PRINCETON PENNSTATE HAWAII

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

Renewable Bio-solar Hydrogen Production from Robust Oxygenic Phototrophs

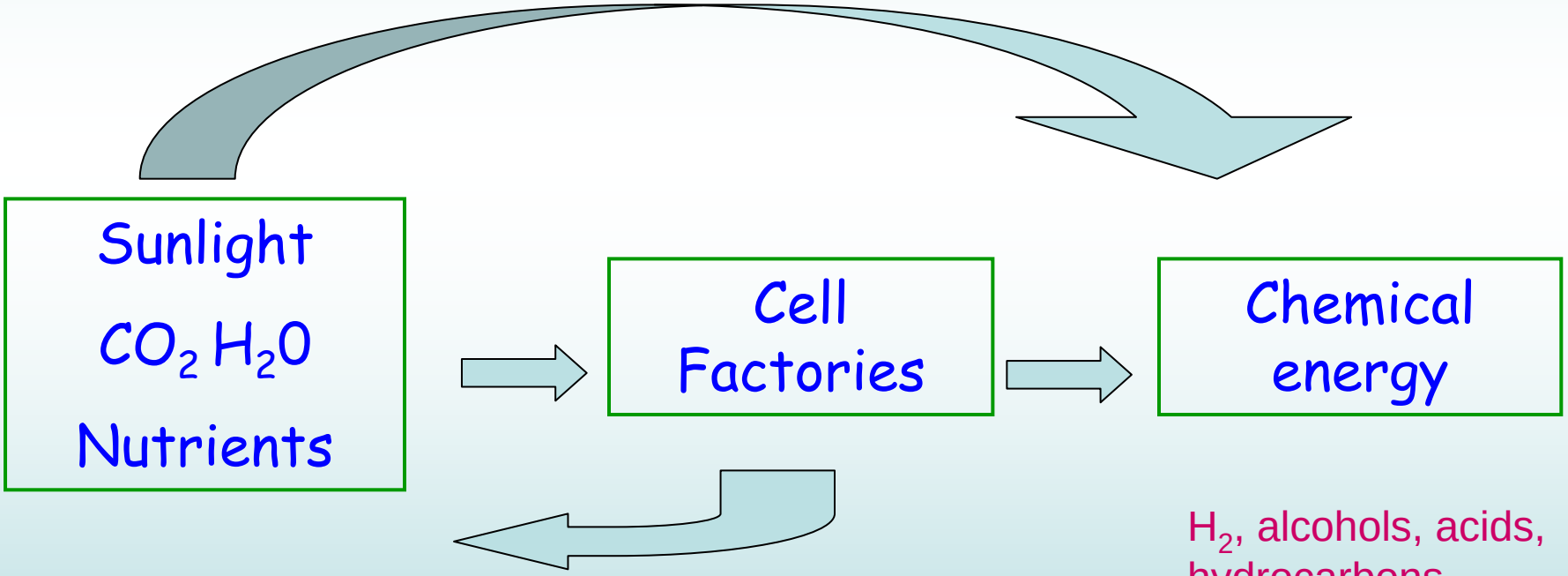
AFOSR MURI Progress Update: January 2007

-BioSolarH_2→ 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 >2006		microbial physiology/ecology
John Peters	MoSU	crystallography & hydrogenases
Robert Austin	PU	microchemostats & cellular evolution

http://www.princeton.edu/~biosolar/

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



Native & GMOs that produce more energy

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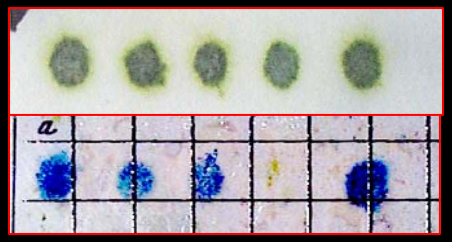
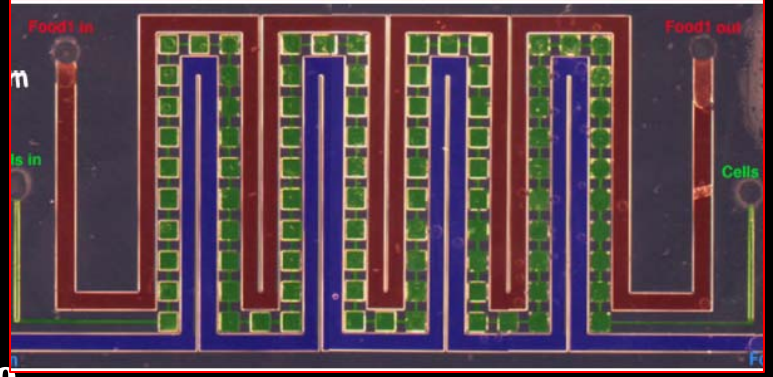
The Goal

