National Renewable Energy Laboratory
Innovation for Our Energy Future

Biological Systems for Hydrogen Photoproduction

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National Renewable Energy Laboratory

May 20, 2009

Project ID # pd_17_ghirardi

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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 H_2 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

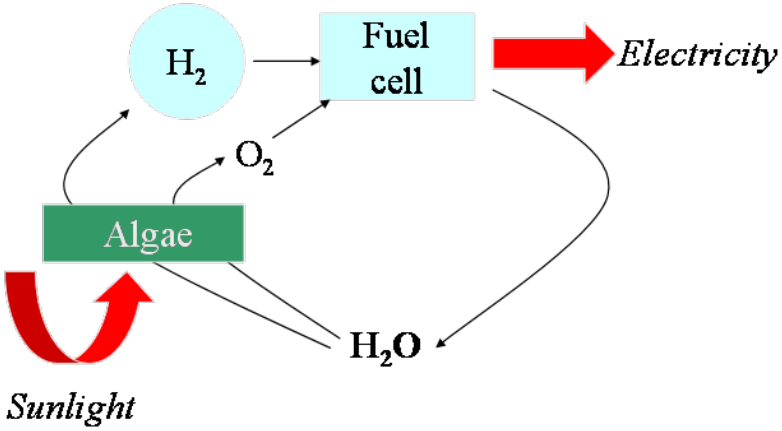
Objectives/Relevance

General: Develop photobiological or integrated photobiological/fermentative systems for large-scale H_2 production.

- **Task 1**: Address the O_2-sensitivity of hydrogenases that prevents continuity of H_2 photoproduction.
- **Task 2**: Utilize an anaerobic H_2-producing method (sulfur deprivation) as a platform to address other factors limiting commercial algal H_2 photoproduction.
- **Task 3**: Integrate photobiological and fermentative systems in different configurations for more efficient H_2 production.

Task 1 – O_2 Sensitivity of Hydrogenases Objectives/Relevance

Develop and optimize *aerobic* photobiological systems for the production of H_2 from water by engineering a H_2-producing catalyst ([FeFe]-hydrogenase) that has an extended half-life following exposure to O_2.



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 – O_2 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 O_2 (Subtask 1).

Develop DNA plasmids to express heterologous wild-type or mutated hydrogenases in *Chlamydomonas reinhardtii* in order to test O_2-tolerance in the alga (Subtask 2).

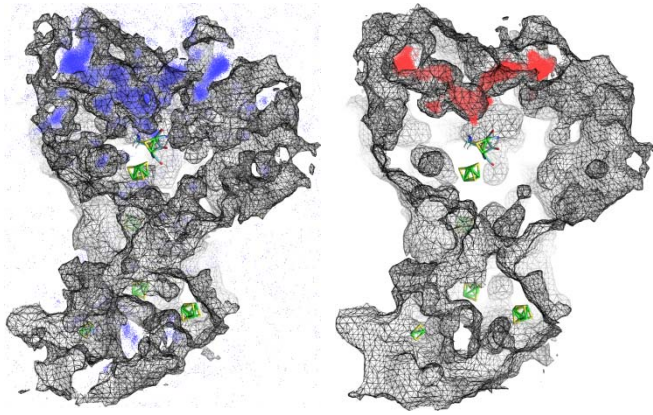
Initiate random mutagenesis/higher-throughput approaches for generation of hydrogenases with increased tolerance to O_2; express mutant enzymes in *E. coli* and test mutants for increased tolerance to O_2 (Subtask 3).

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 *Chlamydomonas reinhardtii*	2/09 (postponed from 08/08)	completed
3.3.7 Design and test a new DNA construct for *E. coli* expression of hydrogenases	12/08	completed

