NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Biological Systems for Hydrogen Photoproduction

2011 Annual Merit Review and Peer Evaluation Meeting

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Project end Date: 10/2011

Project continuation and direction determined annually by DOE

Project ID # PD037

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Overview

Timeline

Project start date: FY00

Project end date: 10/21/2011*

Percent complete: 80%

*Project continuation and direction determined annually by DOE

Barriers

Production barriers addressed

- Rate of H_2 production (AH)
- Continuity of H_2 production (AI)
- Feedstock cost in an integrated system (AT)

Budget

Total project funding: $9,200K

Funding received in FY10: $600K

Funding allocated for FY11: $865K

Partners

Drs. Anatoly Tsygankov and Sergey Kosourov, Institute of Basic Biological Problems, RAS, Pushchino, Russia

Dr. Michael Flickinger, North Carolina State University (unfunded)

Dr. Eric Johnson, Johns Hopkins University

Drs. Iftach Yacoby and Shuguang Zhang, MIT (unfunded)

Objectives/Relevance

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

- **Task 1**: Address the O_2-sensitivity of hydrogenases and competition with the CO_2 fixation pathway, which either limit or prevent continuity of H_2 photoproduction under aerobic, high solar-to-hydrogen (STH) conditions.
- **Task 2**: Utilize a limited STH 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 less costly H_2 production in the short term.

Objectives/Relevance

Parameters	Current Status	2013 Targets	Maximum Potential
Duration of continuous photoproduction • Aerobic, high STH (O_2-tolerant) • Aerobic, limited STH (S-deprivation) • Anaerobic, limited STH (S-deprivation)	 0 10 days 90 days	 30 min	 12 hours indefinite indefinite
O_2 tolerance (half-life in air) • Oxidized conditions • Reduced conditions	 4 min 40 min		
Cost ($/kg H_2) • Aerobic, high STH (O_2-tolerant) • Anaerobic, limited STH (S-deprivation) • Integrated (photo + fermentative)			 $2.99 $6.02 $3.21

Project Milestones

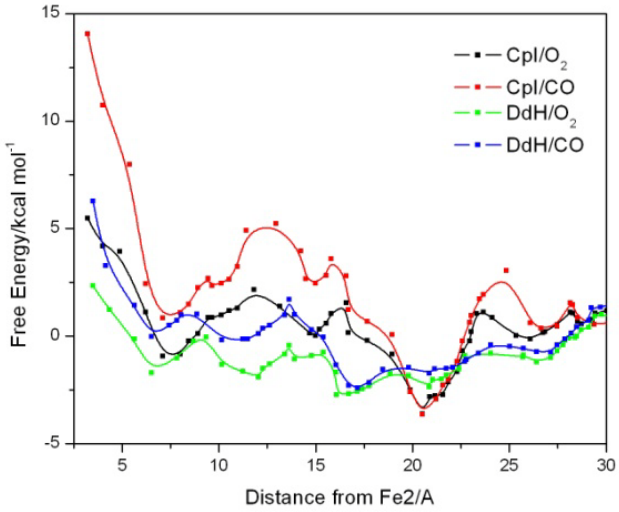
Task 1	Milestone	Due Date	Status
FY10 3.3.6 (carried over to FY11)	Measure the oxygen sensitivity of hydrogenase activity in *C. reinhardtii* transformants expressing an active CaI	03/11	Completed
FY11 3.3.2	Submit data on *in vitro* Fd/H_2ase fusion with MIT	12/10	Completed
Task 2	**Milestone**	**Due date**	**Status**
FY10 3.3.7	Test immobilized ATPase mutants under sulfur-deprived conditions	08/10	Completed
FY11 3.3.3	Demonstrate successful induction of a gene behind a chloroplast inducible promoter	02/11	Completed
FY11 3.3.4	Examine the effect of PEI and/or Ca^{2+} on alginate film stability and culture productivity	02/11	Completed
	Test different gas-to-liquid (or solid) ratios in immobilized algal cell reactors and achieve a 3-fold increase in hydrogen production rates from a photobiological system. ***DOE CPS Agreement Milestone 45590.***	3/11	Completed
FY11 3.3.5	Demonstrate successful characterization of at least two atpE mutants	06/11	In progress
Task 3	**Milestone**	**Due date**	**Status**
FY10 3.3.8 (carried over to FY11)	Determine the carbon mass balance, and H_2 production rates and yields of the scaled-up fermentative system	03/11	In progress
FY11 3.3.6	Determine the efficiency of H_2 photoproduction by S-deprived, photoautotrophic cultures	06/11	In progress

Task 1 – O_2 Sensitivity/Rate of Hydrogenases Objectives, Approaches, and Collaborations

Objectives:

(1) Develop and optimize *aerobic, high-STH* 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.