$$2\ H_2O \rightarrow O_2 + 2\ H_2\ (H^+/e^+)$$



Rapid H_2 & Fluorescence Screening
CSM/NREL H_2 gas sensor



Microarrayed chemostats for directed evolution

Fast repetition rate fluorimeter

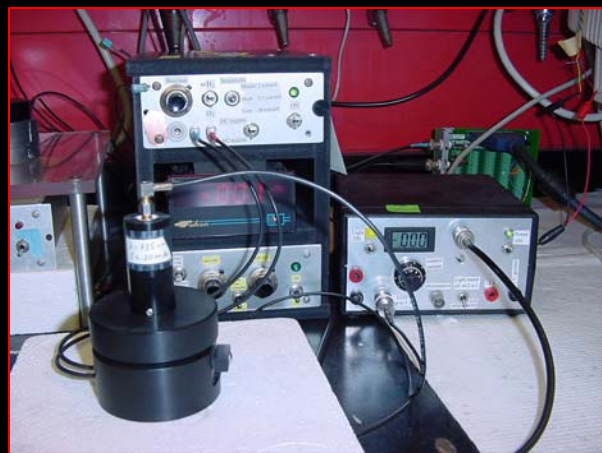
Instrumentation Development

Metabolomics & Proteomics

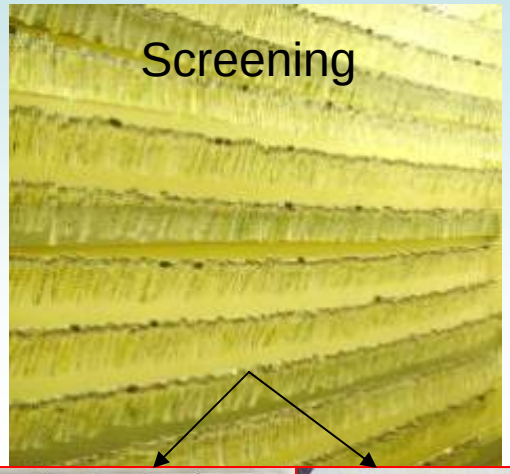
Two LC-MS Engines
Active February 2007

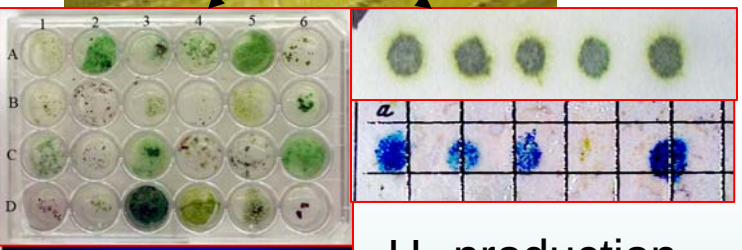
Mass Spectrometry

Dissolved H_2 & O_2
LED + Clark cells

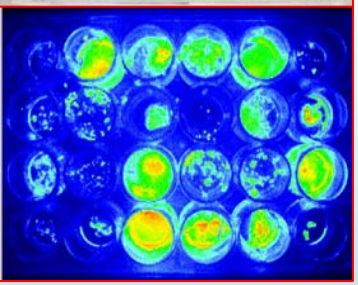


Sampling of Culture Collections in Progress





H_2 production



Photosynthetic Quantum Efficiency

Marine Microbial Ecology and Diversity Center, University of Hawaii

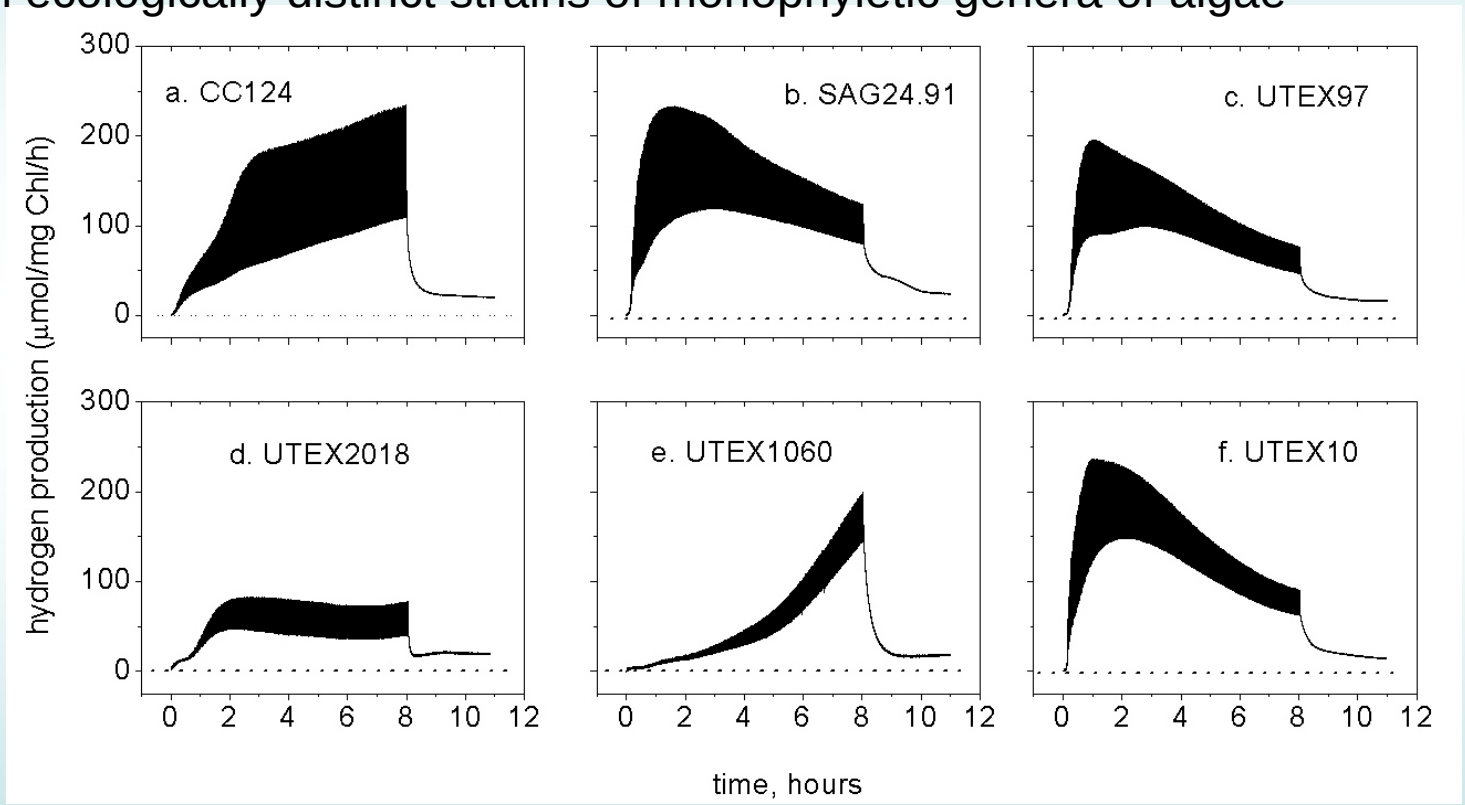
- Patterson Collection.
  - Largest collection of cyanobacteria in the world
  - Approx. 1800 viable strains
  - Difficult to assemble a library of this scope due to sampling rights.
- NREL Collection.
  - 180 strains collected from the US southwest for NREL biodiesel program.
- Mitsui collection.
  - 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-H_2 production and rates of anaerobic induction between ecologically distinct strains of monophyletic genera of algae