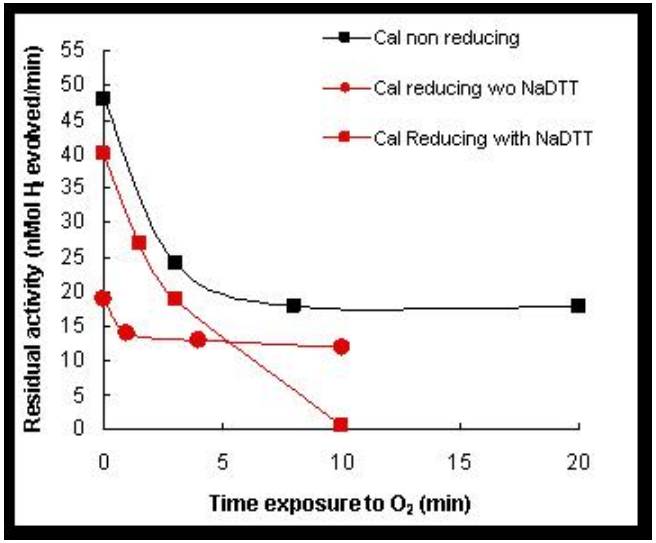
Task 1 – Technical Accomplishments

Subtask 1 – Modeling and Site-Directed Mutagenesis

Earlier computational modeling indicated two gas-diffusion pathways in **[FeFe]-hydrogenases** that allow O_2 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 O_2 inactivation.**



Results: Lack of (■) or short exposure to reductant (■) results in enzymes that are partially insensitive to O_2 inactivation, whereas enzymes exposed to reductant throughout the purification process exhibit classical O_2 inactivation rates (●).

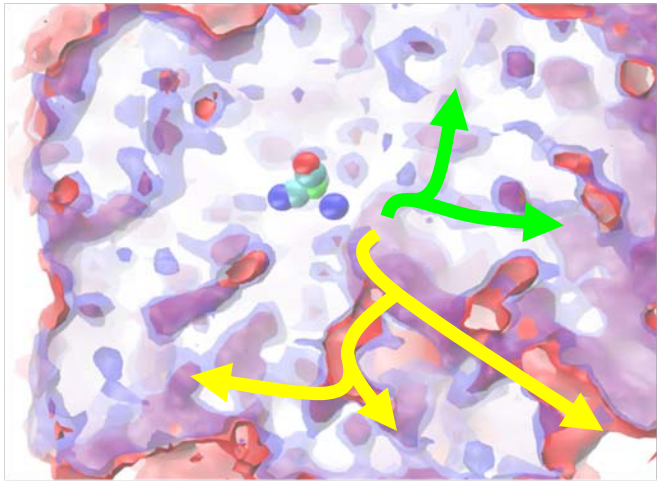


Conclusion and Future Work: We will focus on understanding the factors that affect the O_2 sensitivity of the enzyme before proceeding with the mutagenesis work. We will conduct additional experiments to understand the nature of the different states of the hydrogenase enzyme as well as their relationship to O_2 tolerance.

Task 1 – Technical Accomplishments

Subtask 1 – Modeling and Site-Directed Mutagenesis

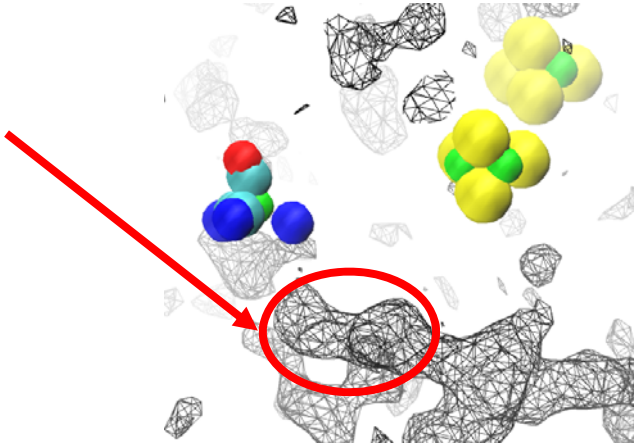
Computational modeling was initiated to determine whether accessibility to the catalytic site was also involved in conferring O_2-tolerance to **[NiFe]-hydrogenases**.



Results: Three pathways for O_2 diffusion (yellow) were identified. Two more pathways (green) were found for H_2, suggesting that H_2 can still migrate out — even if all O_2 pathways are blocked.

