II.E.2 Biological Systems for Algal Hydrogen Photoproduction

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Project Start Date: October 1, 2013
Project End Date: December 31, 2014

Overall Objectives

Develop photobiological systems for large-scale, low-cost, and efficient H_2 production from water to meet DOE's targets (see Table 1).

Fiscal Year (FY) 2014 Objectives

- Quantify the initial and final rates, as well as total H_2 yield following a 30-min illumination of wild-type versus a transformant expressing the more O_2-tolerant *Clostridium acetobutylicum* Ca1 hydrogenase.
- Measure the light conversion efficiency of wild-type and Ca1 transformant under solar intensities.
- Initiate genetic crosses to introduce additional traits to the best H_2-producing Ca1 transformant in order to further enhance its H_2 production capability.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section of the Fuel Cell Technologies Office Multi-Year Research, Development, and Demonstration Plan:

(AO) Rate of Hydrogen Production

(AP) Oxygen Accumulation

Technical Targets

The technical targets for this project are listed in Table 1.

TABLE 1. Progress towards Meeting Technical Targets for Photobiological Algal Hydrogen Production

Characteristic	2014 Status	2015 Target	2020 Target	Ultimate target
Duration of continuous H_2 production under full sunlight intensity	7-30 min[a]	30 min	4 h	8 h
Solar-to-Hydrogen (STH) Energy Conversion Ratio	0.12%	2%	5%	17%

[a]Data variability is responsible for the wide range of values.

FY 2014 Accomplishments

- Demonstrated initial rates of H_2 photoproduction for the Ca1 transformant strain 55 that correspond to about 8% of wild-type, final rates of 10% of wild-type and final net H_2 yield equal to 90% of that measured with the wild-type strain upon 30 min continuous illumination equivalent to solar intensities.
- Estimated a solar conversion efficiency of 0.12% for the mutant versus 0.75% for wild-type strain under solar intensities.
- Demonstrated a 3.6-fold higher average rate of H_2 photoproduction using strains transformed with a linear plasmid containing the Ca1 gene versus strain 55 (transformed with a circular plasmid).

INTRODUCTION

Hydrogen photoproduction is a characteristic of certain microbes, including photosynthetic green algae. *Chlamydomonas reinhardtii* has been a model green alga that has been used to increase our understanding of the H_2 photoproduction process and to test hypotheses regarding factors that need to be addressed to increase and sustain algal H_2 photoproduction capability. One of these factors is the sensitivity of hydrogenases to O_2, a necessary by-product of photosynthetic water oxidation. Other factors include (a) competition for photosynthetic reductant between carbon fixation and hydrogen production, (b) regulatory mechanisms that inhibit electron transport from water to the hydrogenase in the absence of carbon fixation, and (c) the low light saturation of photosynthesis due to the large number of light-harvesting molecules associated with its photosynthetic apparatus.

NREL's approach to address the barriers to H_2 photoproduction consisted of introducing the gene encoding the more O_2-tolerant hydrogenase from the anaerobic bacterium *Clostridium acetobutylicum* into *Chlamydomonas*, followed by integration of known genetic traits that address the other barriers to efficient and sustained H_2 photoproduction.

APPROACH

In previous funding periods, we developed methods to introduce and stably express the Ca1 hydrogenase in a Chlamydomonas strain, *hyd-*, in which the native hydrogenase genes, *HYDA1* and *HYDA2* had been genetically knocked out. We observed that various samples with a positive phenotype (H_2-production measured with the GFP assay[1]) had to undergo a couple of rounds of re-plating to yield homogeneous single colonies. This year, we tested the effect of introducing different combinations of introns into the deoxyribonucleic acid (DNA) construct carrying the Ca1 gene, as well as the efficacy of using linear versus circular plasmid in Ca1 expression. Both approaches have been shown to increase expression of heterologous genes in *Chlamydomonas*. Concomitantly, we started genetic crosses to introduce the *pgrl1* and *tla3* mutations into the *hyd- Chlamydomonas* strain for future crosses with the Ca1 transformant. The *pgrl1* strain was reported to exhibit higher rates of H_2 photoproduction due to the lack of cyclic electron transport (Tolleter, D., et al.) [1]; the *tla3* mutant has a truncated light-harvesting antenna and its photosynthetic rates saturate at much higher light intensities than the wild-type strain (Kirst, H., et al.) [2].

