

II.E.2 Sweet Hydrogen: High-Yield Production of Hydrogen from Biomass Sugars Catalyzed by in vitro Synthetic Biosystems

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- Increase hydrogen production rates two-fold (i.e., 300 mmol H₂/L/h).
- Scale up hydrogen production volume by 10-fold (i.e., 10 mL bioreactor).
- Demonstrate 10-fold volume scale-up with two-fold increase in hydrogen peak production rate (i.e., 300 mmol H₂/L/h) on starch.

Technical Barriers

This project addresses the following technical barriers of biological hydrogen production from the Hydrogen Production section of the Fuel Cell Technologies Office Multi-Year Research, Development, and Demonstration Plan pertaining to dark fermentation.

(AX) Hydrogen Molar Yield

(AY) Feedstock Cost

(AZ) Systems Engineering

Technical Targets

Progress of in vitro enzymatic hydrogen production has been made in achieving the project targets. Table 1 lists the project technical targets (i.e., production cost, productivity, and reactor volume) and where our research and development efforts stand to date. The overall goals of this project are to decrease enzymatic hydrogen production cost, increase its production rate, and scale up its production volume. Our goals would clear up doubts pertaining to enzymatic hydrogen production cost, rate, and scalability for future distributed hydrogen production from renewable liquid sugar solution.

Overall Objectives

- Decrease the production costs 1,000-fold from ~\$10,000/kg (current estimated level) to ~\$10/kg of hydrogen as estimated by using the H2A model by the end of the project.
- Increase the volumetric productivity five-fold from current levels of ~150 mmol H₂/L/h to 750 mmol H₂/L/h.
- Scale up in vitro enzymatic hydrogen production 1,000-fold from 1-mL to 1-L bioreactor.

Fiscal Year (FY) 2016 Objectives

- Decrease hydrogen production costs by co-expression of multi-enzyme in one host, discovery of novel enzymes, and coenzyme engineering.

FY 2016 Accomplishments

- Achieved one of the highest biological H₂ generation peak rates – 320 mmole of H₂/L/h (i.e., ~164 L H₂/L/day).

TABLE 1. Virginia Tech and University of Georgia Status

Targets	Units	June 2016 Project Target	June 2017 Project Target	Year 2020 Plant Gate
Production Cost	\$/kg H ₂	1,000 (estimated)	10	10 (2020 DOE goal for advanced biological generation technologies)
Productivity	mmol H ₂ /L/h	320 (achieved)	750	2,000 (our goal)
Reactor Volume	L of reactor	0.01 (achieved)	1	2,777* (our goal)

*200 kg H₂ per day

- Scaled up recombinant enzyme production 1,000-fold from several milligrams to tens of grams and recombinant hydrogenase production 50-fold from ten milligrams to 500 milligrams.
- Designed and validated biomimetic electron transport chains for accelerating hydrogen peak generation rates five-fold from ~60 mmol to ~300 mmol of H₂/L/h.
- Validated the feasibility of engineering dehydrogenases to work on a low-cost and more stable biomimetic coenzyme, nicotinamide riboside (NR).
- Found out ways to precisely control expression level of each enzyme in multiple-gene coexpression.



INTRODUCTION

Water splitting for hydrogen production is critical for sustainable, renewable hydrogen production. Water electrolysis suffers from high production costs and low electrolysis efficiencies. Water splitting at high temperature requires high temperature thermal energy sources and raises serious material challenges. Water splitting at low or even ambient temperature is highly desired when considering material challenges, availability of high-temperature thermal energy sources, and distributed hydrogen production systems. A few methods conducted at low temperatures are under investigation, including photocatalytic water splitting, photo-electrochemical water splitting, photobiological water splitting, and microbial electrolysis cells. However, they suffer from low hydrogen generation rates and/or low energy conversion efficiencies.