Microalga
Chlamydomonas



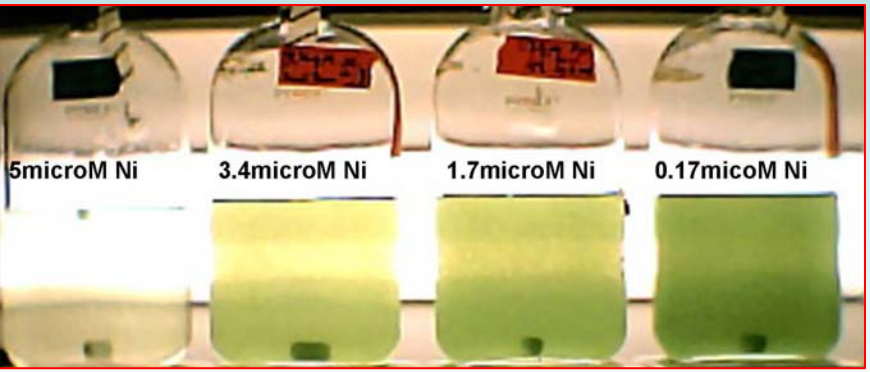
The time profiles reveal the kinetics of induction of photo-H_2 production capacity that occurs following initiation of anaerobisis at time zero of cultures grown on light photosynthetically. Six strains of *Chlamydomonas*

Micronutrients, growth & bioreactor optimization has produced large improvements in H_2 production

1. Sufficiency of Nickel during growth **17X H_2 increase**
2. Duration of prior photoautotrophic growth. **oldies but goodies**
3. Anaerobicity & darkness during fermentation. **strong respiring strains**
4. Selection of salt-tolerant strain. s**ynthesis 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**

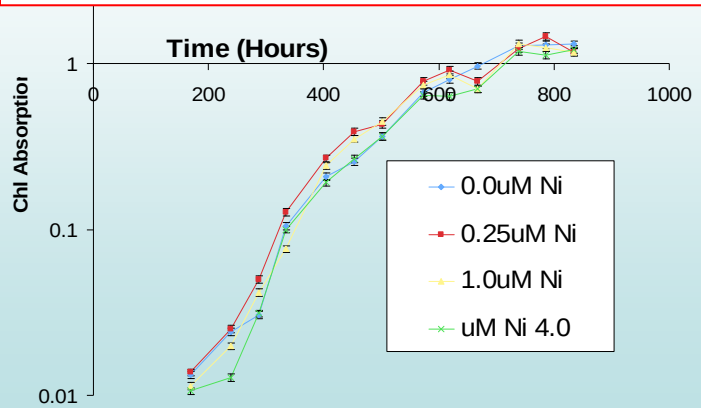
Ni^{2+} Supplementation on Growth & H_2 Production by *A. Maxima*

