

Biological Systems for Hydrogen Photoproduction

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Energy Laboratory**

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Overview

Timeline

Project start date: FY00

Project end date: FY18

Percent complete: N/A

Budget

Funding received in FY08:
\$2,000K

Funding allocated for FY09:
\$840K

Barriers

Production barriers addressed

- Continuity of H₂ production (AI)
- Feedstock cost in an integrated system (AT)

Partners

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

Dr. Michael Flickinger, North Carolina State University

Dr. Mace Golden, GoldenBioenergy, CO

Dr. Eric Johnson, Johns Hopkins University

Objectives/Relevance

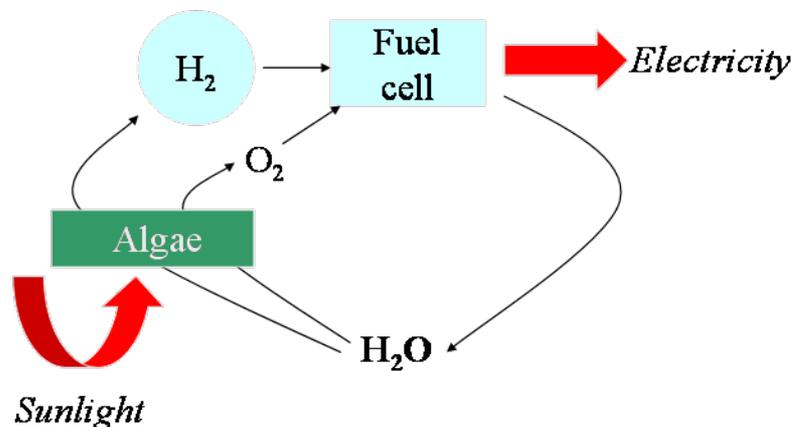
General: Develop photobiological or integrated photobiological/fermentative systems for large-scale H₂ production.

- **Task 1:** Address the O₂-sensitivity of hydrogenases that prevents continuity of H₂ photoproduction.
- **Task 2:** Utilize an anaerobic H₂-producing method (sulfur deprivation) as a platform to address other factors limiting commercial algal H₂ photoproduction.
- **Task 3:** Integrate photobiological and fermentative systems in different configurations for more efficient H₂ production.

Task 1 – O₂ Sensitivity of Hydrogenases

Objectives/Relevance

Develop and optimize *aerobic* photobiological systems for the production of H₂ from water by engineering a H₂-producing catalyst ([FeFe]-hydrogenase) that has an extended half-life following exposure to O₂.



Programmatic milestones

Parameters	Current Status	2013 Target	Maximum Potential
Duration of continuous photoproduction	90 days (anaerobic)		
	cycles of 10 days (immobilized, aerobic)	10 min (aerobic)	12 hours (aerobic)
O ₂ tolerance (half-life in air)	~4 days after exposure to air (bacterial enzyme)		

Task 1 – O₂ Sensitivity of Hydrogenases

Approach/Milestones

Apply rational, site-directed mutagenesis to a bacterial [FeFe]- and [NiFe]-hydrogenases; express mutant enzymes in *E. coli* and test mutants for increased tolerance to O₂ (Subtask 1).

Develop DNA plasmids to express heterologous wild-type or mutated hydrogenases in *Chlamydomonas reinhardtii* in order to test O₂-tolerance in the alga (Subtask 2).

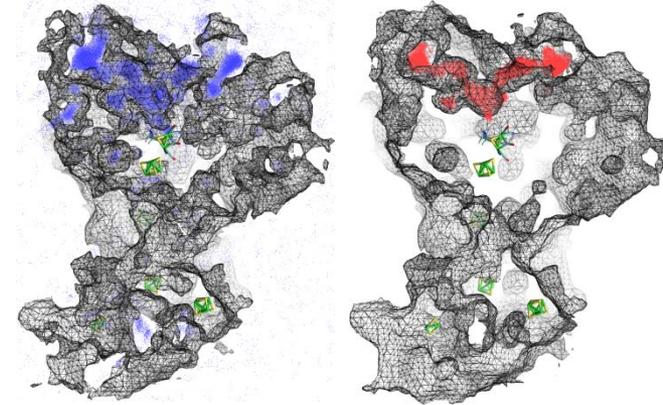
Initiate random mutagenesis/higher-throughput approaches for generation of hydrogenases with increased tolerance to O₂; express mutant enzymes in *E. coli* and test mutants for increased tolerance to O₂ (Subtask 3).