Renewable hydrogen production via water splitting energized by chemical energy stored in biomass is extremely attractive because biomass sugars are the most abundant renewable chemical energy [1]. However, microbial anaerobic fermentation (dark fermentation) suffers from low hydrogen yields, where the theoretical yield is 4 H₂ per glucose molecule according to the reaction: C₆H₁₂O₆ + 2 H₂O = 4 H₂ + 2 CH₃COOH (acetate) + 2 CO₂. Although microbial electrolysis cells enable the utilization of acetate or other organic matter supplemented with an electrical input to split water to generate more hydrogen, this two-step conversion requires two reactors, has decreased energy efficiency compared to the theoretical hydrogen yield (i.e., 12 H₂ per glucose molecule and water) due to electricity consumption, slow hydrogen generation rates, and requires high capital investment of microbial water electrolysis. Recently, we demonstrated in vitro synthetic enzymatic biosystems to generate theoretical yields of hydrogen energized by numerous carbohydrates, such as, starch, cellodextrins, glucose, xylose, and xylooligosaccharides [2]. But some serious barriers to industrial scale-up potential remain,

including (1) enzyme production cost, (2) enzyme stability, (3) coenzyme cost and stability, (4) (slow) reaction rates, and (5) scale-up feasibility [3]. In this project, we propose to address the above issues at the laboratory scale.

APPROACH

The general approach for this project is to apply biochemistry and protein engineering, microbiology, molecular biology, chemistry, and engineer design principles to address technical barriers pertaining to industrial needs of enzymatic hydrogen production (i.e., production costs, reaction rate, and scalability). We have multiple subtasks aiming to achieve each objective. To decrease hydrogen production costs, we co-expressed multiple enzymes in one *E. coli* host and found out the best strategy to precisely control protein expression levels; discovered new hyperthermophilic enzymes; and engineered coenzyme preference of dehydrogenases to biomimetic coenzymes. To increase hydrogen generation rates, we developed kinetic model and identified the rate-limiting steps; constructed novel biomimetic electron transport chains; and built enzyme complexes featuring substrate channeling. To scale up hydrogen production, we scaled up recombinant protein production in *E. coli* and recombinant hydrogenase production, as well as demonstrated hydrogen productions in large bioreactors.

RESULTS

The overall goal of the first phase of this project was to demonstrate 10-fold volume scale-up with two-fold increase in hydrogen peak production rate (i.e., 300 mmol H₂/L/h) on starch. Figure 1A shows the profile of hydrogen evolution from the starch/water solution catalyzed by the synthetic enzymatic biosystem, which was conducted in a 10-mL bioreactor (Figure 1B). The peak hydrogen generation rate demonstrated was 320 mmol H₂/L/h (meeting the Phase 1 go/no-go criteria), which is one of the highest biological hydrogen production rates reported, compared to dark fermentation, photobiological means, and microbial electrolysis cells.

To decrease hydrogen production costs, we have three subtasks: (1) decrease enzyme production costs, (2) discover better enzymes, and (3) engineer the coenzyme preference of dehydrogenases.

Decrease Enzyme Production Costs

To decrease enzyme production costs and the number of *E. coli* hosts for protein production, we co-expressed four enzymes in one host at its maximum recombinant protein production capacity and hoped to precisely control each enzyme expression level for nearly equal enzyme activities, where individual apparent activity is equal to specific

