

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

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- Increase H₂ production rates five-fold (i.e., 750 mmol H₂/L/h)
- Scale up H₂ production volume by 1,000-fold (i.e., 1,000 mL bioreactor).
- Demonstrate 1,000-fold volume scale-up with five-fold increase in H₂ peak production rate (i.e., 750 mmol H₂/L/h) on starch

Technical Barriers

This project addresses the following technical barriers of biological hydrogen production from 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

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 H₂ production cost, increase its production rate, and scale up its production volume. Our goals would clear up doubts pertaining to in vitro H₂ production cost, rate, and scalability for future distributed hydrogen production from renewable liquid sugar solution.

FY 2017 Accomplishments

- Achieved the highest biological H₂ generation peak rate: 550 mmole of H₂/L/h (i.e., ~282 L H₂).
- Scaled up recombinant enzyme production by 10,000-fold from several milligrams to up to tens of grams and recombinant hydrogenase production by 100-fold from 10 mg to 1,000 mg.

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) 2017 Objectives

- Decrease H₂ production costs by use of the first set of hyperthermophilic enzymes, the use of nicotinamide adenine dinucleotide (NAD), and coenzyme engineering

TABLE 1. Virginia Polytechnic/University of Georgia Status

Targets	Units	June 2016 Project Target	December 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	550 (achieved)	750	2,000 (our goal)
Reactor volume	L of reactor	1.0 (achieved)	1.0	15,625* (our goal)

*1,500 kg H₂ per day

- Designed and validated the first NAD-based pentose phosphate pathway instead of natural nicotinamide adenine dinucleotide phosphate (NADP)-based for an ultra-high rate of biohydrogen production.
- Increased the specific activity of 6-phosphogluconate dehydrogenase (6PGDH) on a low-cost and more stable biomimetic coenzyme–nicotinamide mononucleotide (NMN), to comparable activities on NADP.
- Prolong NAD's life-time by 20-fold by linking NAD with dehydrogenases via polyethylene glycol (PEG), greatly decreasing in vitro biohydrogen production costs.



INTRODUCTION

Water splitting for H₂ production is critical for sustainable, renewable H₂ 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 H₂ production systems. A few methods conducted at low temperatures are under investigation, including direct 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. Also, high density hydrogen storage methods are urgently needed.

Renewable H₂ production via water splitting energized by chemical energy stored in biomass (carbohydrates) is extremely attractive because biomass sugars are the most abundant renewable chemical energy and evenly distributed [1]. However, microbial anaerobic fermentation (dark fermentation) suffers from low H₂ yields, where the theoretical yield is 4 H₂ per glucose 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 H₂, this two-step conversion requires two reactors, has decreased energy efficiency compared to the theoretical H₂ yield (i.e., 12 H₂ per glucose and water) due to electricity consumption, slow H₂ generation rates, and high capital investment of microbial water electrolysis. Recently, we demonstrated in vitro synthetic enzymatic biosystems to generate theoretical yields of H₂ energized by numerous carbohydrates, such as, starch, cellodextrins, glucose, xylose, and xylooligosaccharides [2]. But some 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 address these issues on the laboratory scale.

The economic viability of industrial biomanufacturing mediated by in vitro synthetic enzymatic biosystem has been in debate for nearly 10 years. This year, the first industrial example has been demonstrated for cost-competitive production of myo-inositol from starch in 20,000-L bioreactors in China [4]. Now a 5,000-ton plant is under construction.

APPROACH

The general approach for this project is to apply biochemistry and protein engineering, microbiology, molecular biology, chemistry, and chemical engineering 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 H₂ production costs (**Objective 1**), we succeeded in expressing all hyperthermophilic enzymes and purify them by simple heat precipitation, replacing NADP with NAD by coenzyme engineering, and prolonging NAD lifetime (total turnover number [TTN]) by more than 20 times. To increase H₂ generation rates (**Objective 2**), we developed a kinetic model and identified the rate-limiting steps, constructed novel NAD-based biomimetic electron transport chains, and built enzyme complexes or conjugates featuring electron channeling. To scale up H₂ production (**Objective 3**), we scaled up recombinant protein production in *E. coli* and recombinant hydrogenase production, as well as demonstrated H₂ production in 1-L bioreactor.