RESULTS

The first milestone for FY 2014 required us to benchmark the STH conversion efficiency and duration of H_2 production using wild-type versus our best transformant which, at the time, was strain 55. We performed our experiments in the Clark electrode chamber, under illumination from LEDs that emitted 2,000 µEinsteins m^{-2} s^{-1}, which is the equivalent to the photosynthetic active radiation region at one sun intensity (2,500 J/m^{-2} s^{-1}). The uncoupler carbonyl cyanide-p-trifluoromethoxyphenol hydrozone (FCCP) was used to eliminate the down-regulation of electron transport by non-dissipation of the proton gradient. We measured the effect of cell density (represented by Chl concentration) on H_2 photoproduction rates and converted the rates into STH, using the value of 242 kJ for each mole of H_2. Figure 1 shows an STH value of about 0.75% for the wild-type strain and about 0.12% for strain 55. Although the reported STH values are low compared to the programmatic targets, it must be noted that the measurements were done under less than optimal conditions, using a 2 ml-volume "photobioreactor" with an essentially zero headspace, due to technical challenges involved in measuring low rates. The latter prevented fast equilibration of gases between the liquid and gaseous phase, and may have limited the observed rates. Indeed, Kosourov et al. [3] demonstrated that increases in the gas/liquid volume ratio have a significant effect on the rates of H_2 photoproduction.

In order to complete the second quarter Go/No-Go milestone for FY 2014, we measured H_2 photoproduction by wild-type and strain 55 (our best H_2-producing strain from a pool of transformants generated by introduction of a circular plasmid containing the Ca1 gene under regulation of the PsaD promoter) for a total of 30 minutes and estimated initial and final rates, as well as total H_2 yield. The results, which are shown in Table 2, reflect the variability of the measurements, with very high standard deviations. In summary, the initial rate target of 11 mmoles H_2 mg Chl^{-1} h^{-1} was met, the final rate target of 0.06 mmoles H_2 mg Chl^{-1} h^{-1} was not, and the final net H_2 yield value was slightly lower than the target value (equal or higher than wild type, WT). As a result the decision was a No-Go. The high data variability for WT and mutant strain is not very well understood; we have attempted to optimize growth and induction conditions, as well as the experimental set-up, without much success, so far, and this needs to be addressed more carefully in the future.

TABLE 2. Estimated Parameters for WT and Ca1-Expressing Strain 55; Standard Deviations were Calculated from 6 WT and 13 Strain 55 Individual Curves

Strain	Initial Rate	Final rate	Total H_2 Yield
WT (D66)	160 ± 35	-0.47 ± 0.14	0.36 ± 0.20
Strain 55	13.25 ± 7.56	-0.05 ± 0.21	0.31 ± 0.20

As an alternative approach to increase the activity of the Ca1 transformants, we used our previous expression construct (PsaD Ca1+) and added three introns into the Ca1 open reading frame. We also used two commercial plasmids (from Invitrogen) under the regulation of the Hsp70A/RbcS2 promoter/terminators that induce high constitutive expression levels. Into one of the commercial plasmids, we introduced the codon-optimized Ca1 gene carrying an intron in its 5'UTR (pChlamy Ca1); the second plasmid carried additional three introns into the Ca1 open reading frame (pChlamyCa1 introns), similarly to the PsaD $Ca1^+$ construct. Unfortunately, neither of the new intron-containing plasmids resulted in higher Ca1-expressing strains (not shown).

Our next alternative used an excised plasmid (linear) containing no introns, under the regulation of the PsaD promoter ($PsaD_{excised}$). We introduced it into the *hyd- Chlamydomonas* strain, screened transformants using the GFP assay, and selected transformants with high H_2 production rates as measure by the Clark electrode. Two of them were further re-plated and underwent another round of

[1] Green fluorescent protein-based H_2-sensing assay developed by NREL (Wecker et al., Biotechnol. Bioeng. 111, 1332-1340).