Damian Carrieri & Gennady Ananyev

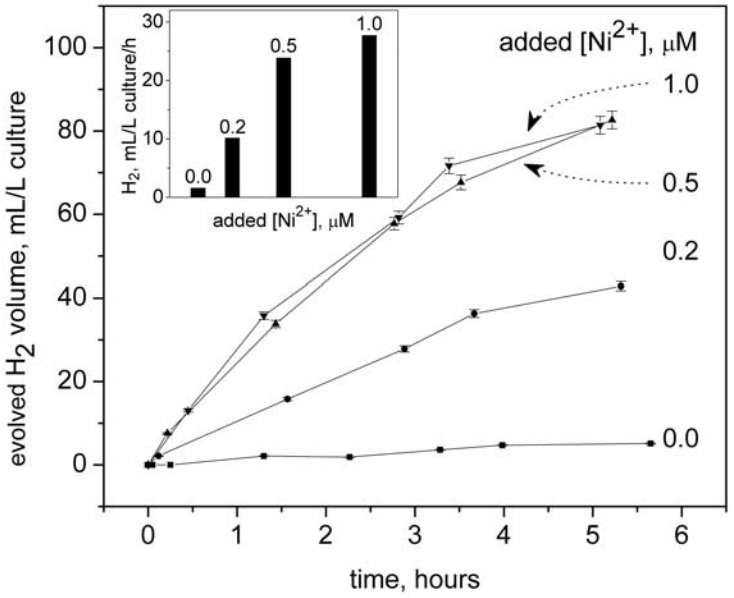


A. [Ni^{2+}] causes chlorosis during initial stage of photoautotrophic growth (130µE/m^2sec).

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

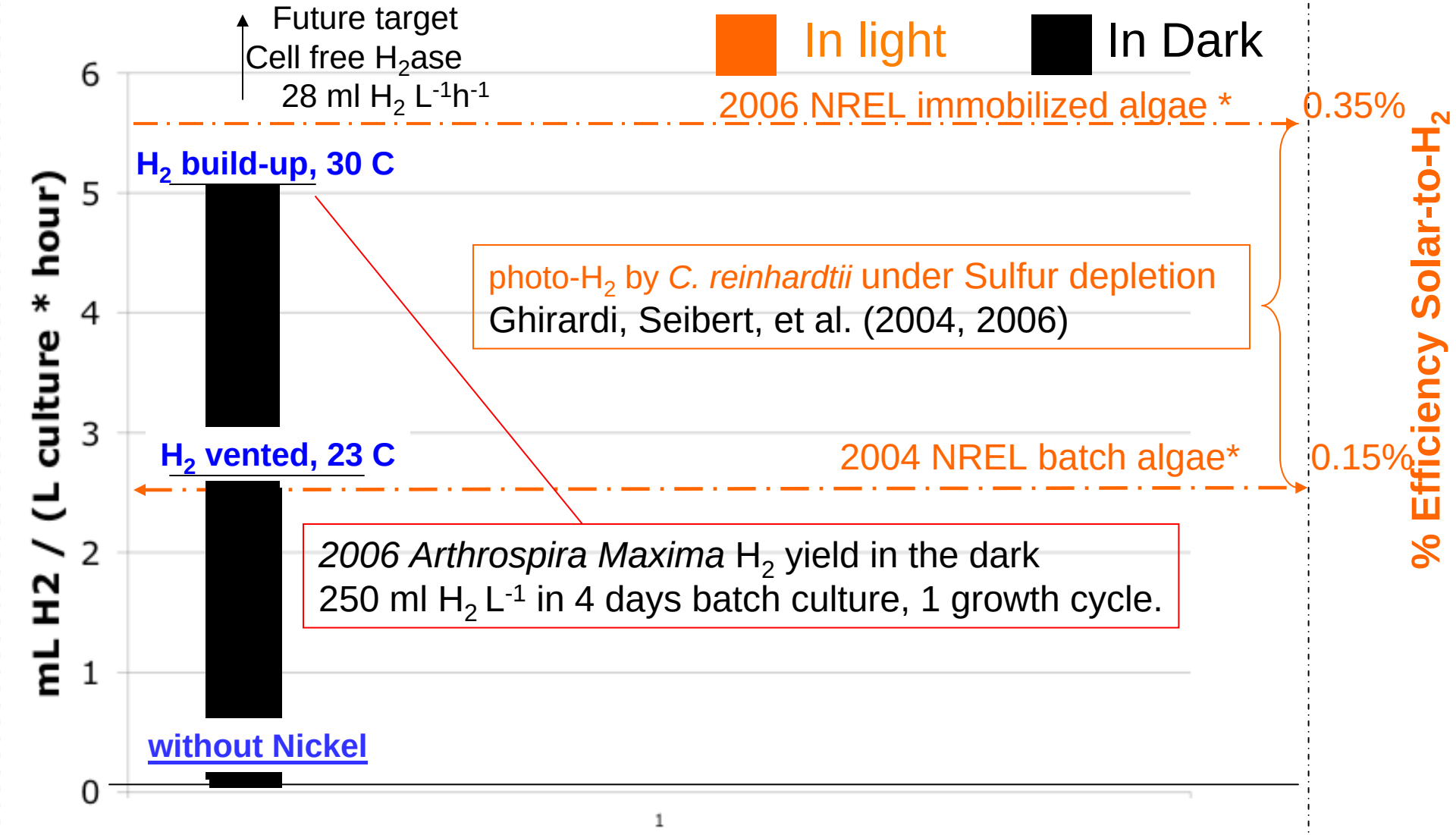


C. Ni^{2+} stimulates cell's capacity to evolve hydrogen by **20 fold!**



$MV_{(reduced)} + 2H^+_{(aq)} \longrightarrow MV_{(oxidized)} + H_2$

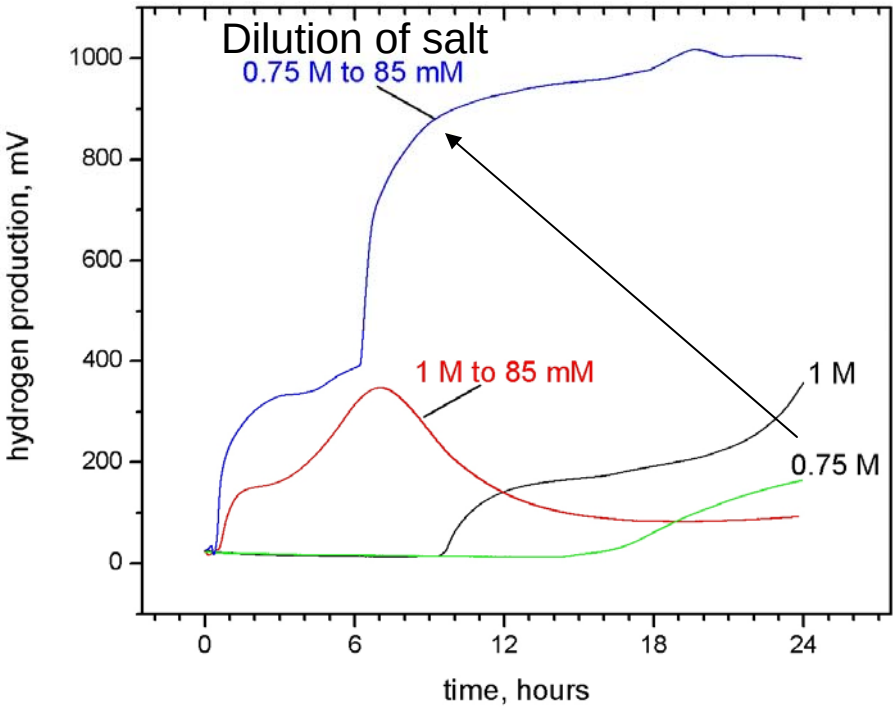
2006 rates & yields of H_2 by cyanobacteria & algal strains



Osmotic Stress by Dilution of Growth Medium Boosts H_2 Yield by 18 X!

Arthrospira maxima acclimated to NaCl