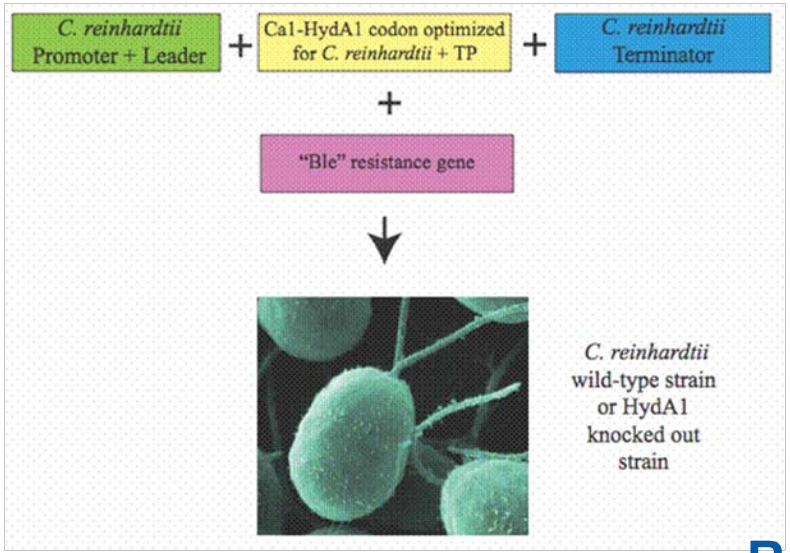
Results: Identified key region for O_2 to further diffuse into the hydrogenase interior and deactivate the [NiFe]-cluster.

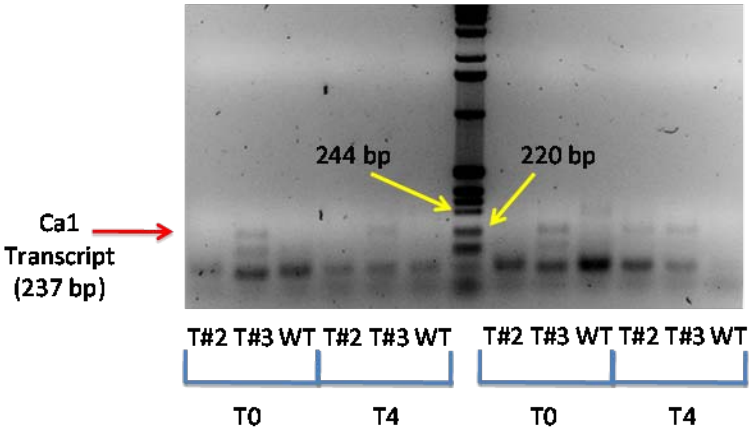
Future Work: Identify specific targets for mutagenesis; implement them and test for changes in O_2 tolerance *in vitro*.



Task 1 – Technical Accomplishments

Subtask 2 – Transformation of Green Algae





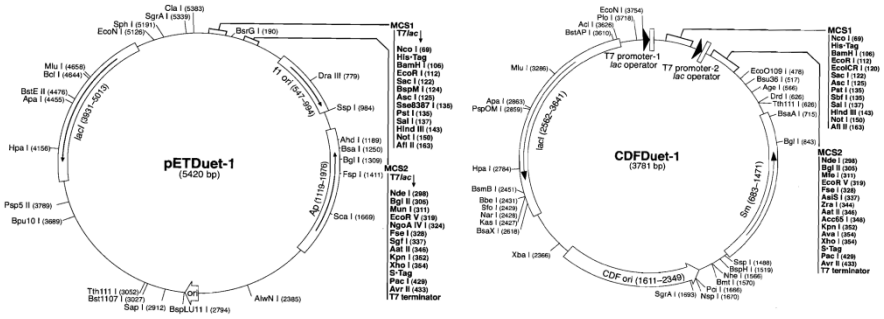
Results: The DNA construct was made and we have demonstrated the presence of Cal gene expression by RT-PCR (see figure above).

Future work: Test the activity and O_2-tolerance of the clostridial hydrogenase in positive transformants and determine physiological effects (if any) or a more O_2-tolerant hydrogenase in photosynthetic organisms.

