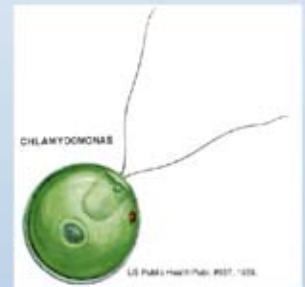


Biological Systems for Hydrogen Photoproduction

2004 DOE Hydrogen, Fuel Cells &
Infrastructure Technologies Program Review



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National Renewable Energy Laboratory
May 24, 2004

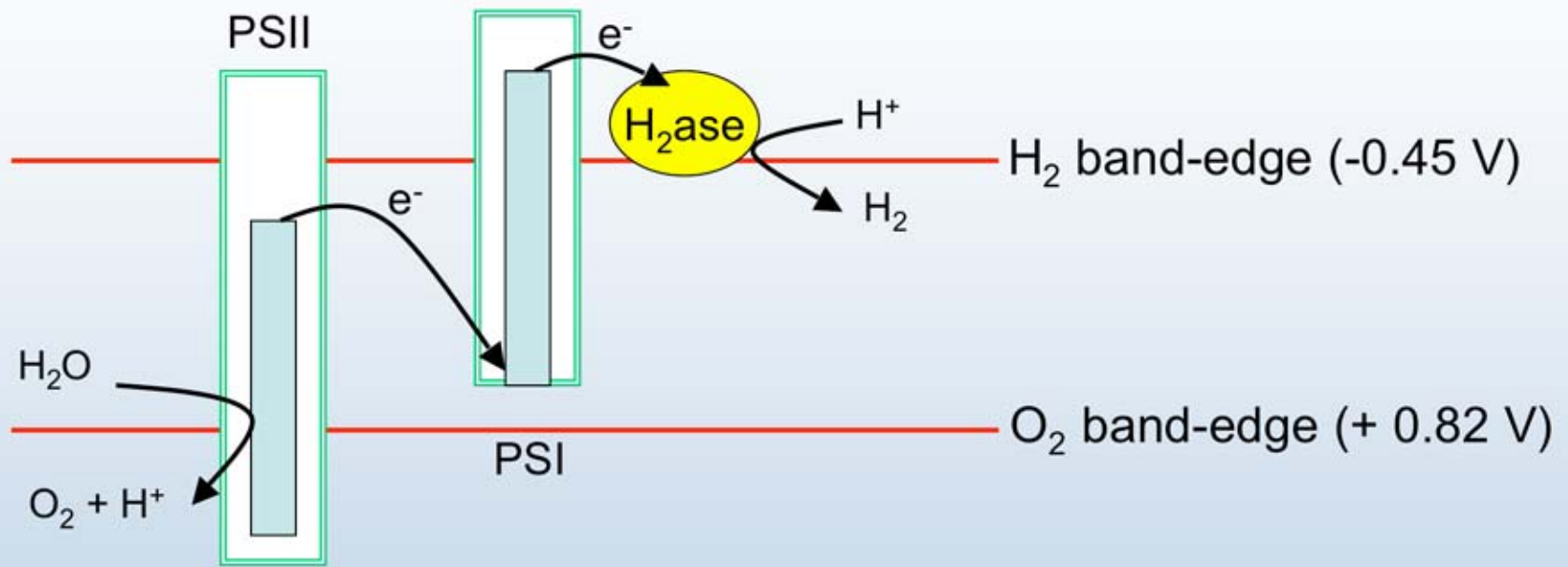
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Project Goal and Budget

- Goal
 - Develop photolytic H₂-production technologies based on microbial H₂O-splitting processes.
- Budget
 - Total Funding in FY04: \$710 K (3 tasks)
 - NREL's Share: \$690 K
 - Subcontractor's Share: \$20 K



Problem to be Addressed and Approaches



Subtask 1. Engineer an algal hydrogenase that is resistant to O₂ inactivation;

Subtask 2. Develop and optimize a physiological method to promote culture anaerobiosis and subsequent H₂-production activity in algae;

Subtask 3. Introduce a bacterial hydrogenase with increased O₂ resistance into a water-splitting photosynthetic cyanobacterial system.