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



Arthrospira exhibits large increases in fermentative H_2 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**

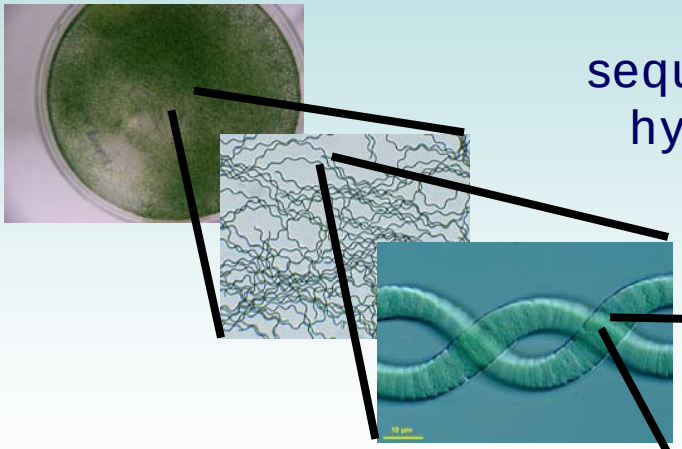
These results with the Princeton microbioreactor indicate promising Applications for larger scale fermentation trials in progress

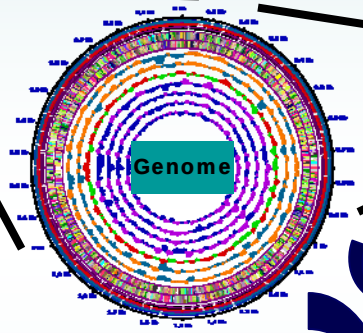
Genetic Opportunities Ahead

- Develop a genetic system for *Arthrospira* sp.
- Determine the optimal conditions for H_2ase expression in cyanobacteria
- Over-express H_2ase(s) in cyanobacteria
- Introduce foreign H_2ase(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 H_2 production