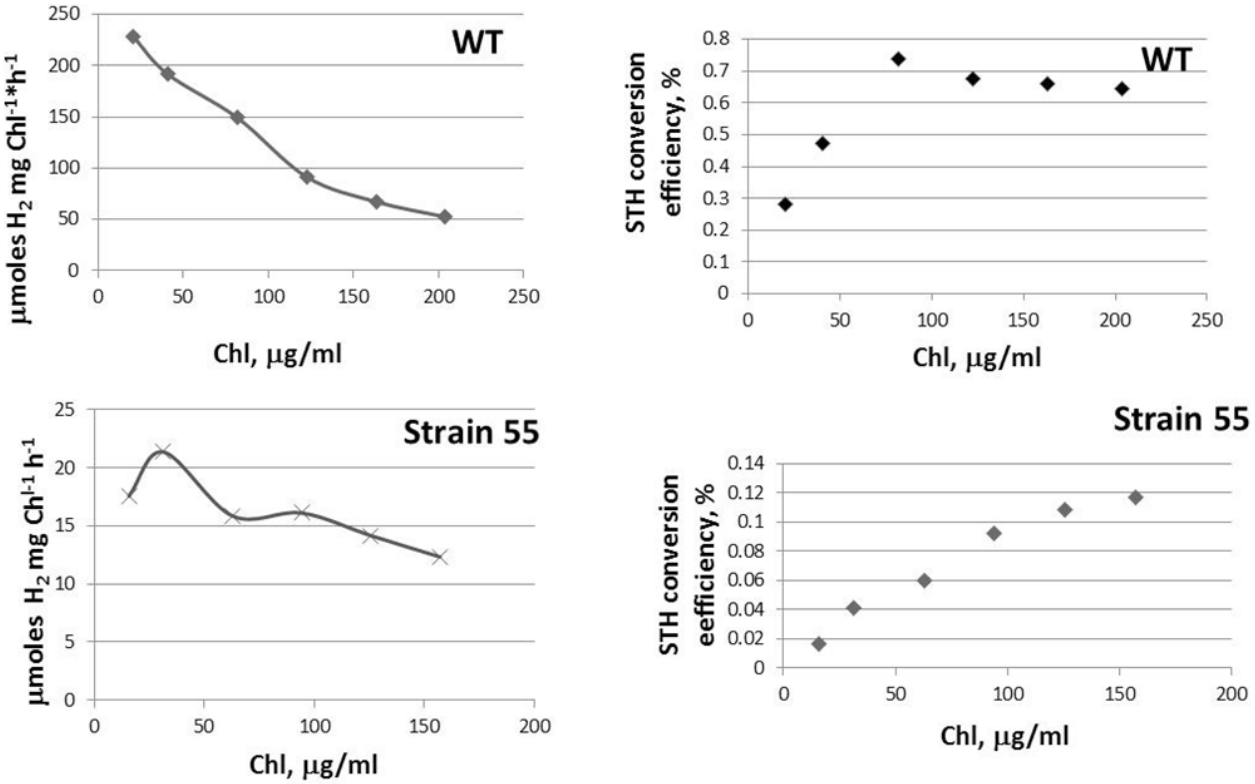

FIGURE 1. Left: Rates of H_2 production (µmoles H_2 mg Chl^{-1} s^{-1}) by representative WT (top) and strain 55 (bottom) as a function of the Chl concentration in the electrode chamber. Right: Estimated STH conversion efficiency of WT (top) and strain 55 (bottom) as a function of Chl concentration in the electrode chamber.