Objectives for FY04

- Identify additional amino acid residues in the algal HydA1 hydrogenase as targets for site-directed mutagenesis aimed at increasing its resistance to O_2 ;
- Extend H_2 production in the continuous system by adjusting algal culture parameters; improve the efficiency of H_2 photoproduction using immobilized algal cultures;
- Initiate studies for expression of the O_2 -resistant *Rubrivivax gelatinosus* bacterial hydrogenase in a photosynthetic cyanobacterium.

Technical Barriers and Targets

Photobiological Hydrogen Generation Barriers:

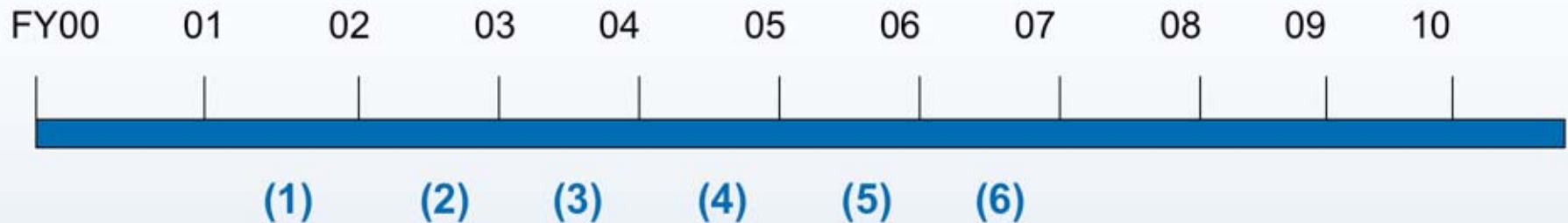
- **K. Continuity of Photoproduction**

DOE Technical Targets for Photobiological H₂ Production:

Parameters	2005	2010
Utilization efficiency of absorbed light	10%	20%
Conversion efficiency of absorbed light to H ₂	0.5%	5%
Duration of continuous H ₂ photoproduction	500 h	1500 h
Cost of low pressure H ₂ at plant gate	\$100/kg	\$30/kg

Meeting the technical targets involves a collaboration between NREL, UCB and ORNL to address the 3 barriers identified by DOE (I, J and K).

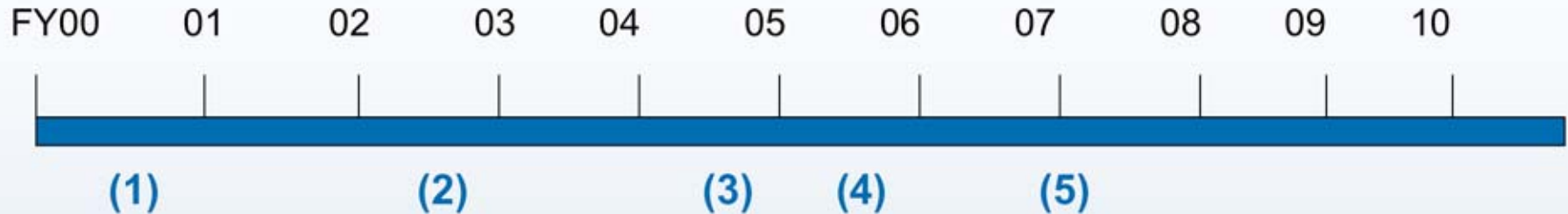
Project Timeline



Subtask 1

- (1) Cloned and sequenced the algal hydrogenase *HydA1* gene;
- (2) Discovered and sequenced a second algal hydrogenase, HydA2;
- (3) Generated the first algal mutant with improved O₂ resistance;
- (4) Published a landmark article on the expression of active algal hydrogenase in the bacterium *E. coli* (joint effort with an Office of Science project); completed simulations of O₂ diffusion in an Fe-hydrogenase and identified targets for mutagenesis;
- (5) Will perform simulations of H₂ diffusion in an Fe-hydrogenase;
- (6) Will generate and test additional hydrogenase mutants for improved O₂-resistance.