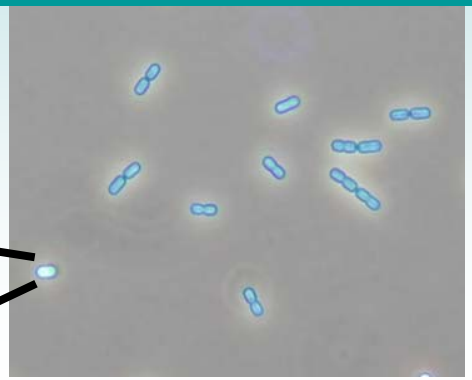
-BioSolarH_2→

Obtain genome sequences from robust, hydrogen-producing oxyphototrophs





Synechococcus sp. PCC 7002



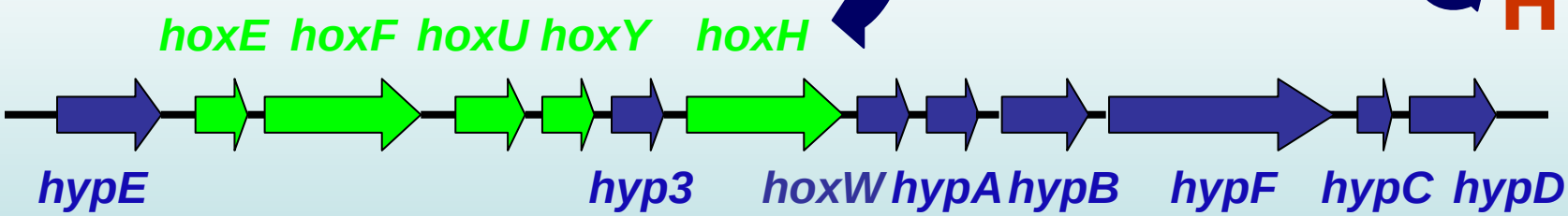
Arthrospira maxima

Develop genetics in this excellent H_2-producer; genome will be sequenced in 2007 by JGI-DOE

Apply "-omics"
Transcriptomics
Proteomics
Metabolomics

Metabolic Engineering

H_2

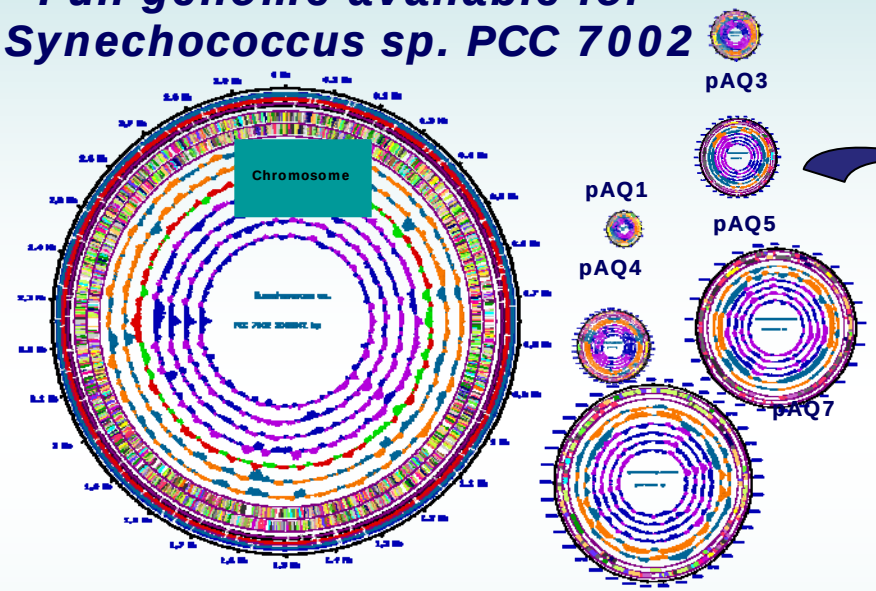


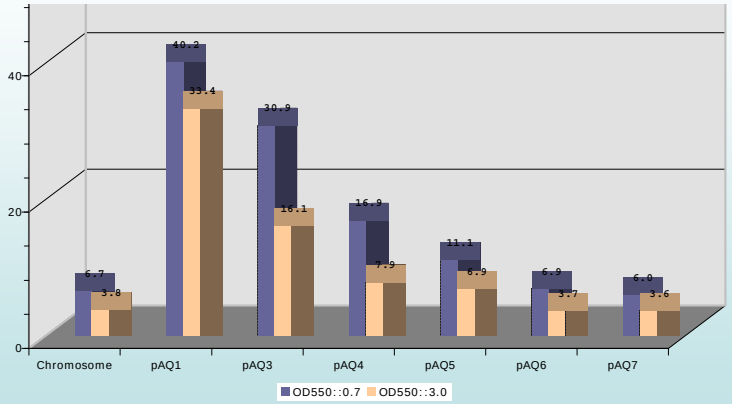
In *Synechococcus* sp. PCC 7002, the genes encoding the Ni-Fe hydrogenase uniquely form an operon of 13 genes

-BioSolarH_2→

Improving H_2 production with proven genetics tools

Full genome available for Synechococcus sp. PCC 7002





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

Identify, and then delete or inactivate, genes for pathways that compete for electrons with H_2ase; enhance glycogen storage

