrogenase uniquely form an operon of 13 genes



Improving H₂ production with proven genetics tools

Full genome available for *Synechococcus* sp. PCC 7002



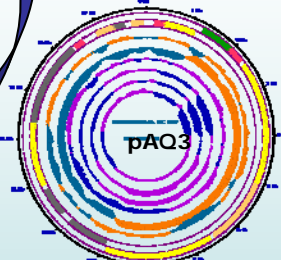
Identify, and then delete or inactivate, genes for pathways that compete for electrons with H₂ase; enhance glycogen storage

LIGHT
grow

Synechococcus sp. PCC 7002 strains for enhanced H₂ production

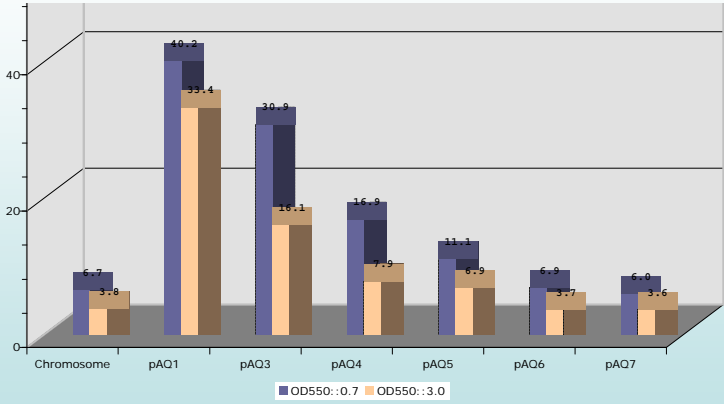
Over-express genes for modified hydrogenases

ferment H₂



Insert hydrogenase structural genes in pAQ1

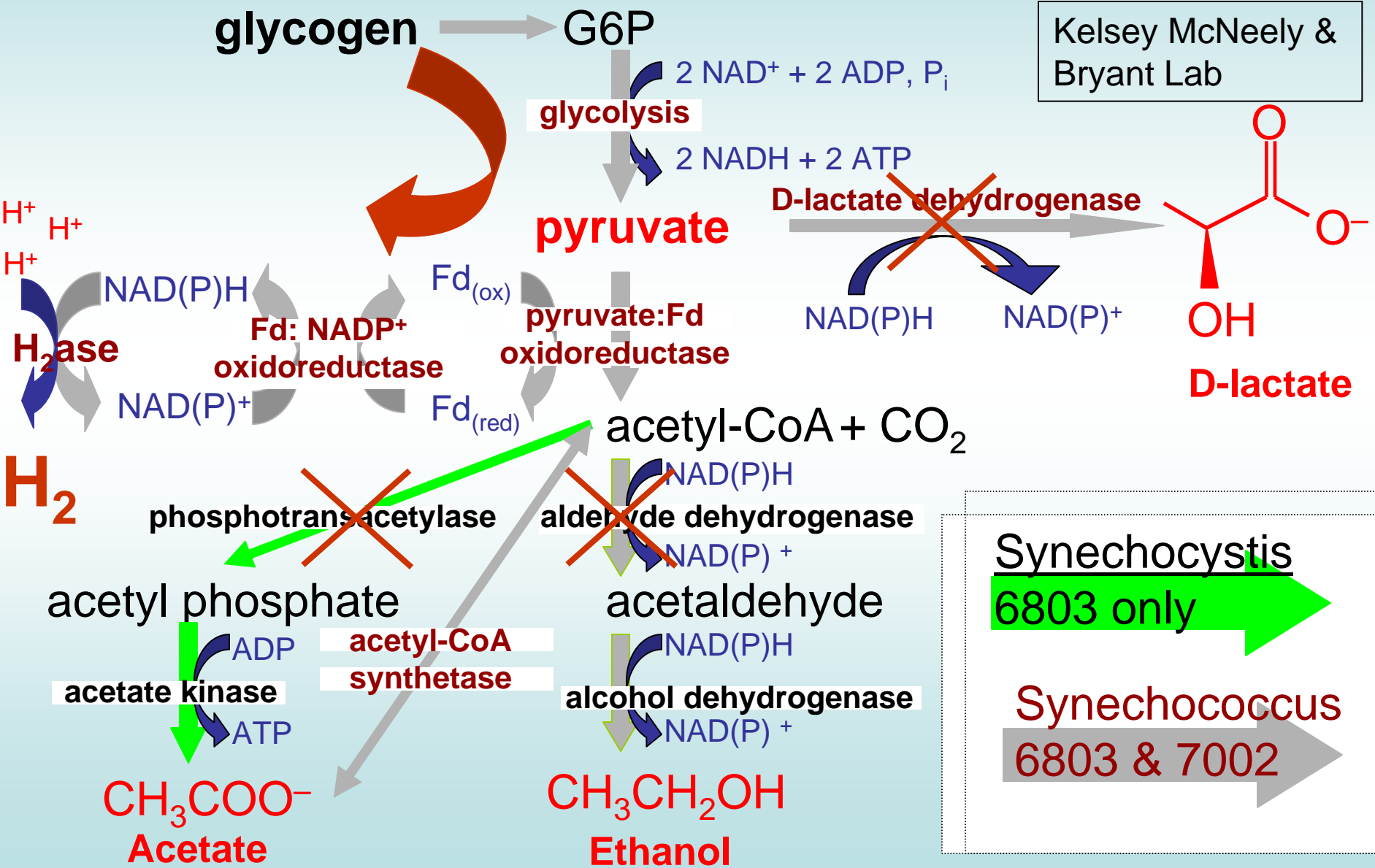
Insert hydrogenase accessory genes in pAQ3



