Final Report

Metabolic Engineering of Light and Dark Biochemical Pathways in Wild-Type and Mutant Strains of *Synechocystis* PCC 6803 for Maximal, 24-Hour Production of Hydrogen Gas

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Introduction

This document constitutes the final report for the above-named project. The project began on October 1, 2005, and, after a one-year extension, was completed September 30, 2009. The project used the cyanobacterial species, *Synechocystis* PCC 6803, to pursue two lines of inquiry, with each line addressing one of the two main factors affecting hydrogen (H_2) production in *Synechocystis* PCC 6803: NADPH availability and O_2 sensitivity. H_2 production in *Synechocystis* PCC 6803 requires a very high NADPH:NADP+ ratio, that is, the NADP pool must be highly reduced,which can be problematic because several metabolic pathways potentially can act to raise or lower NADPH levels. Also, though the [NiFe]-hydrogenase in *Synechocystis* PCC 6803 is constitutively expressed, it is reversibly inactivated at very low O_2 concentrations. Largely because of this O_2 sensitivity and the requirement for high NADPH levels, a major portion of overall H_2 production occurs under anoxic conditions in the dark, supported by breakdown of glycogen or other organic substrates accumulated during photosynthesis. Also, other factors, such as N or S limitation, pH changes, presence of other substances, or deletion of particular respiratory components, can affect light or dark H_2 production. Therefore, in the first line of inquiry, under a number of culture conditions with wild type (WT) PCC 6803 cells and a mutant with impaired type I NADPH-dehydrogenase (NDH-1) function, we used H_2 production profiling and metabolic flux analysis, with and without specific inhibitors, to examine systematically the pathways involved in light and dark H_2 production. Results from this work provided rational bases for metabolic engineering to maximize photobiological H_2 production on a 24-hour basis. In the second line of inquiry, we used site-directed mutagenesis to create mutants with hydrogenase enzymes exhibiting greater O_2 tolerance. The research addressed the following four tasks: 1. Evaluate the effects of various culture conditions (N, S, or P limitation; light/dark; pH; exogenous organic carbon) on H_2 production profiles of WT cells and an NDH-1 mutant; 2. Conduct metabolic flux analyses for enhanced H_2

production profiles using selected culture conditions and inhibitors of specific pathways in WT cells and an NDH-1 mutant; 3. Create *Synechocystis* PCC 6803 mutant strains with modified hydrogenases exhibiting increased O_2 tolerance and greater H_2 production; and 4. Integrate enhanced hydrogenase mutants and culture and metabolic factor studies to maximize 24-hour H_2 production.

Task 1. Evaluate the effects of various culture conditions (N, S, or P limitation; light/dark; pH; exogenous organic carbon) on hydrogen (H_2) production profiles of wild-type (WT) cells and an NDH-1 mutant.

One of the first objectives of the project was to evaluate the effects of various culture conditions (N, S, or P concentrations; light/dark; pH; exogenous organic carbon) on H_2 production profiles of WT cells and an NDH-1 mutant identified as M55. A further objective was to control culture conditions to optimize photobiological production of hydrogen gas.

By optimizing concentrations of key nutrients in the media of *Synechocystis* sp. PCC 6803, we achieved nearly 150-fold greater photofermentative H_2 production than was achieved by analogous, sulfur-deprived cultures, which were well known to produce much more H_2 than cultures grown on complete media. This was associated with a 44-fold increase in glycogen concentration. Using response surface methodology to determine optimum conditions, we found that, instead of completely starving cells of sulfur or another essential nutrient, the highest H_2 production (0.81±0.36 μmol H_2 mg Chl^{-1} h^{-1}) occurred with 0.52 mM NH_4^+, 20.1 μM SO_4^{2-}, and 46 mM HCO_3^-. H_2 profiling experiments provided initial screening of NH_4^+, HCO_3^-, SO_4^{2-}, and PO_4^{3-} concentrations and identified the significant variables in H_2 production to be NH_4^+, SO_4^{2-}, and the interactions of both NH_4^+ and SO_4^{2-} with HCO_3^-. Our results led to the development of an improved nutrient media (identified as EHB-1) for H_2 production by *Synechocystis* sp. PCC 6803 and indicated that optimized amounts of nitrogen and sulfur in the nutrient media are superior to total deprivation of these nutrients for H_2 production. These results were published in the International Journal of Hydrogen Energy (Burrows, EH, Chaplen, FWR, and RL Ely. 2008. *Int. J Hyd. Energy*. **33**:6092-6099).