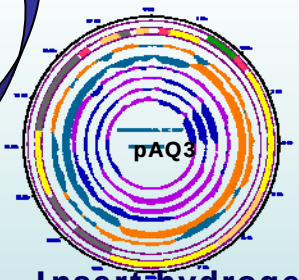
LIGHT

grow

***Synechococcus* sp. PCC 7002 strains for enhanced H_2 production**

Over-express genes for modified hydrogenases

ferment H_2

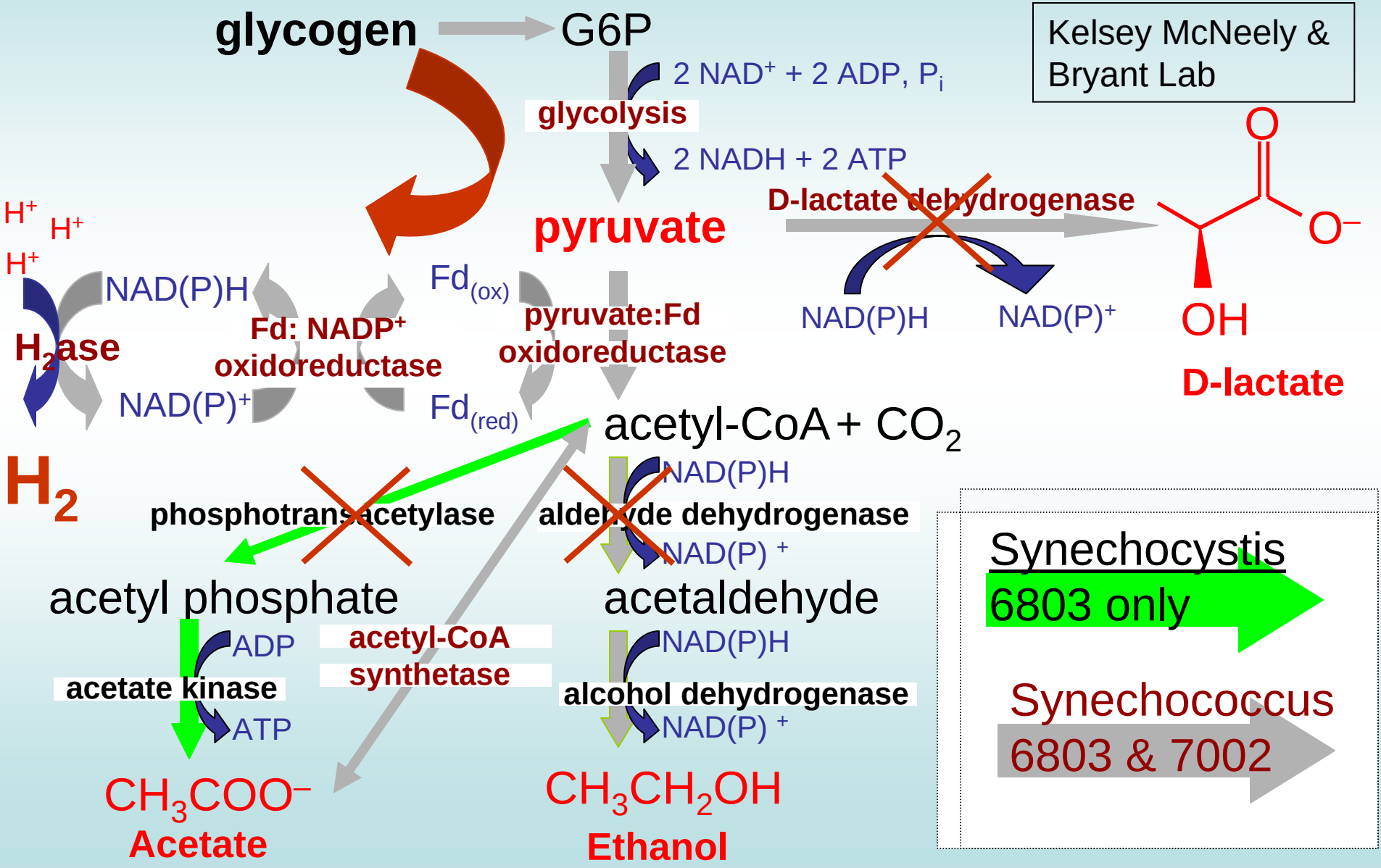


Insert hydrogenase structural genes in pAQ1

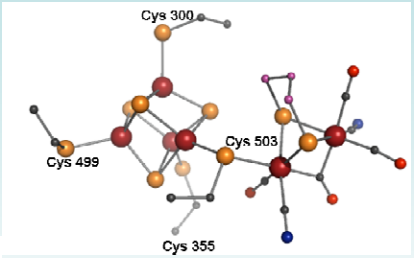
Insert hydrogenase accessory genes in pAQ3

3 Fermentative Gene Knockout Targets based on Genome Sequences

Kelsey McNeely &
Bryant Lab

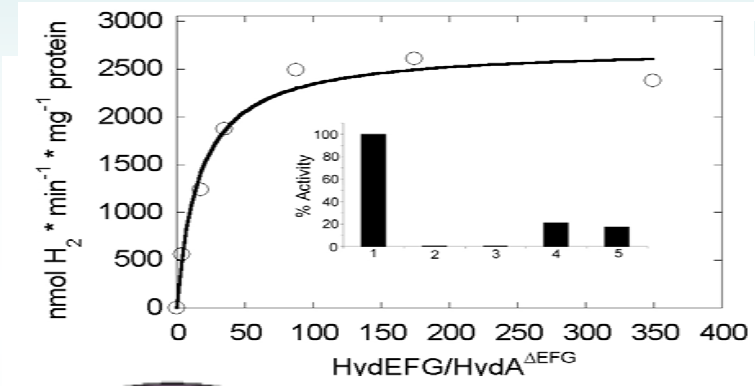


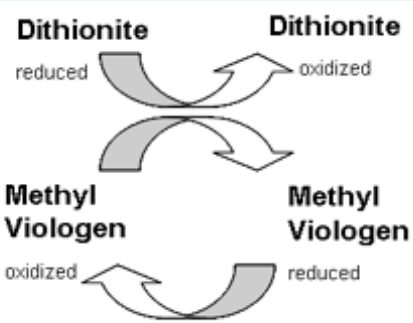
In vitro Hydrogenase activation: a robust platform to investigate hydrogenase maturation