Task 1 – Technical Accomplishments

Subtask 3 – Random Mutagenesis

Mutagenesis → Error-prone PCR → Expression in *E. coli* → Automated GC assay → Screening



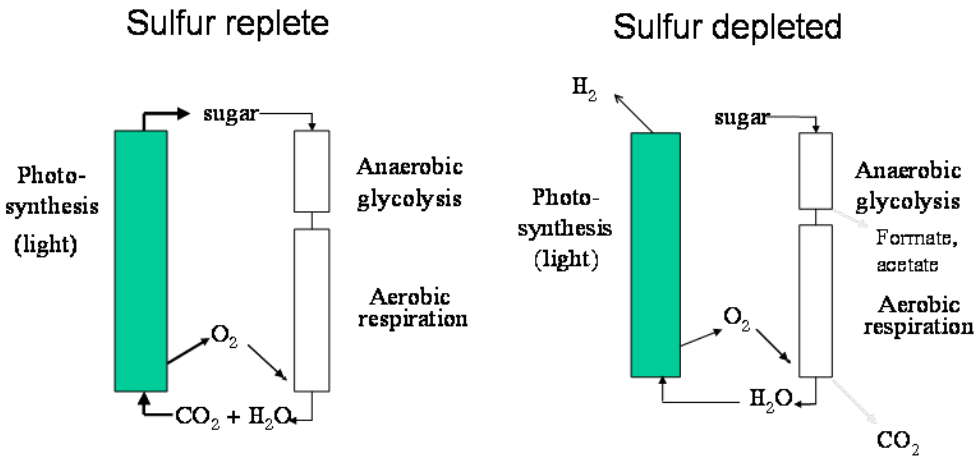
Structural hydrogenase genes and the assembly genes *hydE*, *hydF,* and *hydG* will be incorporated behind the *hya* promoter for expression under anaerobiosis.

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 O_2-tolerant hydrogenases.

Future Work: Test the high-throughput assay; generate random mutagenesis libraries of algal hydrogenases in *E. coli* and screen them for O_2-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.



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Task 2 – Sulfur-Deprivation Platform Approach/Milestones

Determine the light-conversion efficiency of immobilized, H_2-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 *Chlamydomonas reinhardtii*	2/09 (postponed from 08/08)	completed
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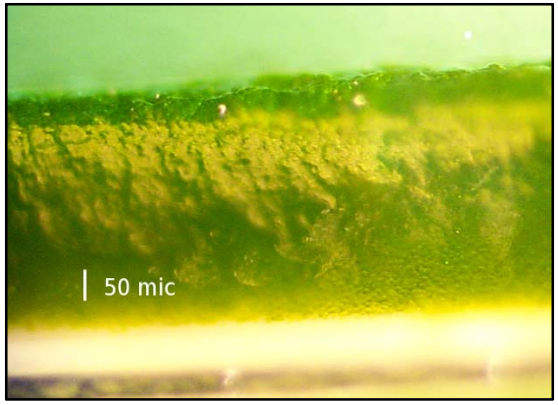
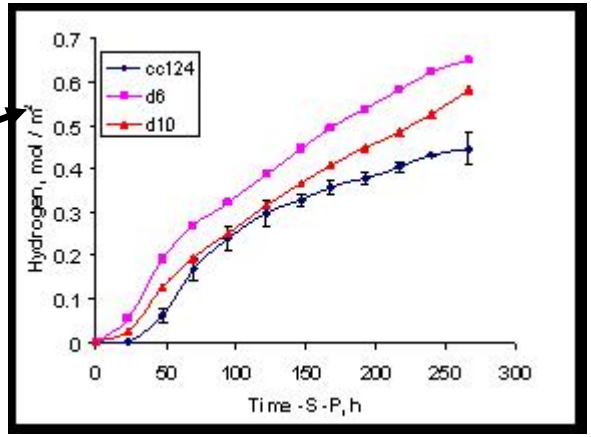
Task 2 – Sulfur-Deprivation Platform Technical Accomplishments

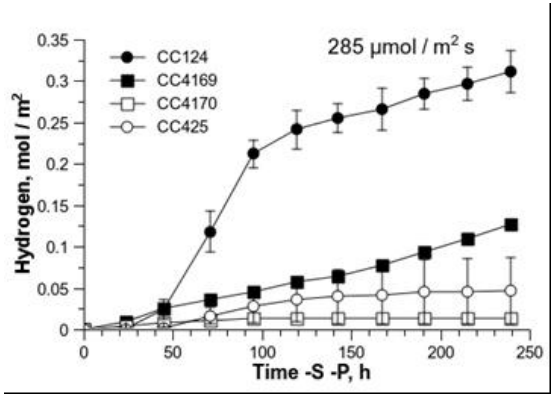


Results: In the past year, we have **demonstrated light-conversion efficiencies of about 1% at 29 μE m^{-2} s^{-1} (solar) and some H_2 production in the presence of O_2** with cells immobilized in alginate films, underscoring the potential of the technique for higher efficiencies and rates.