Ensuing studies sought to optimize the N concentration and pH of culture media to further increase photofermentative H_2 production from *Synechocystis* sp. PCC 6803. The optimization was conducted using two procedures, Response Surface Methodology (RSM), which is commonly used, and a memory-based machine learning algorithm, Q2,

which had not been used previously in biotechnology applications. Both RSM and Q2 were successful in predicting optimum conditions that yielded higher H_2 than previously reported media optimized for N, S, and C (EHB-1 media), which itself yielded almost 600 times more H_2 than *Synechocystis* sp. PCC 6803 grown on standard BG-11 media. RSM predicted an optimum N concentration of 0.63 mM and pH of 7.77, which yielded 1.70 times more H_2 than EHB-1 media when normalized to chlorophyll concentration (0.68±0.43 μmol H_2 mg Chl^{-1} h^{-1}) and 1.35 times more when normalized to optical density (1.62±0.09 nmol H_2 OD_{730}^{-1} h^{-1}). Q2 predicted an optimum of 0.36 mM N and pH of 7.88, which yielded 1.94 and 1.27 times more H_2 than EHB-1 media when normalized to chlorophyll concentration (0.77±0.44 μmol H_2 mg Chl^{-1} h^{-1}) and optical density (1.53±0.07 nmol H_2 OD_{730}^{-1} h^{-1}), respectively. Both optimization methods have unique benefits and drawbacks that are identified and discussed in this study. Detailed results of these studies were published in 2009 paper (Burrows, EH, Wong, W, Fern, X, Chaplen, FWR, and RL Ely. 2009. *Biotechnol Prog.* **25**(4):1009-1014).

Task 2. Conduct metabolic flux analyses for enhanced H_2 production profiles using selected culture conditions and inhibitors of specific pathways in WT cells and an NDH-1 mutant.

Metabolic engineering of *Synechocystis* sp. PCC 6803 strains with the capability of consistent, high-yield, biosolar H_2 production requires continued development of comprehensive mathematical models describing the metabolism underlying H_2 production and linking genomic, proteomic, and metabolomic information. Such models help to organize disparate hierarchical information, discover new strategies, and understand the essential qualitative features of components and interactions in a complex system (Bailey, J.E. 1998. *Biotechnol. Prog.* **14**:8-20). Metabolic flux analysis is an analytical approach used to estimate the fluxes through a biochemical reaction network operating at steady state based on measured inflows and outflows. Analysis of both over- and underdetermined networks is possible, the latter with linear programming. We used metabolic flux analysis to examine the effect of different network parameters and constraints on photoautotrophic H_2 production by WT *Synechocystis* sp. PCC 6803 and by the high H_2-producing mutant, M55. Two different networks were used with both WT and the M55 mutant strain under chemostat growth: 1) an overdetermined network with 24 metabolites and 20 constraints, requiring at least 4 measurements of fermentation parameters for solution; and 2) an underdetermined network with increased

detail for gene knockout simulations, requiring constraints-based approaches for solution (Price, N.D., Reed, J.L. and Palsson, B. Ø. (2004) *Nature Review|Microbiology* **2**:888-897). The inflows and outflows measured for both networks were H_2, O_2, CO_2, glucose, glycogen, ammonium, and biomass production/consumption. The behavior of both model networks was consistent with WT and M55 mutant phenotypes, thus validating the general approach. The models were then used to provide insights into the possible effects of different mutant phenotypes on H_2 production.