(2) Develop and test hydrogenase constructs to direct more photosynthetic electron transport to hydrogenase rather than to FNR and CO_2 fixation to increase rates of H_2 production under aerobic conditions.



Task 1 – O_2 Sensitivity/Rate of Hydrogenases Objectives, Approaches, and Collaborations

Approaches:

(1) Computational simulations to identify energy barriers for O_2 access to the hydrogenase catalytic site

(2) Use simulations to guide site-directed and random methods to generate mutants with higher O_2 tolerance.

(3) Introduce a more O_2-tolerant bacterial hydrogenase into algae.

(4) Express hydrogenase-ferredoxin fusions to increase electron flux to the hydrogenase (informal collaboration with Iftach Yacoby and Shuguang Zhang, MIT).

Tasks 1.1 and 1.2 – O_2 Sensitivity of Hydrogenases Accomplishments

Task 1.1

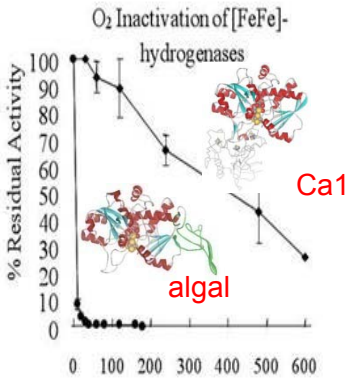
Computational simulations identified differences in the geometries and energies of the gas diffusion barriers protecting the H-cluster in two [FeFe]-hydrogenases with a 1,000-fold difference in the level of O_2 sensitivity.

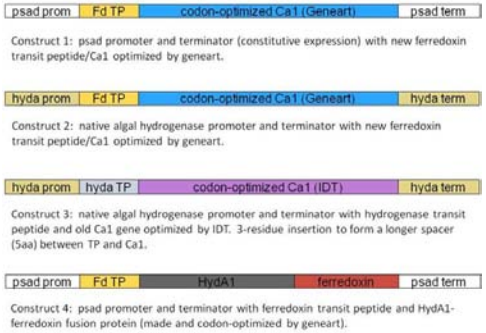
Task 1.2

To change the geometries and increase the energy of accessibility, the regions around the diffusion barriers (not the barrier residues *per se*) are being randomized. These will be expressed and screened in a new high-throughput technique.

Task 1.3 – O_2 Sensitivity of Hydrogenases Accomplishments

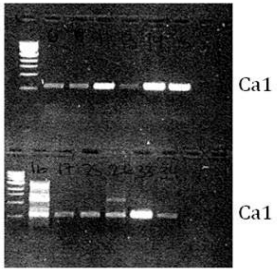
(a) Constructed plasmids for constitutive or inducible expression of the more O_2-tolerant clostridial Ca1 hydrogenase in Chlamydomonas.

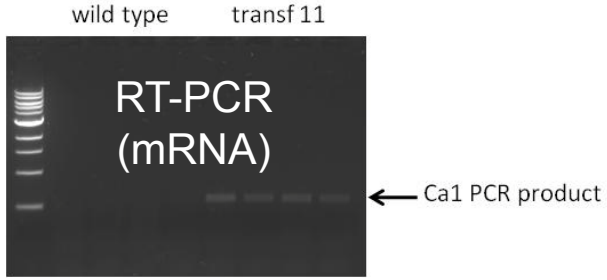




(b) Introduced codon-optimized Ca1 gene in Chlamydomonas (confirmed by PCR) and detected transcription of the gene by RT-PCR.