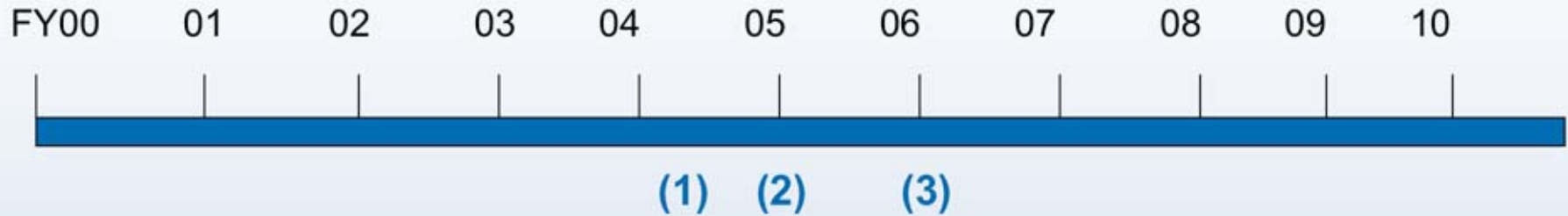
Project Timeline



Subtask 2

- (1) Demonstrated 96 hours of H₂-production per batch operation cycle (with UCB);
- (2) Maintained continuous H₂-production for 240 h at sustained rates;
- (3) Achieved 6 months of continuous H₂-production at variable rates (*exceeding the 2010 technical target for duration of production*); immobilized sulfur-deprived algal cells for batch H₂ production;
- (4) Will determine the feasibility of immobilized algal cells for cyclic H₂-production;
- (5) Will develop a suspension or immobilized system to produce H₂ continuously at sustained and optimized rates.

Project Timeline



Subtask 3

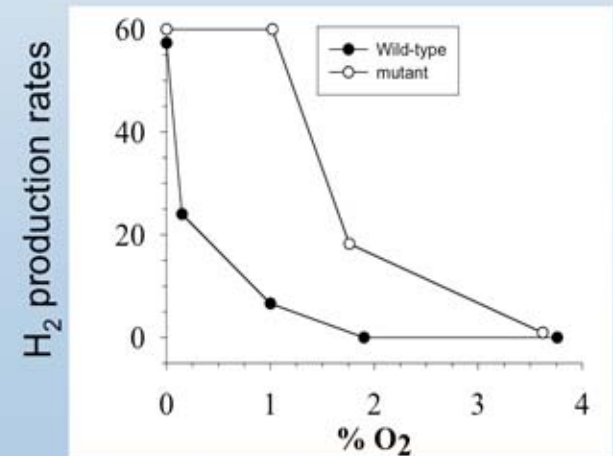
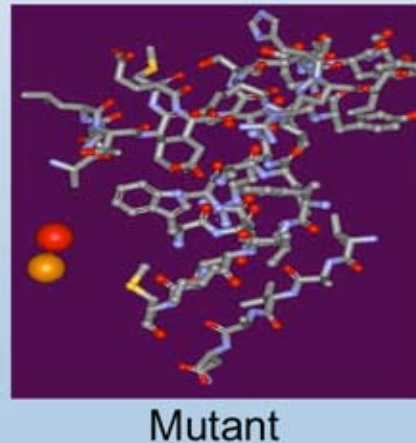
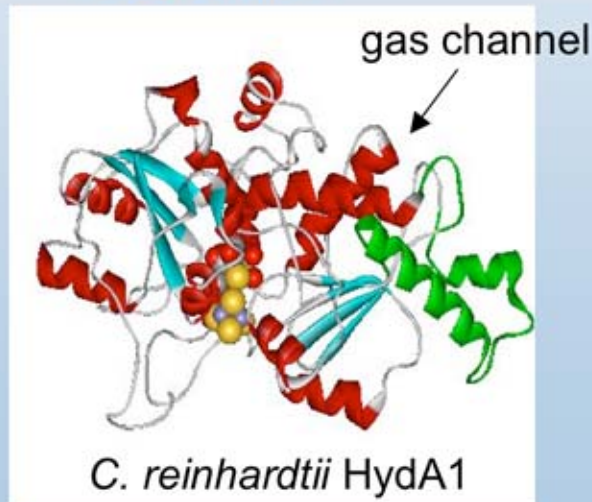
- (1) Started new subtask in January 2004;
- (2) Will create a cyanobacterial hydrogenase knockout mutant;
- (3) Will transfer a bacterial O₂-resistant hydrogenase into the above mutant.