A major factor limiting biosolar H_2 production from cyanobacteria is electron availability to the hydrogenase enzyme. In studies conducted as part of Task 2, we also investigated the effects of five inhibitors of the photosynthetic and respiratory electron transport chains of *Synechocystis* sp. PCC 6803 in order to optimize 24-hour H_2 production using Response Surface Methodology and Q2 (described above). Initial screening showed that cells in full light made the most H_2 in the presence of KCN, that cells in the dark produced the most H_2 when malonate was present in the culture media, and that the two inhibitors worked well together for H_2 production under natural diurnal light/dark cycling. Over 3 days, with 9.40 mM KCN and 1.49 mM malonate we were able to increase H_2 production 30-fold in EHB-1 media previously optimized for N, S, and C concentrations. In addition we measured glycogen concentration over 24 hours with two light/dark cycling regimes in both standard BG-11 and EHB-1 media. Our results indicated that electron flow as well as glycogen accumulation should be optimized in systems engineered for maximal H_2 output (Burrows, EH, Chaplen FWR, and RL Ely. 2011. *Bioresource Technol.* **102**(3):3062-3070).

Task 3. Create PCC 6803 mutant strains with modified H_2ases exhibiting increased O_2 tolerance and greater H_2 production.

For evaluation of H_2 production by modified and WT strains, it was first desirable to develop an assay to carry out high-throughput screening of strains for H_2 production (Schrader, PS, Burrows, EH, and RL Ely. 2008. *Anal. Chem.* **80**(11):4014-4019). The assay we developed is adaptable to various physical configurations, but we used it in a 96-well, microtiter plate format with a lower plate containing H_2-producing cyanobacteria strains and controls, and an upper, membrane-bottom plate containing a color indicator and a catalyst. H_2 produced by cells in the lower plate diffused through the membrane into the upper plate, causing a color change that could be quantified with a microplate reader. We used response surface methodology to optimize the concentrations of the

components in the upper plate. The assay proved to be reproducible, semi-quantitative, sensitive to 20 nmol of H_2 or less, and largely unaffected by oxygen, carbon dioxide, or volatile fatty acids at levels appropriate to biological systems.

We created a *hoxH* mutant of *Synechocystis* sp. PCC 6803, identified as PSS1, by modifying a portion of the enzyme predicted to be adjacent to the active site. Sequence analyses of the genomes of *Ralstonia eutropha*, *Desulfovibrio gigas*, and *Synechocystis* sp. PCC 6803 had shown five conserved regions in the *hoxH* gene, designated as L1 through L5. By deleting 300 base pairs of the gene, we removed all of region L5 and part of L4. We also created a PSS1-ΔhoxH mutant with the hoxH subunit deleted according to Appel et al. (Appel, J., S. Phunpruch, K. Steinmuller, and R. Schulz. 2000. *Arch. Microbiol.* **173:**333-338). We evaluated H_2 production performance of the mutant strains, with and without inhibitors of specific metabolic pathways, in high-throughput screening assay studies, GC vial tests, photobioreactor studies, and membrane inlet mass spectrometer measurements, as described below under Task 4.

Several NiFe hydrogenase enzymes have been characterized as oxygen tolerant (Burgdorf T, O Lenz, T Buhrke, E van der Linden, AK Jones, SPJ Albracht, and B Friedrich**.** 2005. *J. Molec. Microbiol. Biotechnol.* **10:**181-196). One such enzyme is the soluble NiFe hydrogenase from *Ralstonia eutropha*, a lithoautotrophic bacterium (Massanz, C, and B Friedrich. 1999. *Biochem.* **38:**14330-14337). The heterodimeric hydrogenase moiety in *R. eutropha* is very similar to that found in *Synechocystis* (Burgdorf, T, AL De Lacey, and B Friedrich. 2002. *J. Bacteriol.* **184:**6280-6288). Unlike its cyanobacterial counterpart, it is very insensitive to both oxygen and carbon monoxide. This trait was found to be due to increased stability of the active site brought about by the placement of an extra cyanide ligand, performed by an accessory protein HypX (Bleijlevens, B, T Buhrke, E van der Linden, B Friedrich, and SPJ Albracht. 2004. *J. Biol. Chem.* **279:**46686-46691, Jones, AK, O Lenz, A Strack, T Buhrke, and B Friedrich. 2004. *Biochem.* **43:**13467-13477). While the functionality of these two hydrogenase enzymes is quite different, structurally they share significant similarity. Because of these observations, we elected to create a *Synechocystis* sp. PCC 6803 mutant strain, designated as REHX, in which we replaced the gene encoding the wild-type (WT) PCC 6803 hoxH subunit with the *hoxH* gene from the soluble hydrogenase of *Ralstonia eutropha*. We also inserted *hypX*, a gene that encodes an accessory protein essential for oxygen tolerance in *R. eutropha*. The *R. eutropha hoxH* and *hypX* genes were both transcribed into mRNA and hydrogen production was observed, implying that the foreign