PCR (genomic DNA)



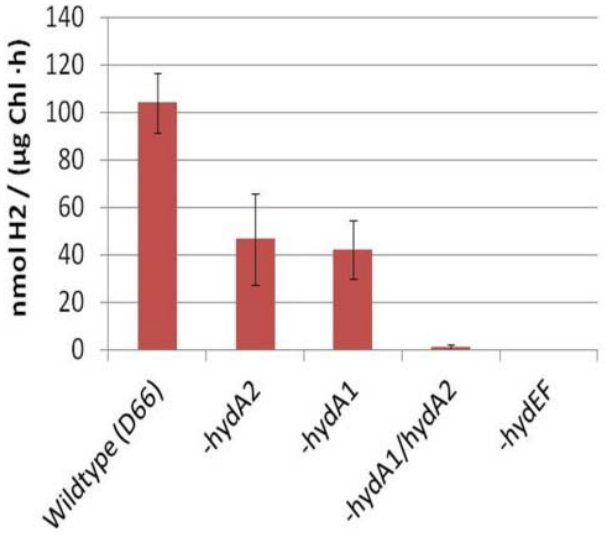


Task 1.3 – O_2 Sensitivity of Hydrogenase Accomplishments

(c) Activity data are ambiguous because of the native algal enzyme background.

(d) Introduce the Ca1 gene in a double hydrogenase mutant (with no native activity) developed under Office of Science (BES and BER) funding.

In progress



FY10 MILESTONE 3.3.6 (carried over to FY11):
Measure the oxygen sensitivity of hydrogenase activity in *Chlamydomonas reinhardtii* transformants expressing an active Ca1 hydrogenase.
Completed.

Task 1.4 – Rate of Hydrogenase Accomplishments and Milestones

INFORMAL COLLABORATION WITH MIT

Created fusions between hydrogenase and ferredoxin to improve photosynthetic reductant flux to the hydrogenase.

FY11 MILESTONE 3.3.2

Publish data on in vitro Fd/H2ase fusion with MIT – manuscript submitted.
Completed.

Task 1 – O_2 Sensitivity/Rate of Hydrogenase Future Work

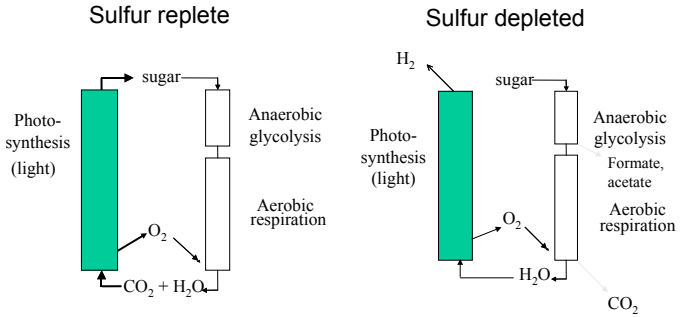
1. **Random mutagenesis**: We will screen new transformants for activity and then measure O_2 tolerance using tools developed from previous funding periods.
2. **Expression of Clostridial hydrogenase in *Chlamydomonas***: Confirm the expression of the Ca1 hydrogenase in the double knock-out hydrogenase mutant; detect the expression of the Ca1 protein with antibodies; complete FY10 milestone 3.3.6; demonstrate that the recombinant Ca1 hydrogenase is linked to photosynthesis in positive transformants.
3. **Hydrogenase-ferredoxin fusions**: The fusion protein will be introduced into *C. reinhardtii* hydrogenase mutant for an *in vivo* evaluation. NREL will continue to collaborate with MIT under a subcontract with the Office of Science.

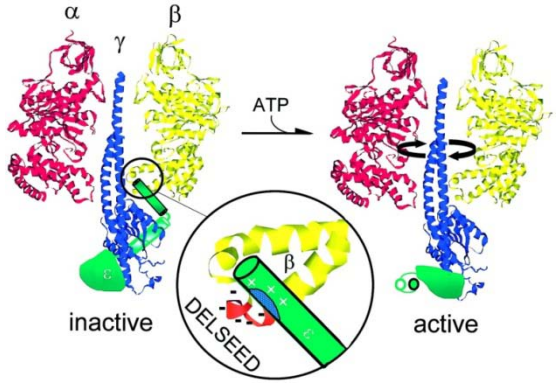
   If the hydrogenase engineering Task 1.1 does not yield results during the remainder of the FY11 funding period, we propose to take advantage of efforts with the Clostridial hydrogenase (Task 1.2), hydrogenase-ferredoxin fusion (Task 1.3), ATP synthase mutants (Task 2) and truncated antenna mutants from Prof. Melis, UCB, and genetically construct a single strain and test H_2 production under sulfur-deprived conditions.