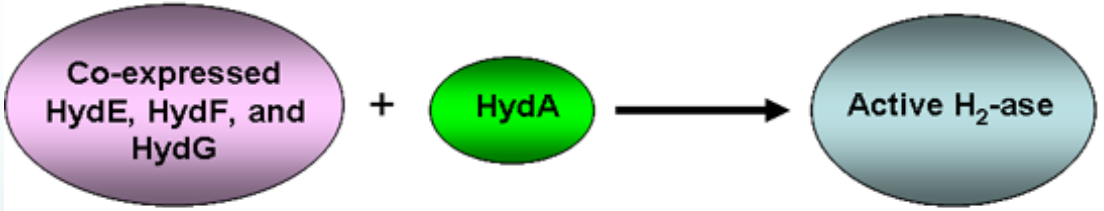


The hydrogenase "H cluster"

- *In vitro* activation of heterologously expressed hydrogenase structural protein HydA (HydA$^{\Delta EFG}$) 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.







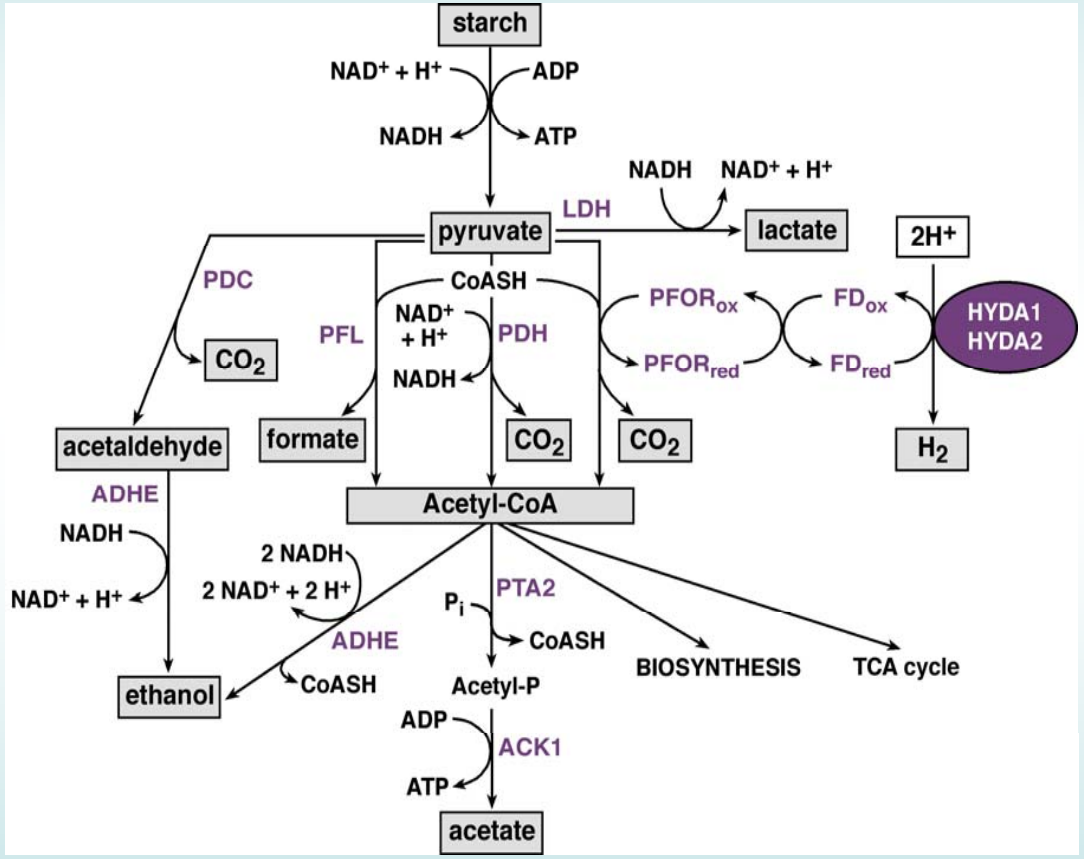
Reaction Sequence:

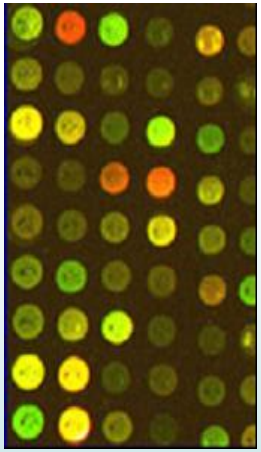
1. Hyd protein extracts are mixed
2. Dithionite added
3. Methyl viologen addd
4. Hydrogenase (H_2-ase) activity assayed by G.C.

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 H_2 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 H_2 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 H_2 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