hoxH gene was translated and that the protein functioned with the unaltered subunits of the WT hydrogenase. H_2 production and oxygen tolerance of REHX were evaluated, with and without inhibitors of specific metabolic pathways, in high-throughput screening assay studies and in gas chromatograph and membrane inlet mass spectrometer measurements, as described below.

Task 4. Integrate enhanced H_2ase mutants and culture and metabolic factor studies to maximize 24-hour H_2 production.

The high throughput screening assay was used to evaluate H_2 production from each cyanobacterial culture under a variety of conditions. Initial experiments indicated greater H_2 production not only by the M55 strain but also, surprisingly, by the PSS1 strain. To maximize photobiological H_2 production, we ran trials to balance the length of light exposure with the subsequent amount of darkness required for the cells to utilize the oxygen produced during photosynthesis to bring about an anaerobic environment and reactivation of the hydrogenase enzyme.

The most common method of measuring hydrogenase activity uses sodium dithionite and methyl viologen. Sodium dithionite scavenges the oxygen, thereby creating an anaerobic environment while at the same time reducing the methyl viologen that acts directly as an electron donor to the hydrogenase enzyme. The enzyme combines the electrons from methyl viologen and protons from its surroundings to generate H_2 gas. We measured hydrogen on a membrane inlet mass spectrometer (MIMS) using either light as an energy source for the generation of reduced electrons or sodium dithionite-reduced methyl viologen as a direct supply of reduced electrons in testing for hydrogenase activity. Our results were consistent with results from previous studies of the WT and M55 strains (Cournac, L., G. Guedeney, G. Peltier, and P. M. Vignais. 2004. J. Bacteriol. **186:**1737-1746). When we applied these tests to the PSS1 strain we observed WT-like behavior when exposed to light, in that there was no marked increase in hydrogen while a sharp increase in oxygen was detected. When sodium dithionite and methyl viologen were added, no hydrogen production occurred. These results indicated that the hydrogenase activity of the PSS1 mutant had been eliminated as traditionally measured using methyl viologen and sodium dithionite.

Another assay, using deuterium exchange reactions to analyze hydrogenase activity more directly, can measure catalytic activity via the exchange of deuterium from D_2 to HD and the formation of H_2, which happen directly at the active site, and bypasses the

complications of intra- and inter-protein electron transport. We found that WT cells consumed D_2 quickly with a concomitant increase in HD and an increase in H_2. The activity of the enzyme attained its maximum rate of approximately 75 μM min^{-1} almost immediately and maintained that rate over the first 6 minutes of the assay whereupon the D_2 was nearly completely consumed, after which the activity dropped sharply and approached zero by minute 10. As expected, the results were nearly identical with those obtained with mutant M55. However, when the deuterium exchange assay was performed on the PSS1 mutant, after dark anaerobic adaptation, essentially no activity was observed. These results substantiated the data from enzyme activity measurements indicating that the PSS1 mutant had lost activity of the hydrogenase enzyme.

In bioreactor studies of H_2 production, WT, M55, and PSS1 organisms behaved as expected (from what we had seen in the membrane inlet mass spectrometer assays, which rely on the enzyme's ability either to utilize a non-biological electron source or to function equally well in both H_2 production and consumption). However, the PSS1 mutant consistently presented confusing results when analyzed using the high-throughput screening assay (which uses a catalyst and indicator dye as a surrogate measurement for H_2 production). This led us to seek another tool, where H_2 could be measured directly, to help resolve the inconsistent observations with the PSS1 mutant. Bioreactor tests were carried out to compare the WT organism with PSS1 in an attempt to further corroborate data from the screening assay with direct measurement of H_2 under similar conditions. At pH 7.5 the WT made more H_2. However, when the pH was raised to 9.0 the H_2 production profiles flipped and the PSS1 mutant showed more H_2 production than the WT, thereby supporting previous findings from the screening assay. In some ways, even with two of the five conserved regions eliminated, PSS1 behaved similarly to WT PCC 6803. However, H_2 production measurements were inconsistent.