RESULTS

The overall goal of the second phase of this project was to demonstrate 1,000-fold volume scale-up with five-fold increase in H₂ peak production rate (i.e., 750 mmol H₂/L/h) on starch. Figure 1 shows the different electron transport chains from glucose 6-phosphate to H₂ via three key enzymes: glucose 6-phosphate dehydrogenase (G6PDH), 6PGDH, and soluble NiFe-hydrogenase (SHI). The peak H₂ generation rate demonstrated was 550 mmol H₂/L/h (Figure 2) and a maximum H₂ generation rates of 530 mmol lasted for up to 3 h (Figure 3). Such biohydrogen rates are one of the highest biological H₂ production rates reported, compared to dark fermentation, photobiological means, and microbial electrolysis cells.

To decrease H₂ production costs (**Objective 1**), we have three subtasks: (1) decrease enzyme production and purification costs, (2) engineer the coenzyme preference of dehydrogenases from NADP to NAD to NMN, (3) stabilize

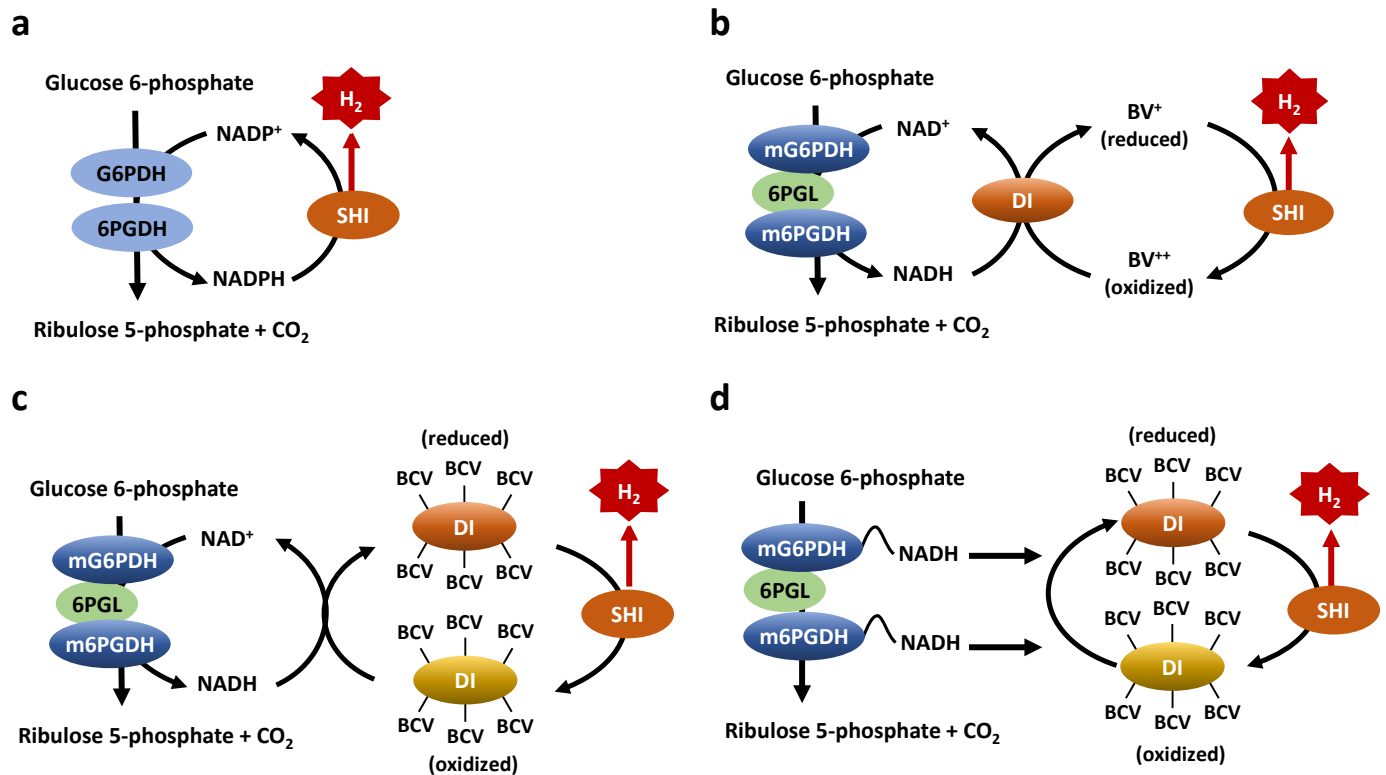


FIGURE 1. Schemes of the electron transport chain (ETC) for in vitro biohydrogen generation, where G6P regeneration via the pentose phosphate pathway is not shown. (a) direct electron transfer from NADPH to H₂ via soluble hydrogenase I (SHI); (b) indirect electron transfer via a biomimetic ETC via engineered NAD-preferred G6PDH (mG6PDH) and engineered NAD-preferred 6PGDH (m6PGDH) via an diaphorase (DI) and an electron mediator benzyl viologen (BV); (c) indirect NAD-based ETC by using a conjugate DI-BCV; and (d) indirect NAD-based ETC by using three conjugates DI-BCV, mG6PDH-NAD, mG6PPDH-NAD.