Technical Accomplishments/Progress

Subtask 1

Background:

- Cloned two algal hydrogenase genes, *HydA1* and *HydA2*; key to current progress
- Modeled the two hydrogenase protein structures by homology to a bacterial Fe-hydrogenase that had been crystallized;
- Identified a gas channel as the putative O₂ access to the catalytic site;
- Modified a residue in the gas channel and observed increased O₂ resistance.



Technical Accomplishments/Progress

Subtask 1

FY04 Results

Molecular dynamics modeling of O₂ diffusion in the Fe-hydrogenase allowed us to identify future targets for site-directed mutagenesis (proprietary information) aimed at preventing O₂ inactivation of the algal enzyme.

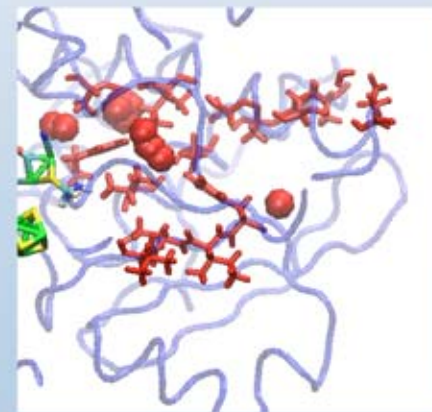
Collaborators: K. Schulten, University of Illinois, and the NREL Computational Sciences Center.



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236 psec



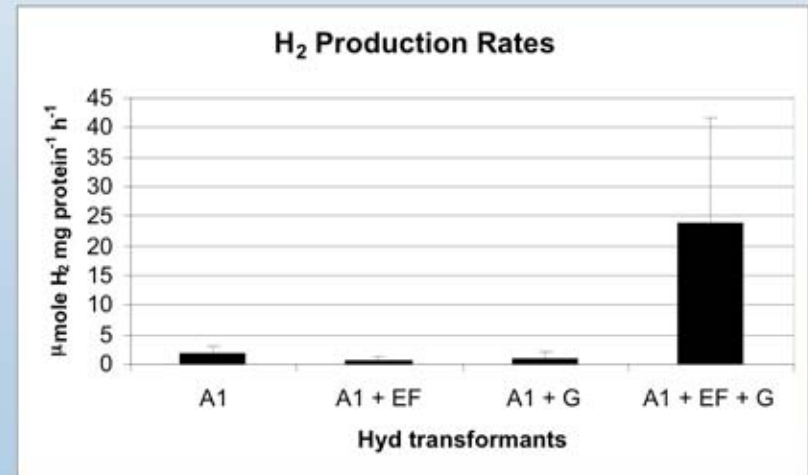
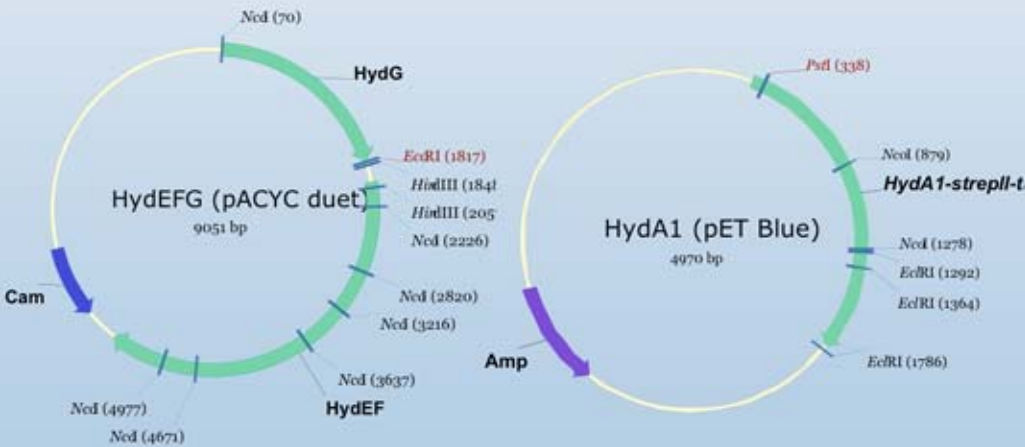
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Technical Accomplishments/Progress