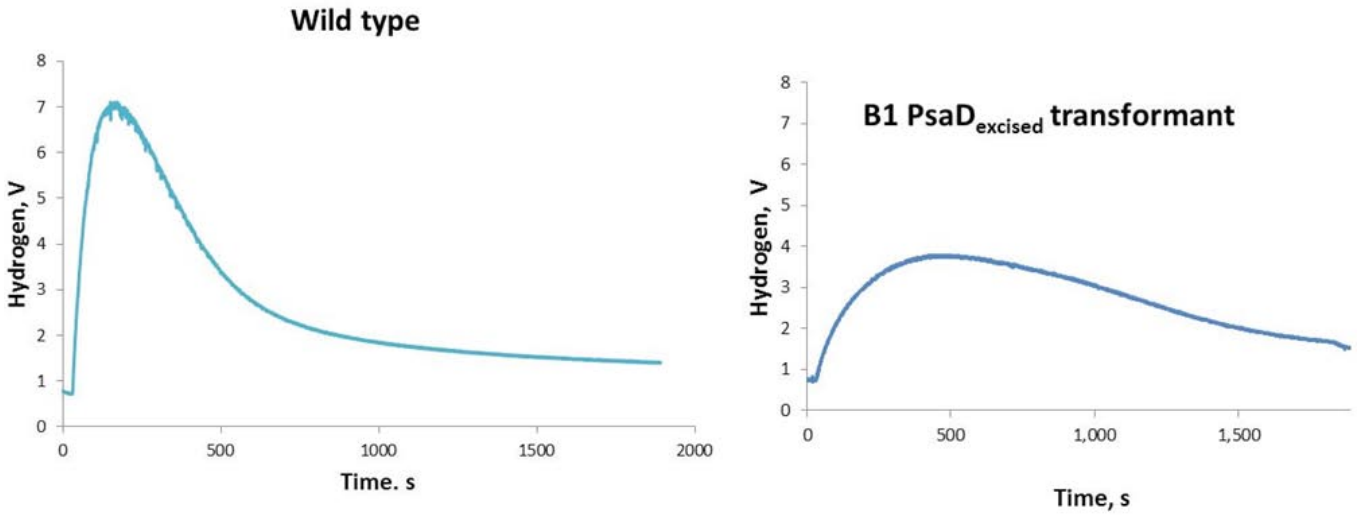

FIGURE 2. Hydrogen levels (measured as volts) in the Clark electrode chamber during a 30-min illumination period. WT (left) and B1 transformant (right). Initial rates were estimated from the slope of the curves during the initial 100 s; final rates represent the slopes during the last 500 s; total H_2 yield was determined by subtracting the voltage at the beginning of the illumination to that at the end of the illumination period and converting the number into moles H_2 per Chl concentration, as discussed in the text. Estimated initial rates determined from the representative curves are 163 and 49.5 mmoles H_2 mg Chl^{-1} s^{-1} for WT and the B1 transformant, respectively.

GFP selection. Transformants B1 and C2 exhibited average rates 3.5-fold higher than strain 55 (transformed with a circular plasmid) and 29% of the average WT initial rates (Figure 2 and Table 3). However, there is considerable H_2

uptake during the 30-min experiment, resulting in a total final H_2 yield that is similar to those of the WT and strain 55. We will use either of the two transformants in our final genetic cross. Nevertheless, the maximum H_2 peak occurs at longer times than that recorded for the WT strain, at about 7.2 vs. 2.6 min, demonstrating that the phenotype of the Ca1-expressing mutant is indeed different from that of the WT strain.

TABLE 3. Total H_2 Yield, Initial and Final Rates of H_2 Photoproduction by Mutant Strains Carrying Excised Plasmid

Strain	Initial rate (µmoles H_2 mg Chl^{-1} h^{-1})	Final rate (µmoles H_2 mg Chl^{-1} h^{-1})	Total H_2 yield/Chl (µmoles H_2 mg Chl^{-1})
B1	29	-0.488	0.102
	49.5	-1.59	0.317
C2	78	-2.336	0.554
	30.6	-1.036	0.24
Average all	**46.8**	**-1.36**	**0.303**
% average WT[1]	29	289	84
% average strain 55[1]	350	2,700	98

[1] See Table 2 for average values used here

CONCLUSIONS AND FUTURE DIRECTIONS

- The data show that it is possible to generate Ca1-expressing mutants with final net H_2 yields comparable to those of the WT strain. The best transformants to date (strains B1 and C2) show initial rates equivalent to about 29% of WT, but do not exhibit significantly higher final net H_2 yields/Chl, although peak production occurs at later timepoints.
- The STH conversion efficiency of mutant (strain 55) and WT strains are currently very low (0.12 and 0.75%, respectively) but could be increased through reactor engineering approaches. We have not yet measure the STH of our latest mutants.
- Intron-containing constructs did not increase the levels of Ca1 expression by *Chlamydomonas*, but the use of linear versus circular plasmids resulted in an average 3.6-fold increase in the initial rates.
- This project will be terminated at the end of the first quarter of FY 2015. The work remaining to be completed in FY 2015 focuses on generating and characterizing the final transformant strain, *pgrl1 tla3 hyd*- Ca1. We expect that the results will direct future research towards improving algal H_2 photoproduction.

FY 2014 PUBLICATIONS/PRESENTATIONS

1. Presentation to the Hydrogen Production Tech Team at PNNL (July 2013).

2. Poster presentation at the International Photosynthesis Conference, St. Louis (August 2013).

3. Invited speaker at the University of Rochester, NY (Dec. 2013).

4. Invited seminar presentation at the University of Colorado (Jan. 2014).

REFERENCES

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2. Kirst, H., Garcia-Cerdan J., Zurbriggen A., Ruehle, T. and Melis, A. *Plant Physiol.* 160, 2251-2260.

3. Kosourov, S., Ghirardi, M.L. and Seibert, M. (2011) *Int. J. Hydrogen Res.* 36, 2044-2048.