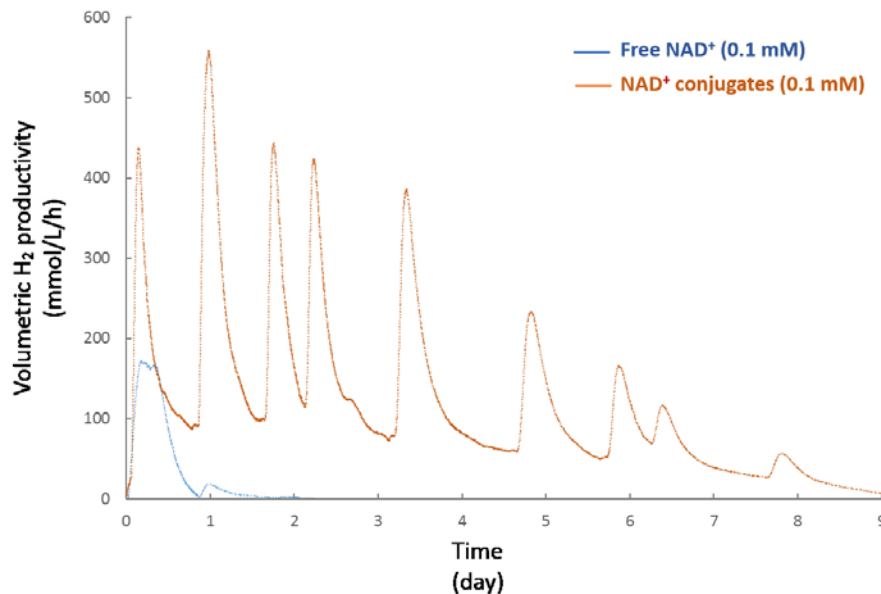


FIGURE 2. The profile of hydrogen generation from maltodextrin at a fed-batch mode via the in vitro pathway comprised of mG6PDH-NAD, mG6PPDH-NAD, DI-BCV and other 12 hyperthermophilic enzymes, in comparison of the pathway based on free NAD at 80°C.

NAD by chemical linkage with dehydrogenases, and (4) complete oxidation of all glucose units of starch.

To decrease enzyme production and purification costs, we succeeded in expressing all hyperthermophilic enzymes in *E. coli*, which are inherently stable at more than 80°C. After high-density fermentation, the cell lysate can be heated at 80°C for 20–30 min. After centrifugation to remove *E. coli* cellular proteins and ultra-filtration to concentrate target enzymes, we can make up to tens of grams of enzymes in one batch fermentation in 6-L fermenter. It is estimated that hyperthermophilic enzyme production costs could be as low as \$50/kg on an industrial scale [4,5]. This suggests that enzyme costs are not a showstopper for the in vitro synthetic biology platform.

To decrease coenzyme costs in in vitro biosystems, we did coenzyme engineering, changing the coenzyme preference of two dehydrogenases (i.e., G6PDH and 6PGDH) from NADP⁺ to NAD⁺ and small-size, low-cost biomimic–NMN. The general strategy for coenzyme engineering is based on a combination of rational design and directed evolution. First, coenzyme preference of both hyper-thermophilic G6PDH and 6PGDH has been changed from NADP to NAD without significant activity discounts (manuscript submitted for publication). Therefore, we are able to construct NAD-based electron transport chains (Figure 1b). Second, we developed a novel high-throughput screening methods for rapid identification of NMN-preferred mutants by using a redox dye without influence from *E. coli* intracellular NAD(P). Three, we applied two rounds of rational design followed by four-round directed evolution. Finally, we obtained the best 6PGDH mutant exhibited the comparative activity on NMN with its wild-type enzyme on its natural coenzyme. It means that we have increased

6PGDH activity on NMN by more than 100-fold. Now we attempt to increase G6PDH activity on NMN. This result suggests a great opportunity of coenzyme engineering by using much cheaper biomimetic coenzymes. Besides its application for in vitro biohydrogen production, this breakthrough will have great impacts on biosynthesis of chiral compounds.