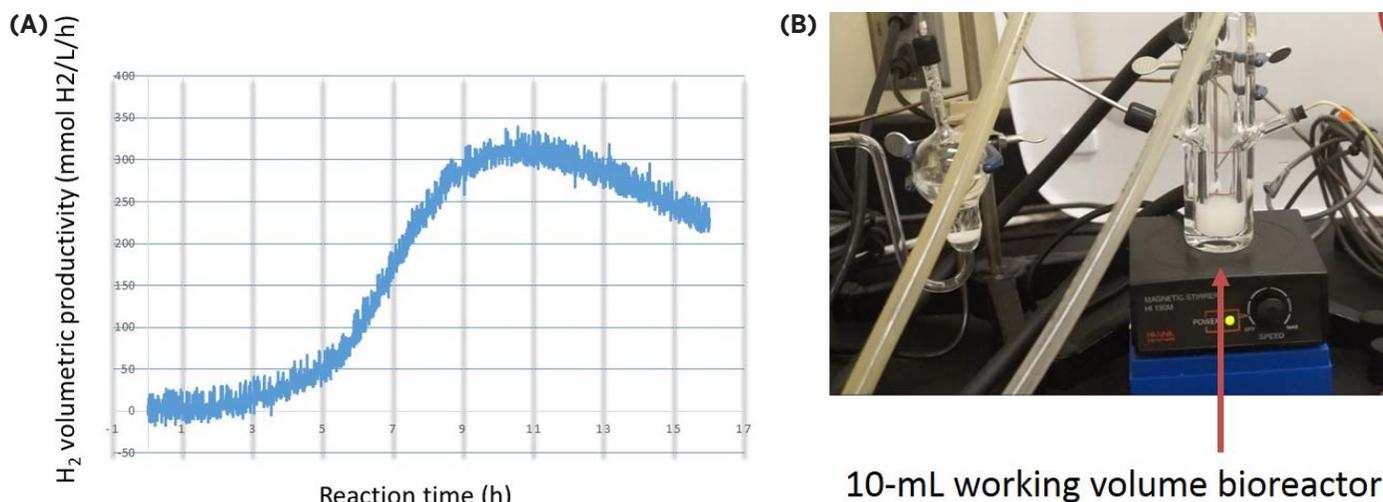


FIGURE 1. The profile of the hydrogen evolution from isoamylase-treated corn starch (A) and photo of 10-mL bioreactor for enzymatic hydrogen production based on starch (B).

activity of individual enzyme multiplied by individual enzyme percentage in the cell lysate. We tested four different strategies, testing one vector (Cases 9, 10, and 11) versus two vectors (Case 8), and different deoxyribonucleic acid transcription regulation mechanisms, whether to put the T7 terminator behind each gene (Cases 8 and 11) or only after the last gene (Cases 9 and 10) and/or T7 promoter before the each gene (Cases 8, 10, and 11) or only before the first gene (Case 9) to control protein expression levels (Table 2). The best strategy for our four enzymes is Case 8 with two vectors, each of which encodes two genes and each gene has its own promoter and terminator (Table 2). Consequently, the calculated apparent activity for each of the enzymes in Case 8, based on the enzyme proportion results from the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the previously tested specific activity for each enzyme, wherein specific activities of α GP, precious group metal (PGM), glucose 6-phosphate dehydrogenase (G6PDH), and 6-phosphogluconate dehydrogenase (6PGDH) are 20, 350, 35, and 16 U/mg, respectively [2], is similar for

all enzymes in the cell lysate being ~ 1.6 U/mL of cell culture (Table 2). This suggests that none of the enzymes were over-expressed relative to the others.

Discover Better Enzymes

To decrease the enzyme costs through the use of enzymes with more stability (i.e., longer lifetime) and higher specific activities (i.e., less protein use in terms of mass), we have cloned nine new hyperthermophilic enzymes to replace previous modestly thermophilic enzymes. Also, all of the enzymes used in this project can be easily purified by heat precipitation, where $\sim 80^\circ\text{C}$ heat treatment can deactivate all of the *E. coli* proteins. In the future, enzymatic hydrogen can be produced by a mixture of the heat-treated cell lysates without costly enzyme purification steps.

Engineer the Coenzyme Preference of Dehydrogenases

To decrease coenzyme costs of in vitro biosystems, we did coenzyme engineering, changing the coenzyme

TABLE 2. Comparison of Four-Enzyme Co-Expression Cases in *E. Coli*. The apparent activities of the individual enzyme in the cell lysates and the total activities of the four-enzyme cell lysates based on xylitol formation.