Milestones	Completion date	Status
3.3.4 Test the performance of immobilized ATPase mutants	6/08	completed
3.3.5 Test the performance of immobilized antenna mutants	10/08	completed
3.3.6 Introduce and test the expression of a clostridial hydrogenase gene in <i>Chlamydomonas reinhardtii</i>	2/09 (postponed from 08/08)	completed
3.3.7 Design and test a new DNA construct for <i>E. coli</i> expression of hydrogenases	12/08	completed

Task 1 – Technical Accomplishments

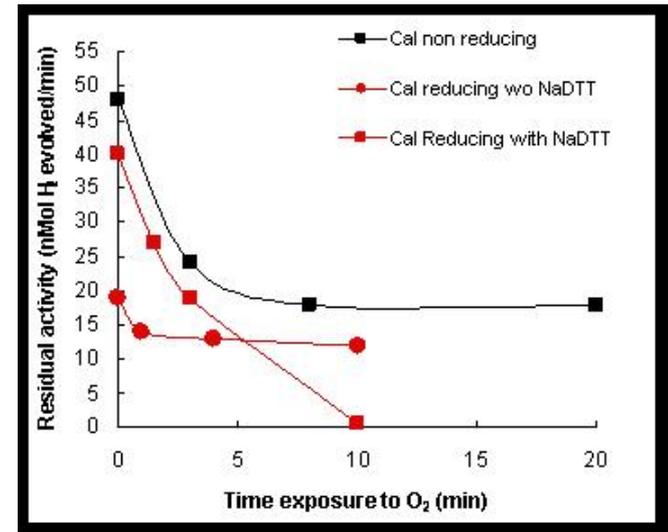
Subtask 1 – Modeling and Site-Directed Mutagenesis

Earlier computational modeling indicated two gas-diffusion pathways in [FeFe]-hydrogenases that allow O₂ to access the active site and inhibit their catalytic activity. Site-directed mutagenesis was initiated, aiming at closing those pathways. However, our studies led to the observation that, **depending on the purification procedures, different populations of hydrogenases show different sensitivity to O₂ inactivation.**



Results: Lack of (■) or short exposure to reductant (■) results in enzymes that are partially insensitive to O₂ inactivation, whereas enzymes exposed to reductant throughout the purification process exhibit classical O₂ inactivation rates (●).

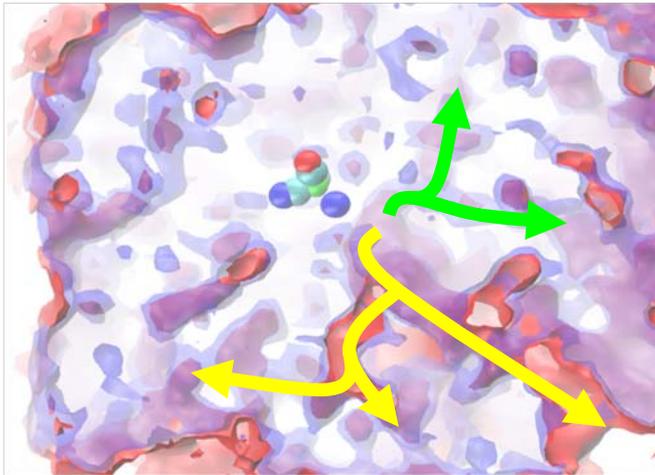
Conclusion and Future Work: We will focus on understanding the factors that affect the O₂ sensitivity of the enzyme before proceeding with the mutagenesis work. We will conduct additional experiments to understand the nature of the different states of the hydrogenase enzyme as well as their relationship to O₂ tolerance.