Task 2 – Sulfur-Deprivation Platform Objectives, Approaches, and Collaborations

Objectives:

- Further optimize and utilize an anaerobic, limited-STH working platform to study biochemical and engineering factors that affect H_2 photoproduction by biological organisms.
- Focus on the effect of an inactive, leaky ATP synthase on the rates.





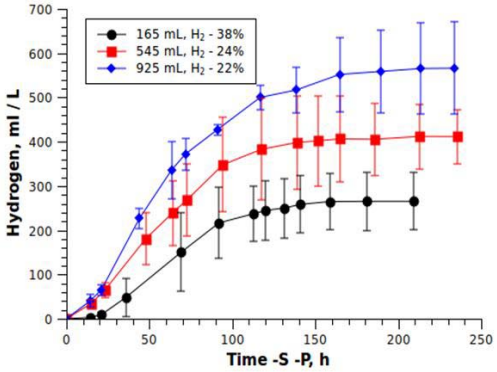
Approaches:

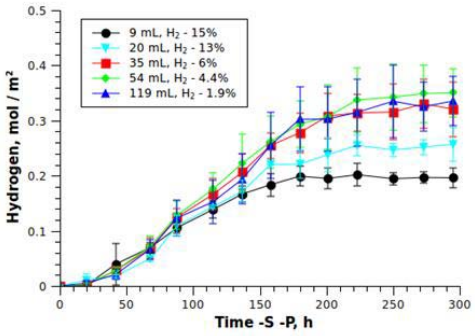
- **Task 2.1**: Optimize the photobioreactor operating parameters (RAS).
- **Task 2.2**: Generate *inducible* ATP synthase mutants (JHU) and test them with the immobilized system (RAS).

Collaborators: Johns Hopkins University (JHU), the Institute of Basic Biological Problems, Russian Academy of Sciences (RAS).

Task 2.1 – Sulfur-Deprivation Platform Accomplishments and Milestones

1. Investigate the effects of different gas-to-liquid ratios in immobilized cell





Gas space volume	Rate of H_2 production (mmoles x mg Chl^{-1} x h^{-1})	Total final yield of H_2
Suspension cultures		
165 mL	4.8	250 mL/L
545 mL	7.3	400 mL/L
925 mL	12.5	560 mL/L
Immobilized cultures		
9 mL	6.1	0.20 mol/m^3
20 mL	9.6	0.24 mol/m^3
35 mL	10	0.33 mol/m^3
54 mL	10	0.36 mol/m^3
119 mL	10	0.35 mol/m^3

2.6-fold increase

1.6-fold increase

Increases in the gas phase yielded the highest reported rate of H_2 photoproduction by sulfur-deprived algae in suspension (**12.5 mmoles mg Chl^{-1} h^{-1}**), and they may not be fully optimized. The rates of cultures immobilized on alginate saturate at about **10 mmoles mg Chl^{-1} h^{-1}**.

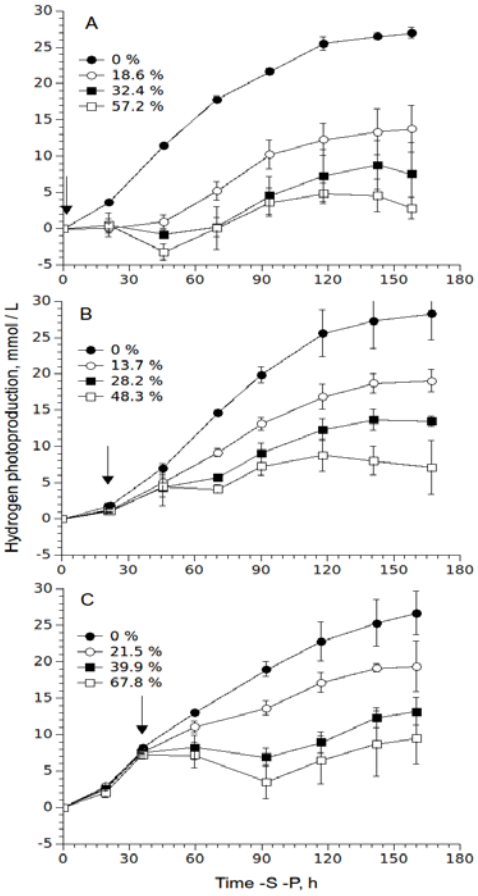
FY11 DOE MILESTONE:

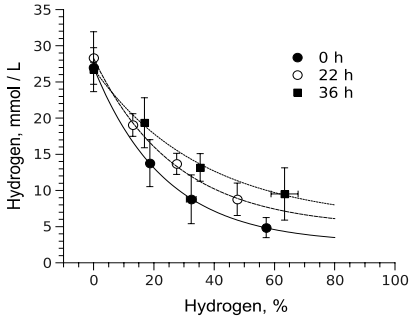
Test different gas-to-liquid (or solid) ratios in suspension and immobilized algal cell reactors and achieve a 3-fold increase in hydrogen production rates from a photobiological system.

Completed.

Task 2.1 – Sulfur-Deprivation Platform Accomplishments and Milestones

2. Determine the effect of H_2 gas pressure on H_2 photoproduction rates by suspension cultures of sulfur-deprived *C. reinhardtii*.



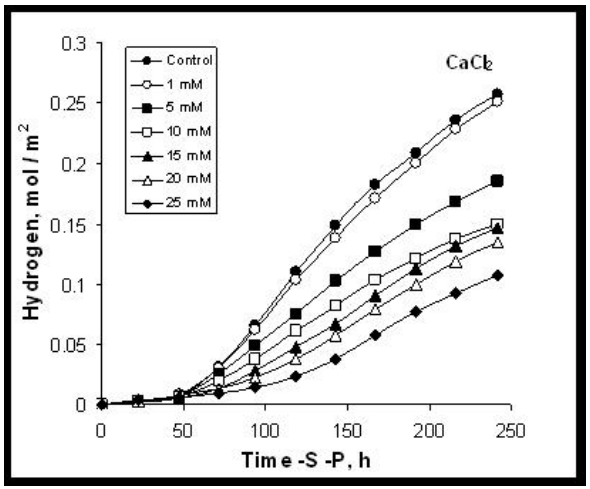


The results confirm the inhibitory effect of H_2 on the reaction and stress the need for gas purging from the photobioreactor to achieve maximum rates of H_2 photoproduction.

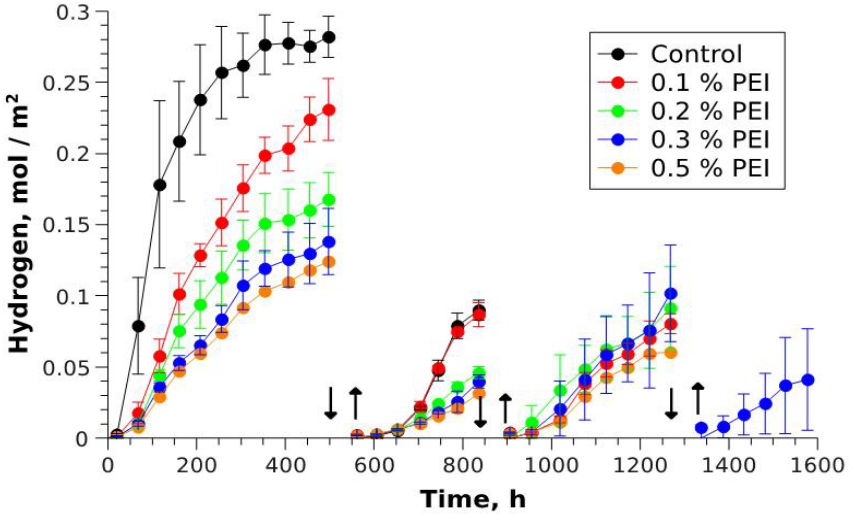
These results and the previous DOE milestone results imply that the cost of gas purging and gas separation must be balanced with the increase in the rates of H_2 production in order to achieve the most economical system.

Task 2.1 – Sulfur-Deprivation Platform Accomplishments and Milestones

Polyethyleneimine were added to increase the mechanical stability of alginate films.