Case Name ^a	Expression ratio (%)					Apparent activity (U/mL) ^b				Total activity (mM) ^c
	α GP	PGM	G6PDH	6PGDH	Sum	α GP	PGM	G6PDH	6PGDH	
Case 8	17	0.75	28	20	66	1.6	1.9	1.9	1.3	24.58
Case 9	30	6.2	4.2	1.6	42	2.8	16	0.29	0.10	15.48
Case 10	28	6.9	3.9	1.3	40	2.6	18	0.27	0.083	15.27
Case 11	16	5.4	3.6	17	42	1.5	14	0.25	1.1	16.67

^a Case 8, two vectors, each of vector encodes two enzyme genes and each gene has its own T7 promoter and T7 terminator; Case 9, 10 and 10, one vector encoding four enzymes. Case 9, one gene-cluster containing *6pgdh*, *g6pdh*, *agp*, and *pgm* (one T7 promoter and one T7 terminator); Case 10, the four T7 promoters for the four genes and one T7 terminator in the end of the four-gene cluster; Case 11, one T7 promoter and one T7 terminator for each gene.

^b apparent activity = specific activity of individual enzyme \times individual enzyme percentage in the cell lysate.

^c total activity of the four-enzyme cell lysate was measured based on the formation of xylitol from xylose.

and confirmed that the rate-limiting step of the whole biosystem is hydrogen generation from NADPH catalyzed by hydrogenase SH1. Inspired by the natural electron mediator ferredoxin protein for high-speed biohydrogen production bacteria, we investigated the use of small-size non-protein electron mediators such as, benzyl viologen (BV), methyl viologen, and neutral red. We discovered that the best electron mediator was BV for NADPH. The biomimetic electron transport chain was from NADPH (generated from the pentose phosphate pathway) to BV catalyzed by NADPH rubredoxin oxidoreductase (NROR), to hydrogen catalyzed by SH1 (Figure 3A). Starting from substrate G6P via the biomimetic enzymatic pathway, the peak hydrogen generation rate was 310 mmol H₂/L/h at 80°C (Figure 3B). We also constructed six G6PDH-6PGDH-SH1 enzyme complexes, which may facilitate electron transfer among adjacent enzymes. Our preliminary data indicates the feasibility of further reaction rate enhancements.

To scale up enzymatic hydrogen production, we conducted high-cell density fermentation in 6-L fermenter and accomplished the cell density of ~50 g dry cell weight per liter. Compared to 1-L flask, we were able to increase enzyme production capability by more than 1,000-fold. Such information suggests that bulk enzyme production costs could be as low as \$50/kg [4]. By changing the promoter and enzyme purification tag of SH1, we increased SH1 production capability by 50-fold. Consequently, we scaled up our hydrogen production in 10-mL reactor.

CONCLUSIONS AND FUTURE DIRECTIONS

Although sweet hydrogen production is still in its early stage. Several conclusions can be made:

- The high biological H₂ generation rates achieved suggests that these in vitro hydrogen generation rates are fast enough to produce hydrogen at stationary hydrogen bioreactors.

- Engineered dehydrogenases were able to work on biomimetic coenzymes and novel high-throughput screening methods for biomimetic coenzyme engineering were established.

Future work includes:

- Further increase hydrogen production rate.
- Enhance activities of dehydrogenase mutants working on biomimetic coenzymes.
- Scale up enzymatic hydrogen production to 1 L.
- Conduct detailed economic analysis of enzymatic hydrogen production by using H2A model.

FY 2016 PUBLICATIONS/PRESENTATIONS

- Moustafa, Hanan M.A., Taha I. Zaghoul, and Y.H. Percival Zhang. "A Simple Assay for Determining Activities of Phosphopentomutase from a Hyperthermophilic Bacterium *Thermotoga Maritima*." *Analytical Biochemistry* 501 (2016): 75–81.
- Moustafa, H.M.W., E-J. Kim, Z.G. Zhu, C-H. Wu, T.I. Zaghoul, M.W.W. Adams, and Y-HP. Zhang. "Water Splitting for High-Yield Hydrogen Production Energized by Biomass Xylooligosaccharides Catalyzed by an Enzyme Cocktail." *ChemCatChem* (2016): DOI: 10.1002/cctc.201600772, in press.
- Kim, J-E., R. Huang, H. Chen, C. You, and Y-H.P. Zhang. "Facile Insertion of a Large-Size Random Gene Mutagenesis Library into *Escherichia Coli* Plasmid without Restriction Enzymes." *Biotechnology Journal* (2016): DOI: 10.1002/biot.201600121.
- Huang R., Chen H., Zhong C., Kim J-E., Zhang Y-H.P. "High-throughput screening of coenzyme preference change of thermophilic 6-phosphogluconate dehydrogenase from NADP⁺ to NAD⁺." *Scientific Reports* (2016), accepted.
- Invited keynote talk. "Biomufacturing 4.0: in vitro synthetic enzymatic biosystems beyond the cells' constraints." International Metabolic Science Conference. Shanghai Jiao-Tong University. Shanghai, China (12/15/2016).