Activity of the hydrogenase enzyme in both the WT *Synechocystis* sp. PCC 6803 and the REHX mutant was analyzed by several methods using the MIMS. To discern the cells' ability to produce hydrogen directly from the splitting of water, the first method looked at cells that had been anaerobically adapted, then exposed to light. A barely detectable amount of hydrogen was produced by the WT organism and the REHX mutant, and the mutant appeared to make slightly less oxygen than the WT, 38 μM vs 31 μM, given the same amount of light. With sodium dithionite and methyl viologen present, hydrogen production by WT cells continued to increase over the 20 minute period to a maximum of nearly 22 μM while hydrogen production in the REHX mutant reached a

maximum of 8 μM after just 2.5 minutes and then began to slowly decline. It is not known for certain where methyl viologen interacts with the hydrogenase enzyme, though it is believed to react with the small subunit (hoxY), which contains 3 [FeS] clusters responsible for transporting electrons to and from the active site. Our previous studies with PSS1 suggested that alterations of the active site subunit could result in an active enzyme lacking the ability to interact with reduced methyl viologen, which would render the traditional sodium dithionite/methyl viologen assay questionable for measuring the activity of a mutated hydrogenase. Perhaps such a phenomenon be due to misalignment of the [FeS] clusters between the small subunit and the large subunit resulting from a conformational shift brought on by the addition of a structurally different, large subunit.

Deuterium exchange reactions were performed in order to analyze the hydrogenase activity more directly. Full enzyme activity was ensured by anaerobic adaptation of the cells. The wild type cells consumed D_2 quickly with a concomitant increase in HD to 28 μM over 3 minutes and an increase in H_2 to 49 μM over 5.5 minutes. The activity of the enzyme attained its maximum rate (of approximately 75 μM min^{-1}) almost immediately and maintained that rate over the first 6 minutes of the assay, whereon the D_2 was nearly completely consumed and the activity dropped sharply and approached zero by minute 10. The REHX mutant behaved quite differently, utilizing only a portion of the D_2 during the assay, with a continuously increasing H_2 concentration over the ten minutes to 56 μM and a maximum HD concentration of 22 μM reached in 4.5 minutes, followed by a slow decline. The calculated enzyme activity also showed substantial differences, with a steady increase over nearly 4 minutes to reach a maximum activity of 30 μM min^{-1}, which then was sustained over the remainder of the assay time period. These results, together with the sodium dithionite/methyl viologen assay results, suggested that the hoxH subunit from *R. eutropha* was translated and an active protein was made. However, under the conditions of these assays the activity of the enzyme appeared to be much lower than that of WT cells.

The low hydrogen production rate detected in the REHX mutant by deuterium exchange reactions raised questions. It was not known whether the REHX hydrogenase would occupy the native location of the native hydrogenase or whether the electron source for the enzyme would be the same. Using selected respiratory inhibitors provided a means to evaluate the interconnectedness of the novel hydrogenase and the source(s) of electrons, it was found that the REHX mutant showed a different inhibitor response profile for hydrogen production compared to M55 and PSS1- ΔhoxH. REHX produced

more hydrogen in the presence of KCN and malonate, while hydrogen production decreased when electron flow to PSI was inhibited. Therefore, the REHX mutant hydrogenase appeared similar to the native hydrogenase regarding its source of electrons. The REHX mutant was found to produce more hydrogen when grown in BG11 media; decreasing nitrogen and sulfur had negative impacts on hydrogen production in this mutant. All strains showed the lowest levels of hydrogen production in the presence of DCMU and DBMIB, indicating that hydrogen production was greatly dependent on the flow of electrons through PSI.