$CaCl_2$ is needed to induce polymerization of alginate. However, $CaCl_2$ > 5mM concentration inhibits H_2 photoproduction rates and does not enhance mechanical stability (not shown).



An increase in the PEI concentration inhibits H_2 photoproduction but increases the mechanical stability of the film, up to 0.3 % PEI (more cycles). The highest total H_2 yield was obtained in films pre-treated with 0.1 % PEI (0.41 mol / m^2).

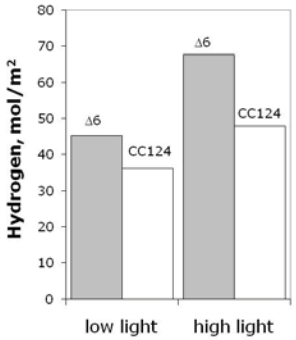
FY11 3.3.4 MILESTONE:

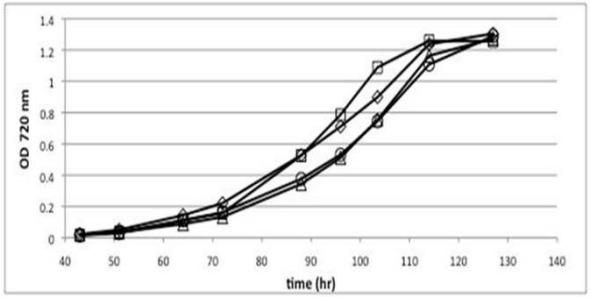
Examine the effect of PEI and/or Ca2+ on alginate film stability and culture productivity.

Completed.

Task 2.2 – Sulfur-Deprivation Platform Accomplishments

1. Generate and test ATP synthase mutants with altered ATP synthase; introduce the mutation into an algal strain containing non-mutated enzymes to allow for normal growth under aerobic conditions with a carbon source.





Growth curves for wild-type and different ATP synthase mutants in the light on TAP medium (not selective).

2. Design a plasmid for expression of the altered ATP synthase, using the orange fluorescent protein (mKO) as a marker behind either the atpA constitutive promoter or the psbD inducible promoter. In progress

Task 2 – Sulfur-Deprivation Platform Milestones

FY10 MILESTONE 3.3.7

Test immobilized ATPase mutants under sulfur-deprived conditions.

Completed.

FY11 MILESTONE 3.3.3

Demonstrate successful induction of a gene behind a chloroplast inducible promoter.

In progress.

FY11 MILESTONE 3.3.4

Demonstrate successful characterization of at least two atpE mutants.

In progress.

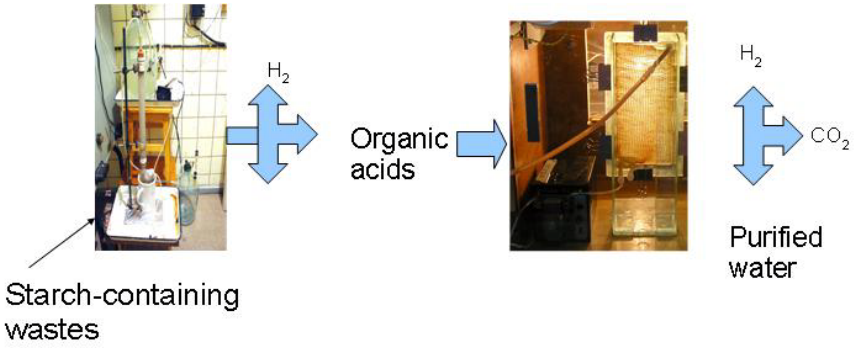
Task 2 – Sulfur-Deprivation Platform Future Work

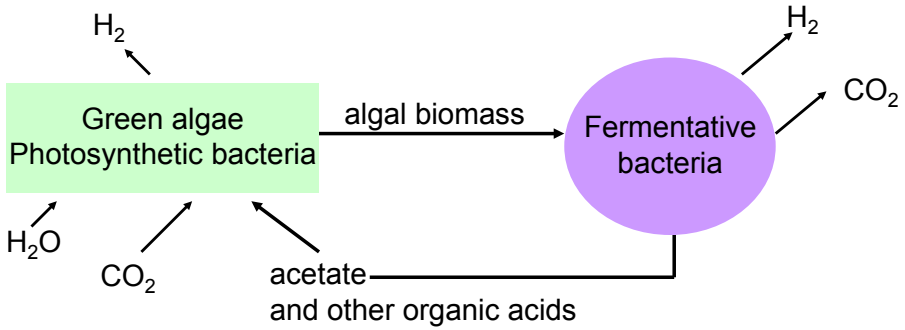
1. **Operational parameters using alginate-immobilized algae:** Perform long-term experiments using cycles of sulfate/phosphate re-addition; run the photobioreactors under continuous flow of medium with added sulfate/phosphate.