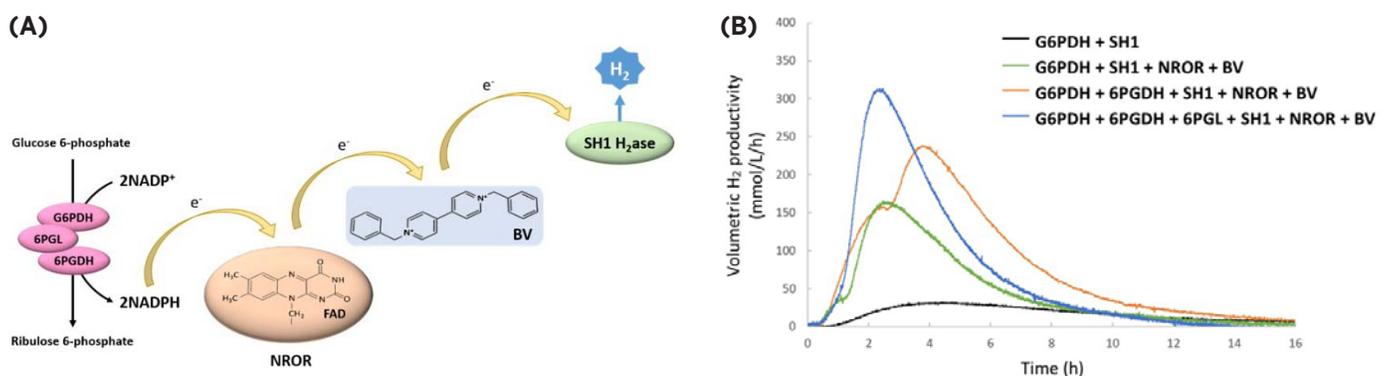


FIGURE 3. Scheme of the biomimetic electron transport chain from G6P to NADPH to H₂ via BV, where the enzymatic cocktail contains G6PDH, 6PGDH, 6PGL, DI, and SH1 (A) and the high-speed H₂ evolution profile by using the enzyme cocktail: G6PDH, 6PGDH, 6PGL, deionized water and SH1, plus BV, on glucose 6-phosphate (B).

6. Invited talk. “Biomufacturing 4.0: in vitro synthetic enzymatic biosystems beyond the cells’ constraints.” Tianjin University Sino-USA Synthetic Biology Conference. Tianjin, China. (12/04/2015)
7. Invited keynote talk. “In vitro metabolic engineering would solve key problems of to the food-energy-water nexus.” Metabolic Engineering Summit 2015, Beijing, China (12/02/2015).
8. Invited plenary talk. “Beyond cell’s constraints: in vitro synthetic enzymatic biosystems for next generation biomanufacturing.” Division 15A speaker for lenary symposium of the AIChE National Conference 2015. Salt Lake City, UT, USA. (11/08/2015)
9. Invited keynote talk. “Biomufacturing 4.0: in vitro Synthetic Enzymatic Biosystems.” The 8th Sino-US Joint Conference on Chemical Engineering. Shanghai, China. (10/23/2015).

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3. Zhang, Y-H.P. “Production of Biofuels and Biochemicals by in Vitro Synthetic Biosystems: Opportunities and Challenges.” *Biotechnol. Adv.* 33 (2015): 1467–1483.
4. Demain, A.L., and G. Vaishnav. “Production of Recombinant Proteins by Microbes and Higher Organisms.” *Biotechnol. Adv.* 27 (2009): 297–306.