Task 1 – Technical Accomplishments

Subtask 1 – Modeling and Site-Directed Mutagenesis

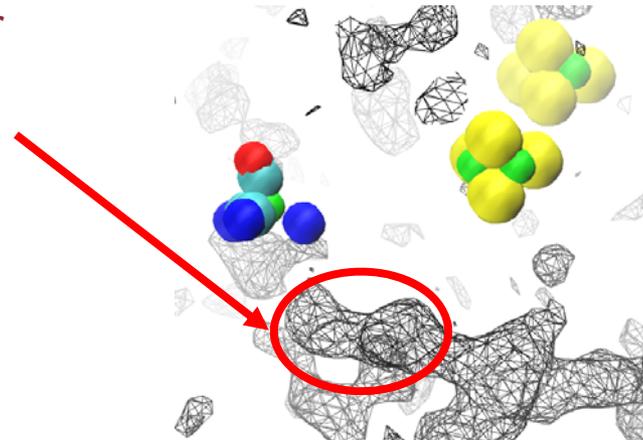
Computational modeling was initiated to determine whether accessibility to the catalytic site was also involved in conferring O₂-tolerance to [NiFe]-hydrogenases.



Results: Three pathways for O₂ diffusion (yellow) were identified. Two more pathways (green) were found for H₂, suggesting that H₂ can still migrate out — even if all O₂ pathways are blocked.

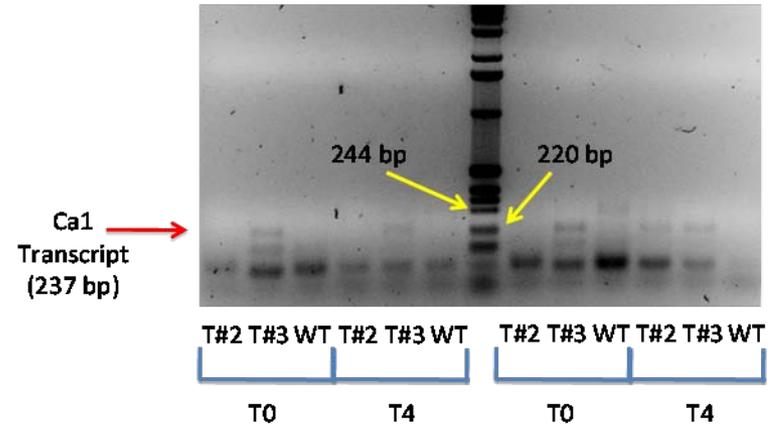
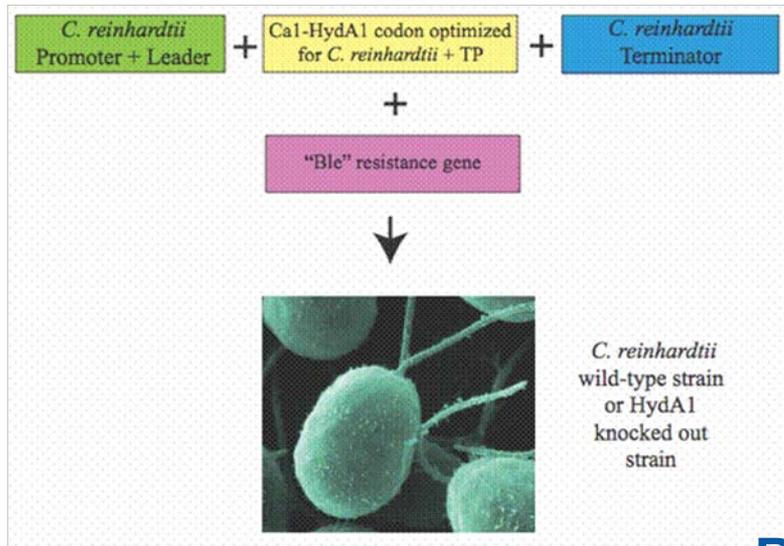
Results: Identified key region for O₂ to further diffuse into the hydrogenase interior and deactivate the [NiFe]-cluster.

Future Work: Identify specific targets for mutagenesis; implement them and test for changes in O₂ tolerance *in vitro*.



Task 1 – Technical Accomplishments

Subtask 2 – Transformation of Green Algae

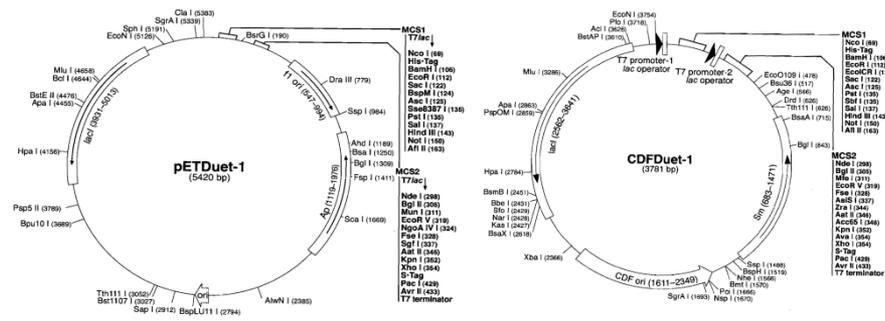


Results: The DNA construct was made and we have demonstrated the presence of Ca1 gene expression by RT-PCR (see figure above).