To decrease coenzyme costs in in vitro biosystems, we linked NAD with G6PDH and 6PGDH through chemical linkage via polyethylene glycol. Figure 2 shows the profiles of H₂ generation by using NAD-linked dehydrogenases and free NAD. Free NAD degraded rapidly, having a TTN value of 5,000 at 80°C. When NAD was linked with dehydrogenases (Figure 1d), its TTN value was increased to more than 100,000 by more than 20-fold, where maltodextrin was re-added a lot of times. Also, this in vitro synthetic systems can produce hydrogen 80°C for more than one week (Figure 3).

To increase starch utilization efficiency, we applied three supplementary enzymes (that is, isoamylase debranching amylopectin, 4-glucosyltransferase extending maltose to long-chain amylopectin, and polyphosphate glucokinase utilizing glucose) to achieve in vitro complete oxidation of starch for the first time. As a result, starch is the best hydrogen storage compound as compared to other carbohydrates (Figure 4). Starch has a gravimetric hydrogen storage of more than 14% (wt/wt), where water is recycled from proton exchange membrane fuel cells.

To increase volumetric productivity of H₂ (Objective 2), we increased reaction temperature to 80°C by using the first set of hyperthermophilic enzymes. Furthermore, we designed a biomimetic ETC from NADH generated from glucose 6-phosphate by NAD-preferred dehydrogenases to benzyl viologen catalyzed by diaphorase (DI) to H₂ catalyzed by

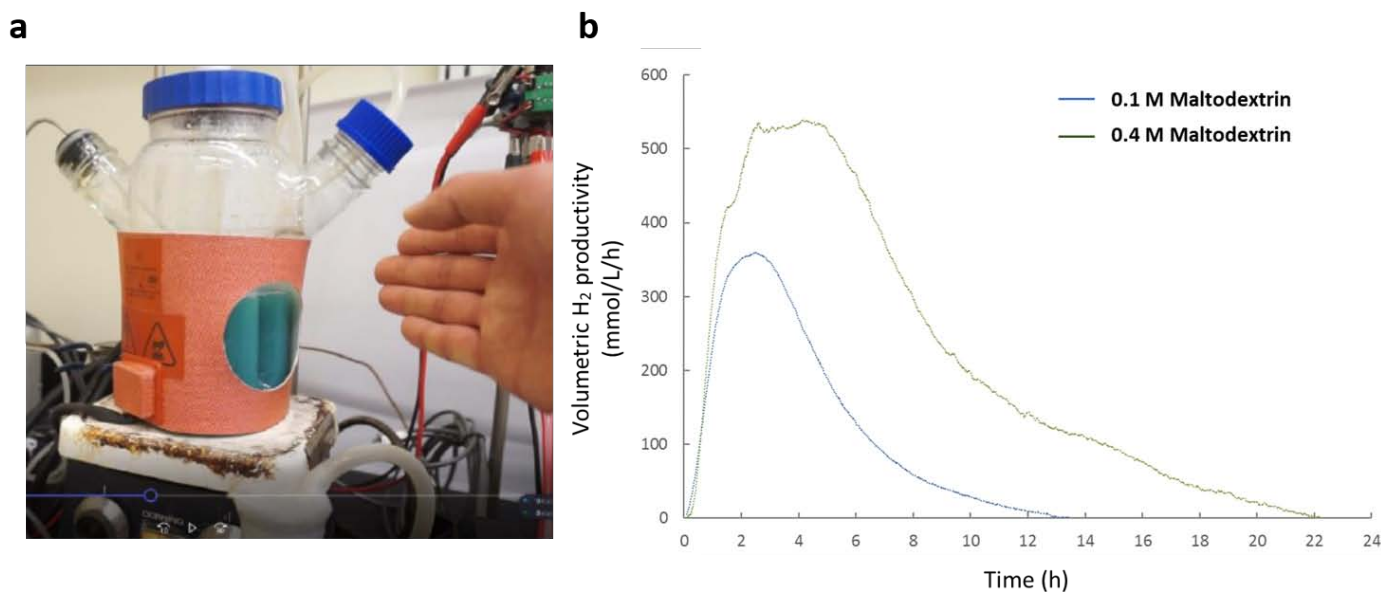


FIGURE 3. Picture of 1-L biohydrogen generation demonstration from starch (a) and the profile of hydrogen generation from starch (b).