Subtask 1

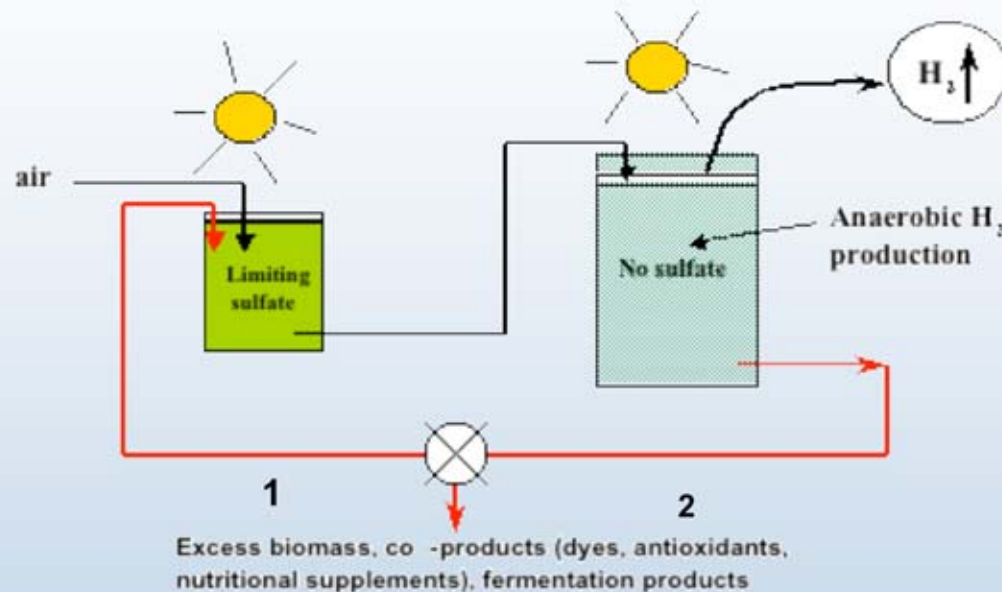
FY04 Results

We discovered how to produce an active algal [Fe]-hydrogenase in *E. coli* by co-expressing it with assembly genes identified under a DOE Office of Science project. This discovery allows us obtain large amounts of active recombinant algal (and other Fe-) hydrogenases, thereby accelerating our ability to generate and test site-directeded mutants.



Technical Accomplishments/Progress

Subtask 2

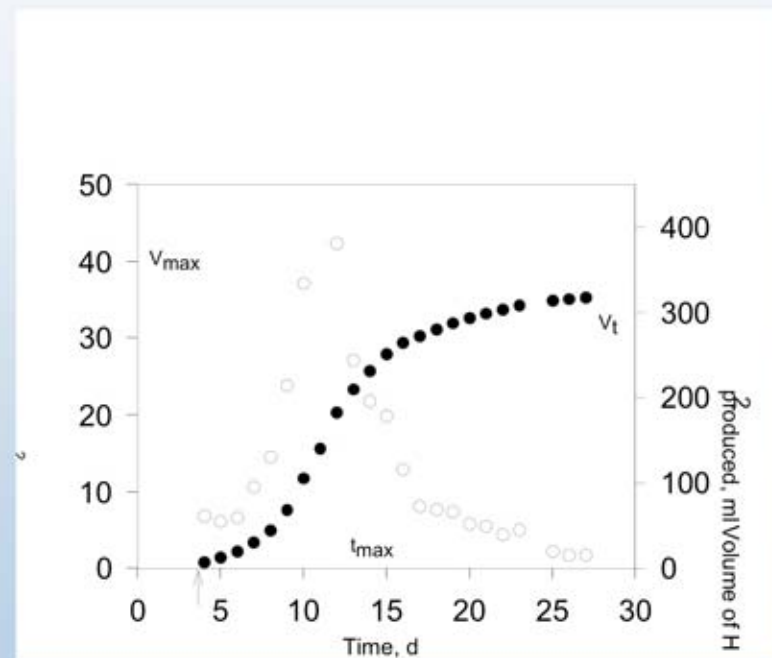
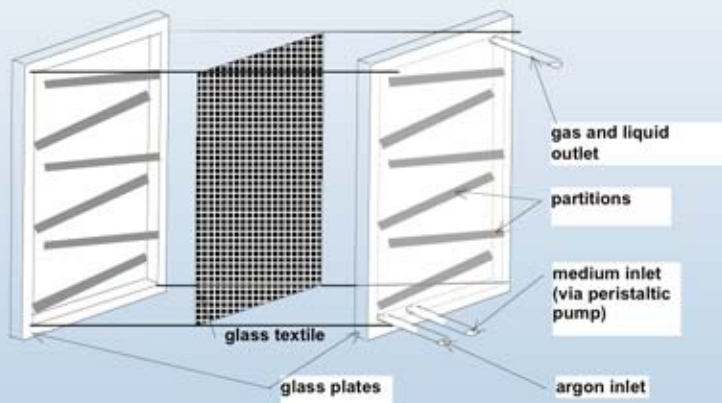