Future work: Test the activity and O₂-tolerance of the clostridial hydrogenase in positive transformants and determine physiological effects (if any) or a more O₂-tolerant hydrogenase in photosynthetic organisms.

Task 1 – Technical Accomplishments

Subtask 3 – Random Mutagenesis



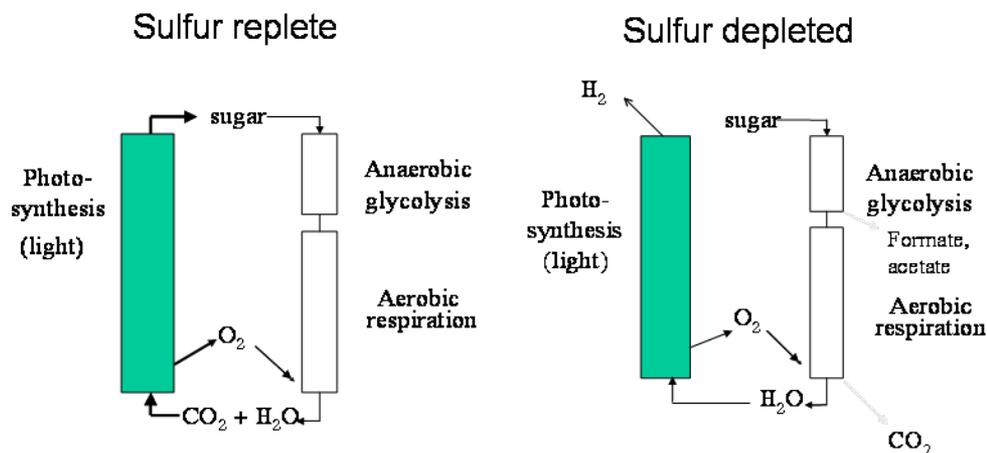
Results: Designed DNA constructs for expression of recombinant [FeFe]-hydrogenases in *E. coli* and integrated these constructs into an appropriate vector; successfully expressed recombinant hydrogenases in *E. coli* concomitant with their maturation genes, using Novagen's pET-Duet vectors; designed a high-throughput chemochromic assay for screening O₂-tolerant hydrogenases.

Structural hydrogenase genes and the assembly genes *hydE*, *hydF*, and *hydG* will be incorporated behind the *hya* promoter for expression under anaerobiosis.

Future Work: Test the high-throughput assay; generate random mutagenesis libraries of algal hydrogenases in *E. coli* and screen them for O₂-tolerant mutants.

Task 2 – Sulfur-Deprivation Platform Objectives/Relevance

Use the anaerobic, sulfur-deprivation platform (Melis et al., 2000) to address biochemical and engineering issues related to photobiological H_2 production.



Parameters	Current Status	2013 Target	Maximum Potential
Duration of continuous photoproduction	90 days (anaerobic)		
O_2 tolerance (half-life in air)	cycles of 10 days (immobilized, aerobic) ~4 days after exposure to air (bacterial enzyme)	10 min (aerobic)	12 hours (aerobic)

Task 2 – Sulfur-Deprivation Platform

Approach/Milestones

Determine the light-conversion efficiency of immobilized, H₂-producing *C. reinhardtii* cultures under different growth conditions.

Test the performance of ATPase mutants under sulfur deprivation.

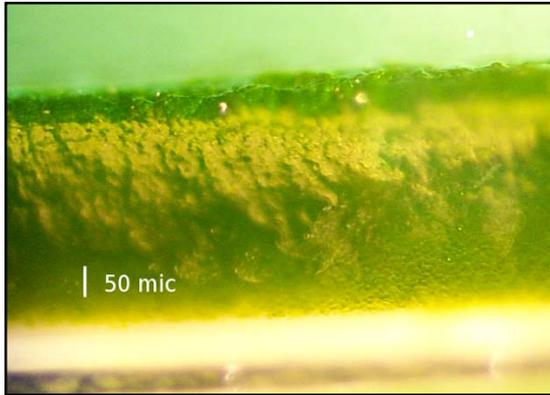
Test the performance of truncated antenna mutants under the same conditions.