Actual copy numbers for chromosome and plasmids in *Synechococcus* sp. PCC 7002 cells in mid-exponential and early stationary phase



3 Fermentative Gene Knockout Targets based on Genome Sequences

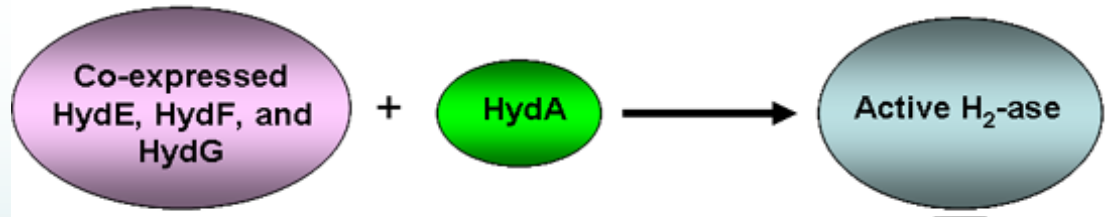
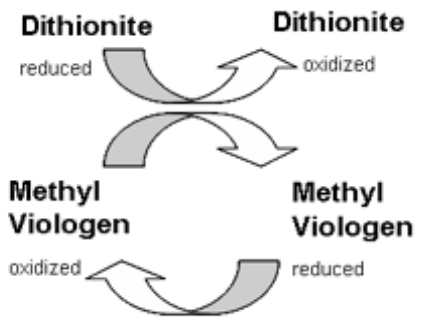
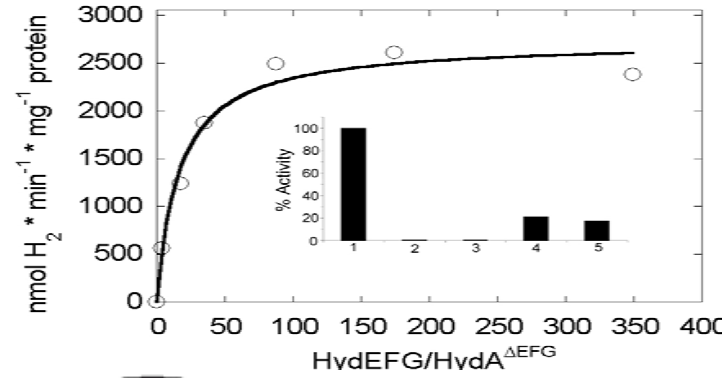
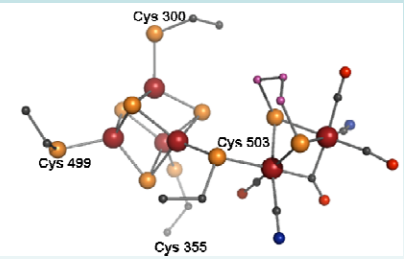


In vitro Hydrogenase activation: a robust platform to investigate hydrogenase maturation

• *In vitro* activation of heterologously expressed hydrogenase structural protein HydA (HydA^{ΔEFG}) by the addition of co-expressed accessory proteins HydE, HydF and HydG.

• A robust system amenable to in depth study and characterization of hydrogenase maturation.

The hydrogenase “H cluster”



- Reaction Sequence:**
1. Hyd protein extracts are mixed
 2. Dithionite added
 3. Methyl viologen added
 4. Hydrogenase (H₂-ase) activity assayed by G.C.