- We found that a light intensity of $50 \mu\text{E m}^{-2} \text{s}^{-1}$ and a D_{1-2} dilution rate of 0.02 h^{-1} yielded the highest H₂-photoproduction rate at an input of $60 \mu\text{M}$ sulfate;
- The overall duration of H₂ production was extended to 6 months (DOE's continuity technical target for 2010 is 1500 hours or about 2 months).

Technical Accomplishments/Progress

Subtask 2

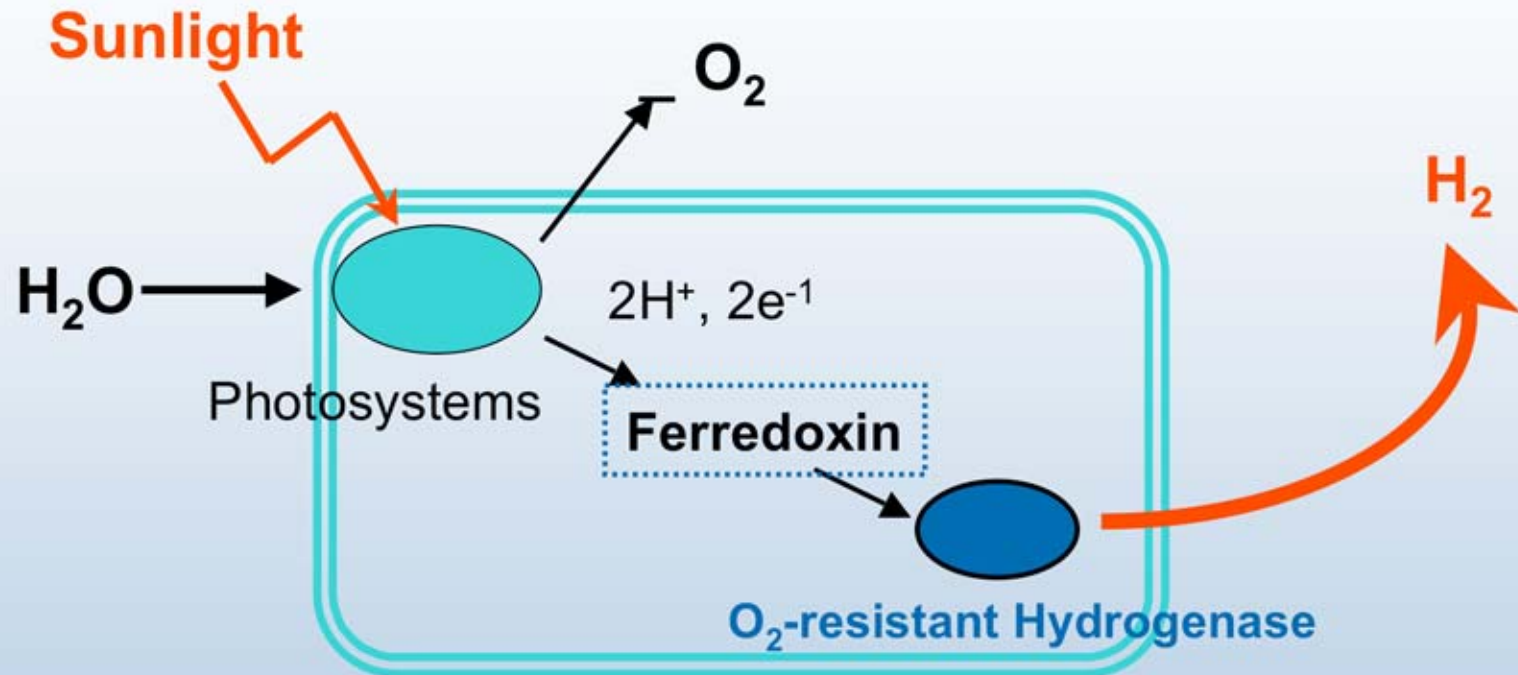
Algae were immobilized on fiberglass surfaces and sulfur-deprived. H_2 production was observed for a 6X longer period of time and at a 1.7X higher rate per liter of photobioreactor than batch suspension cultures.