Improve the mechanical stability of the alginate film.

Milestones	Completion date	Status
3.3.4 Test the performance of immobilized ATPase mutants	6/08	completed
3.3.5 Test the performance of immobilized antenna mutants	10/08	completed
3.3.6 Introduce and test the expression of a clostridial hydrogenase gene in <i>Chlamydomonas reinhardtii</i>	2/09 (postponed from 08/08)	completed
3.3.7 Design and test a new DNA construct for <i>E. coli</i> expression of hydrogenases	12/08	completed

Task 2 – Sulfur-Deprivation Platform

Technical Accomplishments

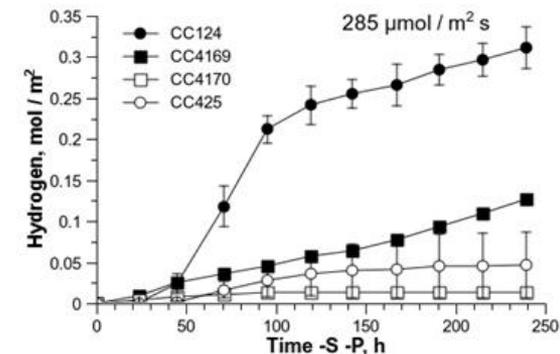
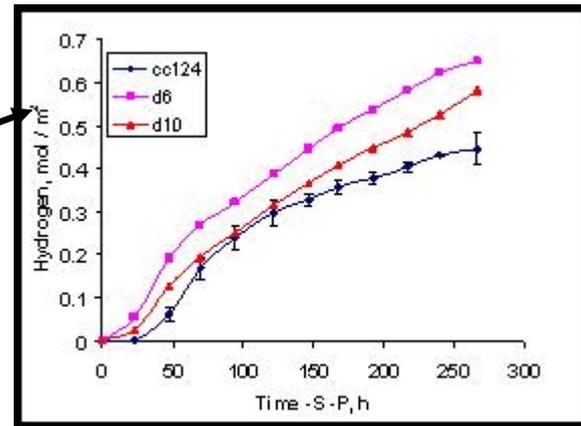


Results: In the past year, we have demonstrated light-conversion efficiencies of about 1% at $29 \mu\text{E m}^{-2} \text{s}^{-1}$ (solar) and some H_2 production in the presence of O_2 with cells immobilized in alginate films, underscoring the potential of the technique for higher efficiencies and rates.

Results:

- Demonstrated 30%–45% more H_2 gas production using an ATPase mutant (not limited by the formation of a proton gradient).
- Showed higher and sustained rates of H_2 production by a truncated antenna mutant under $1/10^{\text{th}}$ of a sun.

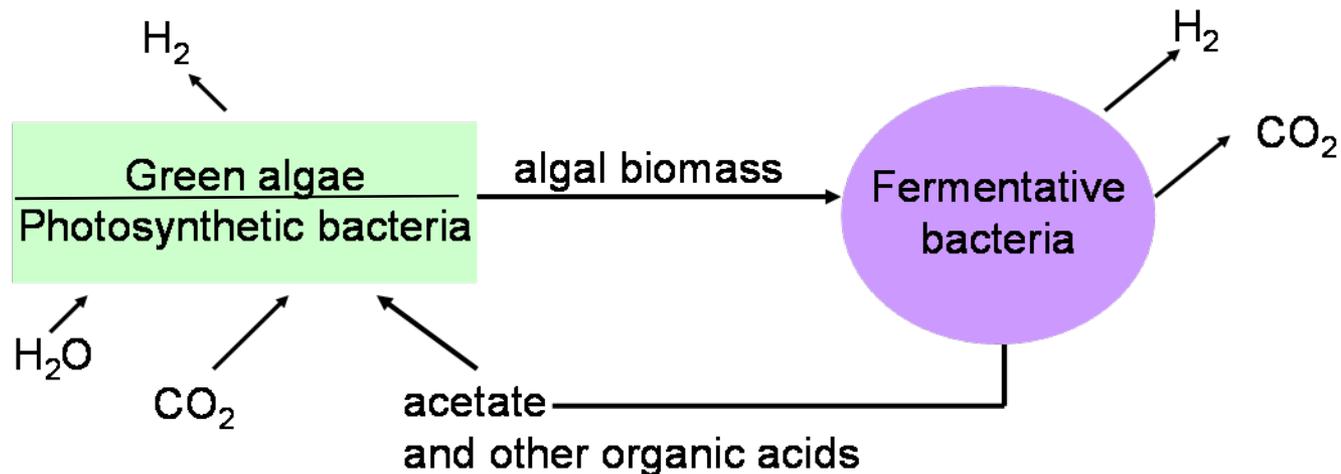
Future Work: Further investigate the performance of the ATPase mutants and initiate a new collaboration to utilize them more efficiently; test other truncated antenna mutants; continue to improve the stability of the alginate films.