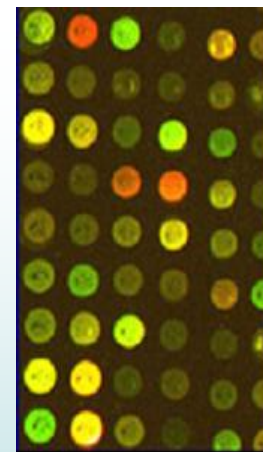
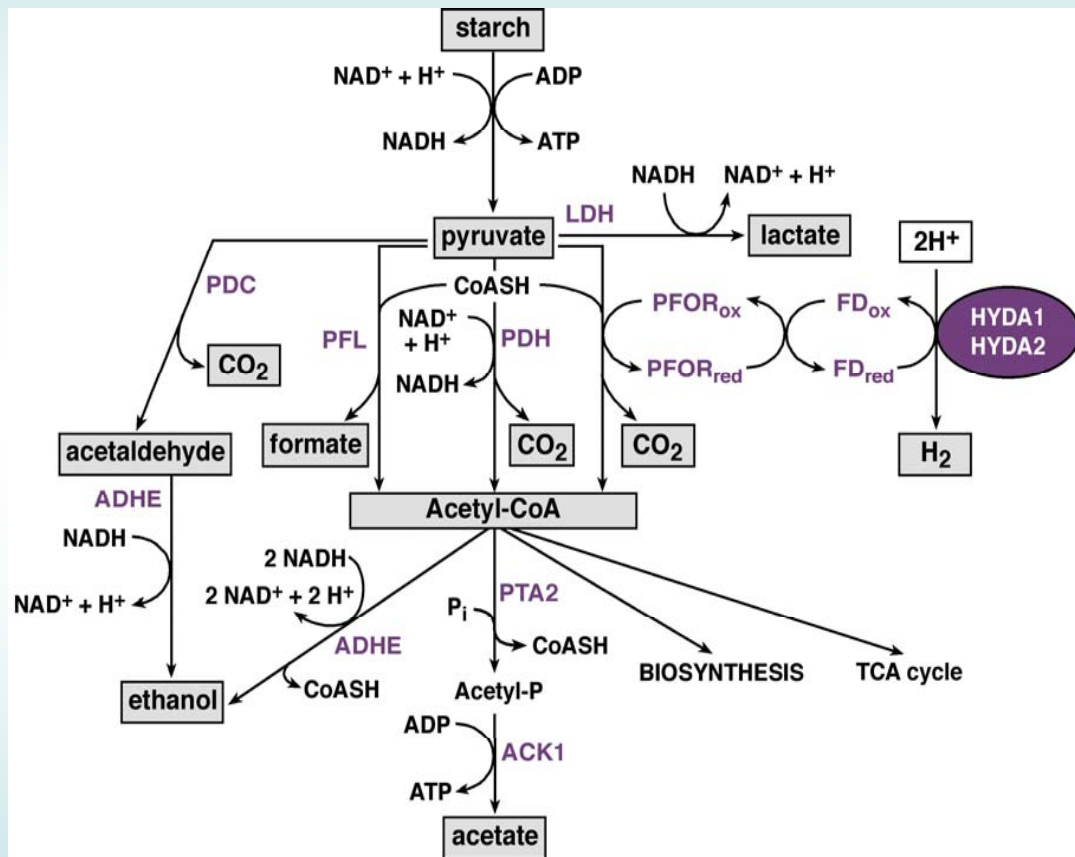
In vitro activation is the first step in the characterization of the biochemical reactions involved in “H Cluster” biosynthesis and hydrogenase maturation. These studies are critical to the effective genetic engineering of organisms expressing [FeFe] hydrogenase

Analysis of *C. reinhardtii* transcriptome under H₂ producing conditions

- Levels of over 500 transcripts change significantly.

- Several hundred are of unknown function.

- Novel targets potentially influencing hydrogenase activity have been identified.



Analysis of the transcriptome during H₂ production is essential to understand hydrogenase activity in the context of whole-cell metabolism. Pathways of electron transfer are being analyzed and targets to enhance H₂ production identified.



Application of gene-shuffling for the rapid generation of novel [FeFe]-

- Gene shuffling protocol was identified, optimized and used to rapidly generate libraries of unique [FeFe]-hydrogenases

- Generates a high percentage of active enzymes in *E. coli*

- User friendly and requires a single set of maturases