Suspension cultures: 250 ml H_2 gas in 4 days at an average rate $2.5 \text{ ml } L_{\text{PhBR}}^{-1} \text{ h}^{-1}$
Immobilized cultures: 380 ml H_2 gas in 25 days at an average rate $4.3 \text{ ml } L_{\text{PhBR}}^{-1} \text{ h}^{-1}$

Technical Accomplishments/Progress

Subtask 3



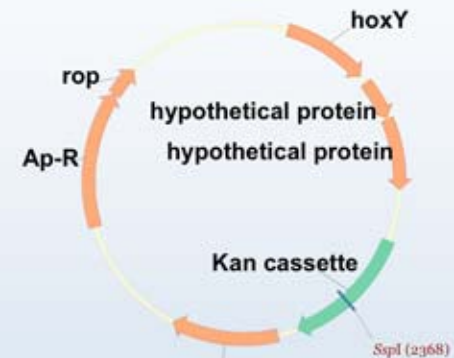
Cyanobacterial Recombinant

- A complementary approach to surmount the O₂-sensitivity issue

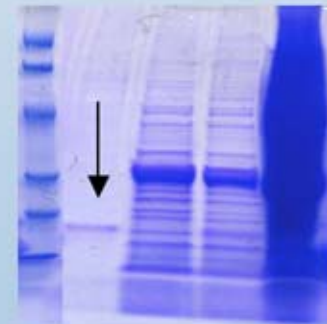
Technical Accomplishments/Progress

Subtask 3

- Constructed a plasmid to exchange the host native gene with an inactive hydrogenase gene to meet our FY04 milestone (create a hydrogenase knockout in the cyanobacterial host).
- Constructed an expression system in *E. coli* to mass produce cyanobacterial ferredoxin, the electron mediator between photosynthesis and hydrogenase. This will facilitate experiments to test the linkage of the two systems *in vitro* to complement the genetic work *in vivo* (milestone FY05).



incomplete hoxH
pSMART LC-Amp containing
Hox amplicon with mutated
hoxH (hoxH::KanR)



Project Safety

- Safety Vulnerability Techniques

- A hazard identification and control program is employed to identify possible failure modes and associated risks. Redundant engineering and procedural controls are used to ensure that acceptable levels of risk are not exceeded.
- Hydrogen safety is being addressed *via* gas sensor monitoring, and potential H₂ and O₂ gas mixtures are prevented *via* active catalytic systems.
- Gas pressure build up inside the bioreactors is prevented by appropriate venting.
- Genetically modified organisms are physically contained and disposed of by heat or bleach treatment; NREL Radiation Safety Program guidelines for the utilization and disposal of radioactive materials are applied.

- Management of Change

Safety techniques to address systems that simultaneously produce H₂ and O₂ are being evaluated and will be implemented in the future as mutant organisms become available.

- Lesson Learned

Monitor the build-up of gas pressure inside bioreactors and ensure that any accumulated gas is continuously and properly vented.

Interactions and Collaborations

Collaborations:

Dr. A. Tsygankov, Russian Academy of Sciences, Pushchino, Russia

Non-Program funded interactions:

Dr. K. Schulten, Beckman Institute/ University of Illinois

NREL Computational Sciences Center

Institute of Biological Energy Alternatives (IBEA)

Publications:

4 manuscripts published, 3 in press, 5 submitted and 3 in preparation.