Task 3 – Integrated System

Objectives/Relevance

Integrate photobiological with fermentative organisms to more efficiently utilize the solar spectrum and the substrates/products from each reaction for H₂ production.



Task 3 – Integrated System Approach/Milestones

- Determine whether a H₂-producing, fermentative consortium can utilize spent algal and photosynthetic bacterial biomass (Subtask 1).
- Find a consortium of organisms that is able to ferment organic wastes, produce organic acids to support photosynthetic bacteria growth, and produce H₂ at high efficiency (Subtask 2).



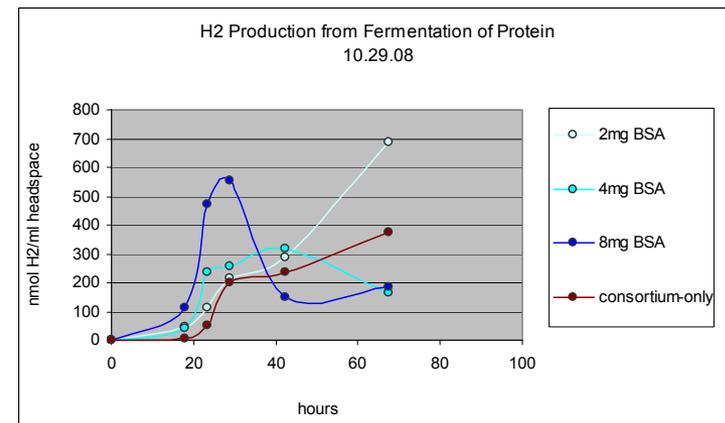
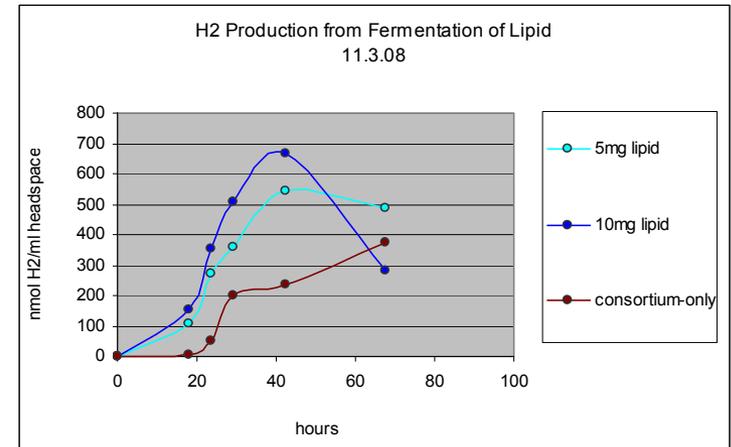
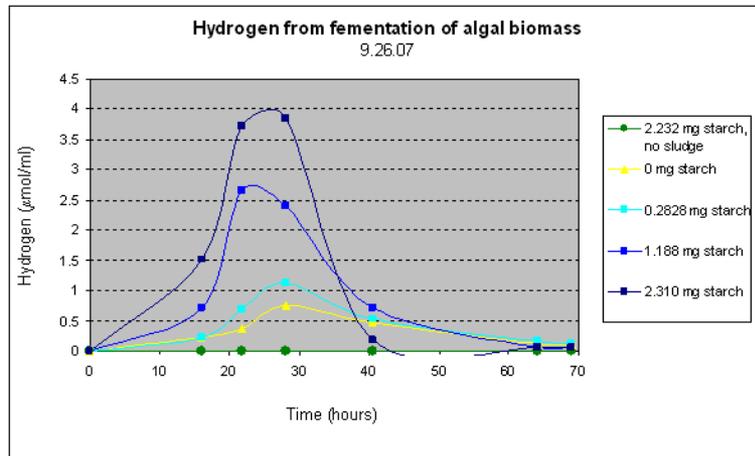
The continuation of this subtask will be determined by the results of a techno-economic analysis by Directed Technologies, Inc., that is being completed.