Results:

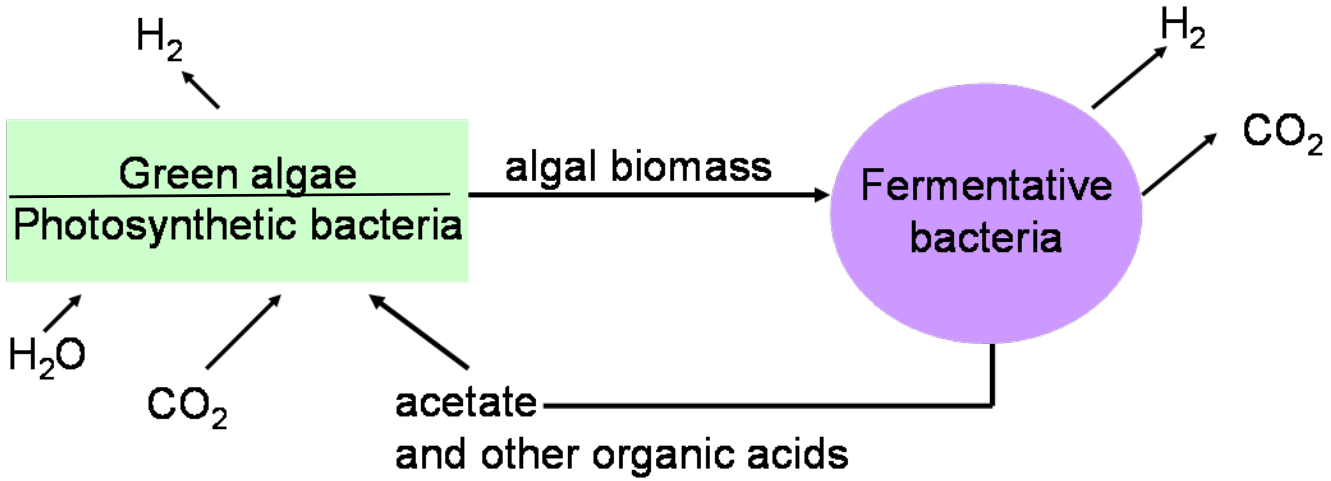
- **Demonstrated 30%–45% more H_2 gas production using an ATPase mutant** (not limited by the formation of a proton gradient).
- **Showed higher and sustained rates of H_2 production by a truncated antenna mutant** under 1/10th of a sun.



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 H_2 production.



Task 3 – Integrated System Approach/Milestones

- Determine whether a H_2-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 H_2 at high efficiency (**Subtask 2**).

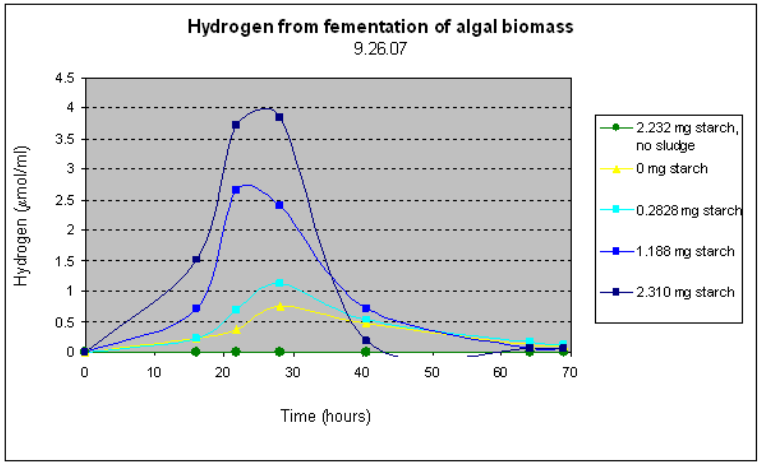
The continuation of this subtask will be determined by the results of a techno-economic analysis by Directed Technologies, Inc., that is being completed.