2. **Continue to design and test the performance of *Chlamydomonas* inducible transformants carrying a leaky ATP synthase**: Transformants will be tested for growth, photosynthetic activity, and H_2 production capability.

Task 3 – Integrated Systems Objectives

Objective: 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 Systems Approaches and Collaborations

Approaches:

- Integrate fermentative H_2 production from potato waste to photosynthetic H_2 production by anaerobic, purple non-sulfur bacteria (RAS).
- Integrate sulfur-deprived, alginate-immobilized algal H_2 production to fermentative H_2 production by an anaerobic consortium isolated from wastewater sludge, using information from potato waste approach.

Collaborator: Institute of Basic Biological Problems, RAS

Task 3 – Integrated Systems Accomplishments and Milestones

1. Design, test and optimize a two-chamber reactor for integrated fermentation of potato waste with photobiological non-oxygenic H_2 production.

   A membrane was installed between the two chambers, connecting them through holes for diffusion of organic acids.

   Issues encountered: low diffusion of VFAs limits photoproduction of H_2; improper sealing of the membrane did not prevent contamination; inhibition of photosynthetic bacterial growth by factors present in the fermentative effluent; nitrogen sources for photosynthetic bacteria need to be tightly controlled.

2. Independent successive cultivations with pH correction and addition of N_2 gas. Tested different amounts of feedstock, different dilutions of fermentative effluent before transfer to photobioreactor, and a new strain of *Rhodobacter capsulatus*.

Yields of 55% (based on 12 moles H_2/glucose) were obtained with 2% starch (from potato waste), 75-95% dilution of fermentative effluent before feeding the *R. capsulatus* strain N7.

Task 3 – Integrated Systems Accomplishments and Milestones

3. Set up new photobioreactors and determine the carbon mass balance, H_2 production rates and yields of the integrated system using algal biomass (FY10 Milestone 3.3.8).

   In progress, after delayed installation of new photobioreactors.

Task 3 – Integrated Systems Future Work

1. Finish up research on the potato-waste system.

2. Scale up and further optimize fermentation of suspended and immobilized algal biomass by the fermentative consortium, using new Sartorius fermenters; meet FY10 Milestone 3.3.8.

3. Revisit the use of photoautotrophic cultivation conditions for sulfur-deprived cultures, aimed at increasing H_2 yields and decreasing cost by eliminating organic carbon substrate; meet FY11 Milestone 3.3.6).

Summary

Task 1:

- Identified the distance between H-cluster-binding residues as the next target for mutagenesis; created random mutants and started screening for activity.
- Successfully introduced the more O_2-tolerant Ca1 hydrogenase gene in wild-type Chlamydomonas; biphasic kinetics of O_2 inactivation were observed under certain conditions, demonstrating Ca1 activity in combination with native hydrogenase activity; shifted Chlamydomonas host to a double hydrogenase knock-out strain without native activity, generated new transformants, and are in the process of analyzing these transformants.
- Created fusions between ferredoxin and hydrogenase and submitted a manuscript for publication with MIT.

Task 2:

- Observed that an increase in the photobioreactor's gas phase increases rates of H_2 photoproduction by up to a factor of 3; H_2 accumulation in the headspace, on the other hand, leads to decreased rates; results highlight the need for gas purging. Demonstrated optimal levels of PEI and Ca^{+2} that result in enhanced film stability.
- Continued to optimize plasmids for inducible expression of an altered ATP synthase subunit in Chlamydomonas to allow increased rates of electron transport to the hydrogenase.

Task 3:

- Finalized the optimization of an integrated fermentative/photosynthetic reactor system using potato waste as feedstock, achieving yields of 55% conversion from glucose to H_2.
- Initiated studies of an integrated system using algal biomass as feedstock.