(No milestones in FY09)

Task 3 – Technical Accomplishments

Subtask 1 – Fermentation of Algal Biomass

Results: A fermentative consortium was shown to be capable of metabolizing algal biomass, purified algal lipids (DGDG), and purified proteins, co-producing H₂ gas and organic acids, with a final yield of 0.45 μg H₂/mg biomass (non-optimized).



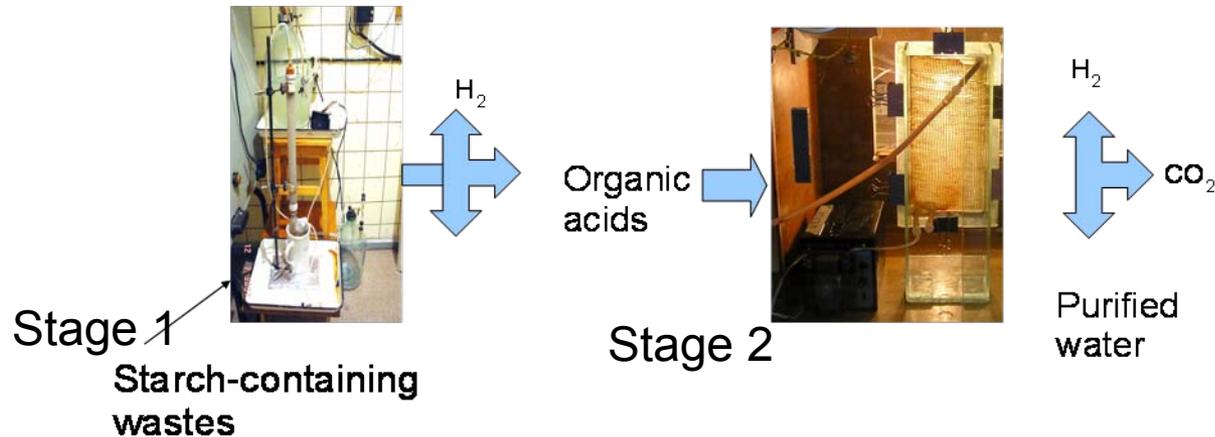
Future work: Test whether the consortium can metabolize alginate films; scale up fermentation to 200 ml and optimize it.

Task 3 – Technical Accomplishments

Subtask 2 – Fermentative and Photosynthetic Bacteria

Results:

1. Demonstrated H_2 production by fermentative organisms using starch-rich potato wastes, accompanied by production of organic acids.
2. Showed H_2 production by photosynthetic bacteria using organic acids from fermentation of potato wastes.



Future work: Integrate fermentation of potato waste with photosynthetic bacterial H_2 production and determine the total efficiency of the process, using dilute wastes.

Collaborations

- **Task 1:**

Dr. Mace Golden, Golden Bioenergy, CO (random mutagenesis and high-throughput assay).

- **Task 2:**

Dr. Eric Johnson, Johns Hopkins University (ATPase mutants).

- **Task 3:**

Dr. Anatoly Tsygankov, Russian Academy of Sciences, Pushchino, Russia (fermentation of potato wastes, photosynthetic bacterial H₂ production).

Summary

Task 1: (1) We continued to investigate the O₂ sensitivity of the two redox states of the [FeFe]-hydrogenase using recombinant enzymes; (2) the expression of a clostridial hydrogenase gene in *C. reinhardtii* was successful, and we will test the expressed enzyme's activity and O₂ tolerance next; and (3) a high-throughput screening for O₂-tolerant recombinant hydrogenases was developed and will be tested.

Task 2: (1) The alginate-immobilization process was used to successfully test the H₂-producing performance of ATPase and truncated antenna mutants.

Task 3: (1) The fermentative consortium at NREL was shown to metabolize algal starch, lipid, and protein at a non-optimal yield of 0.45 μg H₂/mg biomass; and (2) the consortium is also able to utilize algal biomass isolated from a variety of growth conditions, both fresh and frozen (damaged), without the need for pretreatment.