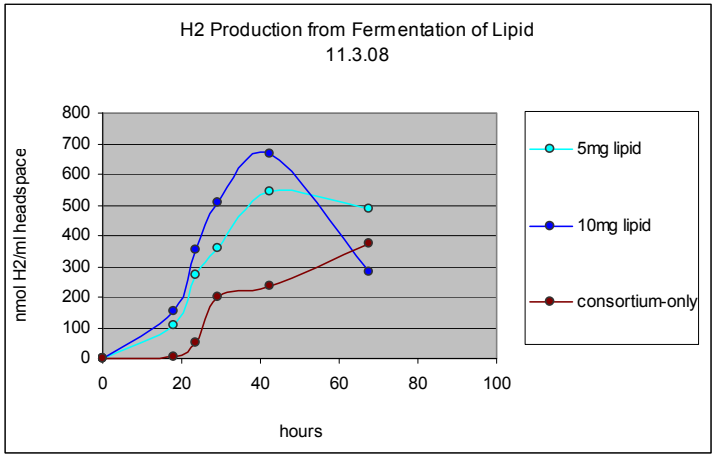
(No milestones in FY09)

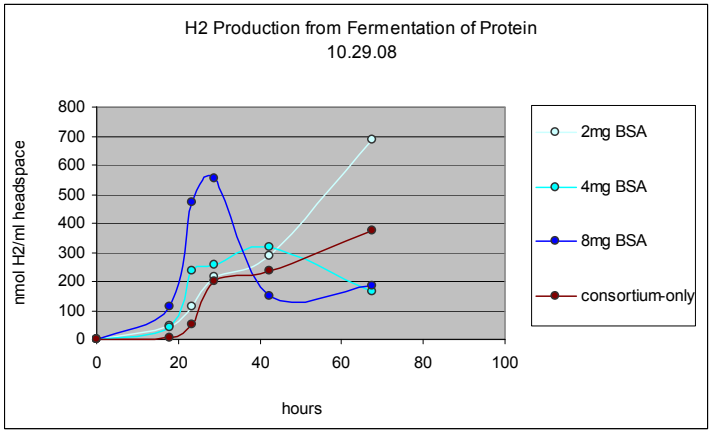
Task 3 – Technical Accomplishments

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 H_2 gas and organic acids, with a final yield of 0.45 μg H_2/mg biomass (non-optimized).







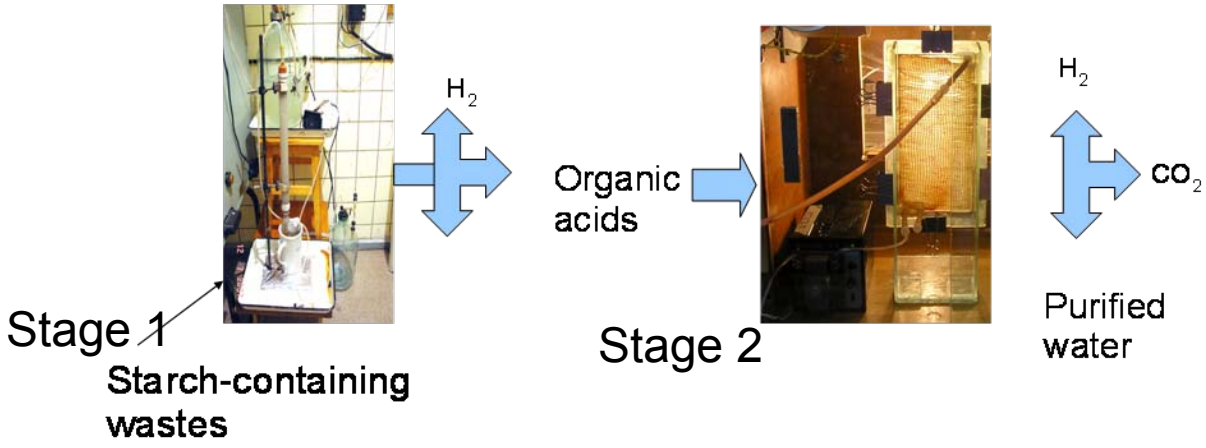
Future work: Test whether the consortium can metabolize alginate films; scale up fermentation to 200 ml and optimize it.

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 H_2 production).

Summary

Task 1: (1) We continued to investigate the O_2 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 O_2 tolerance next; and (3) a high-throughput screening for O_2-tolerant recombinant hydrogenases was developed and will be tested.

Task 2: (1) The alginate-immobilization process was used to successfully test the H_2-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 H_2/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.