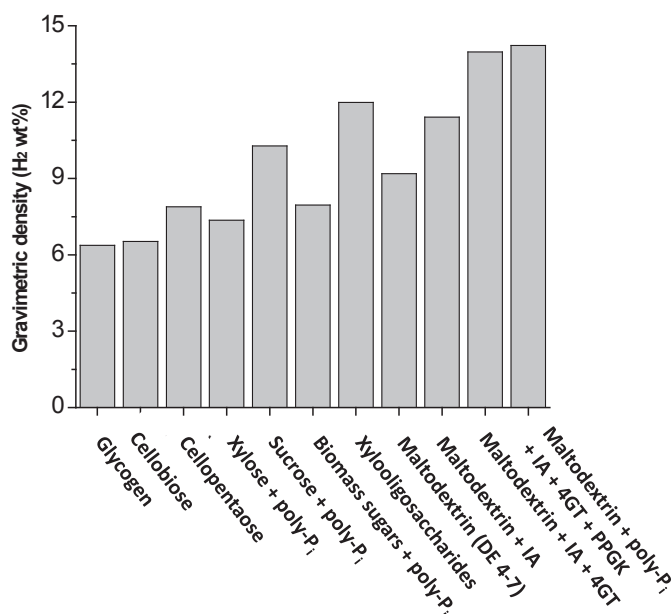


FIGURE 4. Comparison of gravimetric hydrogen storage densities (H₂% wt/wt) for different carbohydrates via different pathways.

soluble [FeNi]-hydrogenase 1 (SHI) (Figure 1b). This ETC can increase hydrogen generation rates by approximately five times relative to direct ETC from Nicotinamide adenine dinucleotide phosphate (NADPH) to H₂ (Figure 1a). What is more, the conjugation of benzyl viologen (BV) and DI, yielding DI-BCV (Figure 1c), doubled H₂ generation rates. We also constructed enzyme complex (called metabolon) comprised of mG6PDH, DI, and SHI, which increased electron transfer rates by nearly one order of magnitude. But this metabolon was not stable at 80°C. We applied glutaraldehyde to stabilize the metabolon greatly. The combination of the use of hyperthermophilic enzymes, biomimetic ETC, and DI-BCV allows us to achieve ultra-rapid production of H₂, being 550 mmol H₂/L/h. In the past 10 years, our lab has increase in vitro biohydrogen production rates by 1,000-fold. We believe that we could increase biohydrogen production rates by another order of magnitude if we have enough funding support.

To scale up enzymatic H₂ production (Objective 3), 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 500-mL flask, we were able to increase enzyme production capability by more than 10,000-fold. Such information suggests that bulk enzyme production costs could be as low as \$50/kg [4,5]. By changing the promoter and enzyme purification tag of SHI, we increased SHI production capability by 100-fold. Consequently, we scaled up our H₂ production in 1-L reactor (Figure 3a).

CONCLUSIONS AND UPCOMING ACTIVITIES

Although sweet H₂ production is still in its early stage. Several conclusions can be made:

- The highest biological H₂ generation rates achieved suggests that these in vitro hydrogen generation rates are fast enough to produce H₂ at stationary hydrogen bioreactors.
- Successful coenzyme engineering from NADP to NAD to NMN along with NAD-linkage suggests that we are able to decrease coenzyme costs greatly, at least by a factor of 100.
- Complete oxidation of starch suggests that starch could be the best solar fuel as a high-density hydrogen carrier with a gravimetric density of up to 14% H₂ wt/wt (Figure 4).

Future work includes:

- Further increase hydrogen production rates toward the ultimate goal of on-board hydrogen generation for hydrogen fuel cell vehicles.
- Enhance activities of dehydrogenase mutants working on biomimetic coenzymes.
- Finish detailed economic analysis of enzymatic H₂ production by using the H2A model.

FY 2017 PUBLICATIONS/PRESENTATIONS

1. Huang R, Chen H, Zhang Y-HP*. 2016. High-throughput screening of coenzyme preference change of thermophilic 6-phosphogluconate dehydrogenase from NADP⁺ to NAD⁺. *Scientific Reports* 6:32644.
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8. Zhang Y-HP. Constructing the electricity-carbohydrate-hydrogen cycle for a carbon-neutral future (plenary talk). SuNEC 2016, Sun New Energy Conference, Sicily, Italy. Sept. 7, 2016.
9. Biodegradable high-energy density sugar biobattery. China Bioindustry and Capital Conference, Tianjin, China. October 25, 2016.
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