Patent applications submitted/granted:

1 application submitted.

Responses to Previous Year Reviewers' Comments

1. *“Encourage priority on immobilized algae cultures to go from +S to –S without centrifugation”*. **The feasibility of using immobilized cultures for H₂ production was demonstrated this year. More cost-efficient immobilization matrices are one of the foci of next year’s research.**
2. *“Is transfer of hydrogenase enzyme system from *R. gelatinosus* worth trying?”; “Focus on hydrogenase from *Rubrivivax* into cyanobacteria as planned”*. **These encouraging comments have resulted in the funding of Subtask 3, a new subtask in FY2004.**
3. *“Very long term R&D and very basic”; “... may need to be funded out of basic R&D”; “Will this meet the President’s goal?” “...may need to be funded out of the Office of Science as longer term objectives”*. **There are clear benefits in using parallel applied and exploratory approaches to biological H₂ production, as evidenced by the results reported this year in Subtask 1 regarding the heterologous expression of the algal hydrogenase in the bacterium *E. coli*.**

Responses to Previous Year Reviewers' Comments (continued)

4. *“Curiously schizophrenic – the H₂ production being measured “indirect biophotolysis” yet the engineering is all aimed at “direct”...Are the researchers clear on the distinction? Is the economist who gave the remarkable \$ value?” “They will have very different costs”*

Direct biophotolysis: H₂ from H₂O

- (a) Simultaneous H₂ and O₂ production (Subtask 1)
- (b) Spatially separated sulfur-deprived cultures (Subtask 2)

Indirect biophotolysis: H₂ from starch

Estimated costs of \$2.34/kg (W. Amos, NREL) are based on an optimized spatially separated system

Future Work

Subtask 1

- Model H₂ gas diffusion to ensure that it will not be inhibited in current and future O₂-resistant mutants;
- Continue iterative process of (a) O₂-gas-diffusion computational simulations and (b) experimental generation and testing of the O₂-resistance of hydrogenase mutants expressed in *E. coli*.

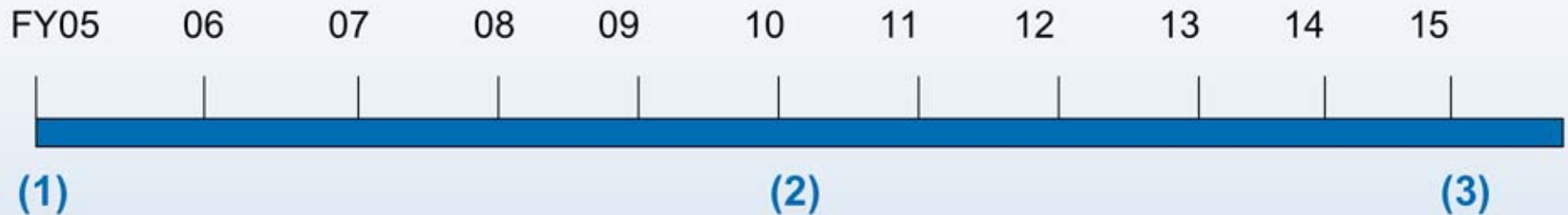
Subtask 2

- Continue optimization of H₂ production in the continuous system by investigating the effect of other culture parameters (e.g., limiting sulfate concentration and pH) ;
- Engineer a cyclic H₂-production system using fiberglass immobilized cultures;
- Initiate studies on the feasibility of low-cost matrices for cell immobilization (proprietary information);
- Perform an economic analysis of immobilized algal systems.

Subtask 3

- Construct a hydrogenase knockout mutant in *Synechocystis*;
- Link cyanobacterial photosynthesis to the oxygen-resistant bacterial hydrogenase, *in vitro* and ultimately *in vivo*.

Future Work (continued)



NREL, UCB and ORNL team consolidated goal:

- (1) “Virtual” optimized organism; H₂ cost \$100/kg;
- (2) Combined triple mutant organism; H₂ cost \$30/kg;
- (3) Optimized organism; H₂ cost \$2/kg.

Exploratory research will continue in parallel to address other related issues