Efficient Cell-Free Hydrogen Production from Glucose
A Feasibility Study
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Investigators
James R. Swartz, Professor, Chemical Engineering and Bioengineering; Phil Smith, Jon Kuchenreuther, and Alyssa Bingham, Graduate Researchers, Chemical Engineering.

Abstract

Our objective is to develop scaleable technology that will efficiently convert glucose and xylose, the primary products of cellulosic biomass digestion, into hydrogen. This project was designed to investigate feasibility. Key challenges were: a) demonstrating effective production of a complex hydrogenase under anaerobic conditions, and b) demonstrating the effective coupling of the hydrogenase with an enzymatic pathway beginning with glucose and using the pentose phosphate pathway (a pathway that will also accept xylose as a substrate). By engineering the production organism and the expression protocol, we have increased hydrogenase production at least 10-fold higher than ever before reported to achieve specific accumulation levels predictive of commercial feasibility. By increasing the concentrations of pathway enzymes, we have demonstrated hydrogen production rates that suggest commercial hydrogen productivities approximately 10-fold greater than current ethanol productivities on the basis of MJ of fuel value per liter of capacity per unit time. While the energy conversion efficiency of the enzymatic pathway is very high, the overall efficiency of energy content conversion from glucose and xylose to hydrogen will depend upon the durability of the enzyme pathway. There is good reason to believe that pathway durability can be engineered to deliver energy conversion efficiencies greater than current ethanol production technology.

Introduction and Background

The proposed energy conversion pathway is outlined in Figure 1 with glucose 6-phosphate as the substrate. The glucose will be phosphorylated using ATP and

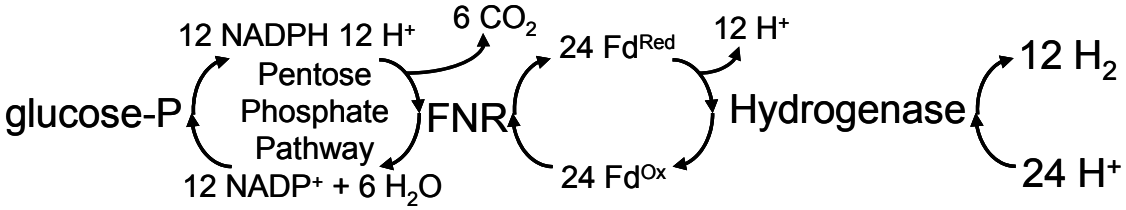


Figure 1: The pathway for conversion of chemical energy from glucose to hydrogen using the pentose phosphate pathway, ferredoxin nucleotide reductase (FNR) and an [Fe-Fe] hydrogenase such as the CpI enzyme from *Clostridium pasteurianum.*

glucokinase. Each carbon atom in glucose will be converted to CO_2 by the pentose phosphate pathway while transferring four electrons to two $NADP^+$ molecules. FNR, or ferredoxin nucleotide reductase, is an FAD containing enzyme that transfers the electrons from NADPH to ferredoxin. The scheme shown indicates a ferredoxin that is a single electron carrier. Thus, 24 transfers would be required if all the electrons were transferred to produce 12 molecules of hydrogen. The required number of protons will be generated by earlier steps in the pathway suggesting that the pH will be relatively stable.

Previous researchers had shown that this pathway is feasible (Woodward et al, 2000 and Zhang, 2007), but had only demonstrated very low rates of hydrogen production while sparging the reactors at high gas flow rates to kept the hydrogen concentration very low to encourage the forward reaction. These reports suggested scientific feasibility but the experiments used expensive purified enzymes and had very slow volumetric production rates. They also used a single enzyme that accepted NADPH and produced hydrogen thus avoiding the need for the ferredoxin intermediate. However, this enzyme is a Ni-Fe hydrogenase with a significantly lower specific activity than that of the [Fe-Fe] hydrogenase. As we approached the problem, a key concern (in addition to the low rates) was the low thermodynamic driving force between NADPH and hydrogen. To counteract this, we realized that we would need to keep the NADPH concentration relatively high and the hydrogen concentration relatively low for a commercially viable pathway.

To judge economic feasibility for our proposed technology, we elected to use current corn to ethanol technology as our comparison benchmark. Although this approach remains controversial, some 13 million gallons of ethanol were produced from corn in 2009. The productivity estimate for this technology is based on a typical yield of 10% ethanol from a 60 hour fermentation (including turnaround time for the batch process). Although the exact conversion efficiency varies depending upon the capital invested towards energy conservation for each facility, we estimate a 70% overall efficiency.

Key issues were the feasibility for efficiently producing the complex [Fe-Fe] hydrogenase and the feasibility of obtaining commercially viable volumetric rates of hydrogen production. These are addressed in the following sections.

Results

Production of the hydrogenase enzyme. The structure of the hydrogenase (CpI) we plan to use is shown on the right side of Figure 2 (on the following page). It has a complex iron sulfur active site (the H-cluster) which includes 3 carbon monoxide and two cyanide moieties as well as a dithiol bridging ligand. It also has four additional iron sulfur centers to deliver electrons to the active site. Its assembly requires three helper proteins called maturases. Because of the complexity of the enzyme and its maturation pathway, it has been very difficult to produce in recombinant organisms. By optimizing a fermentation protocol that combines an aerobic growth phase and an anaerobic production phase, Jon Kuchenreuther demonstrated efficient expression and maturation of two different hydrogenases in *E.coli*, the HydA1 enzyme from *Chlamydomonas reinhardtii* and the CpI enzyme from *Clostridium pasteurianum*, both shown in Figure 2.

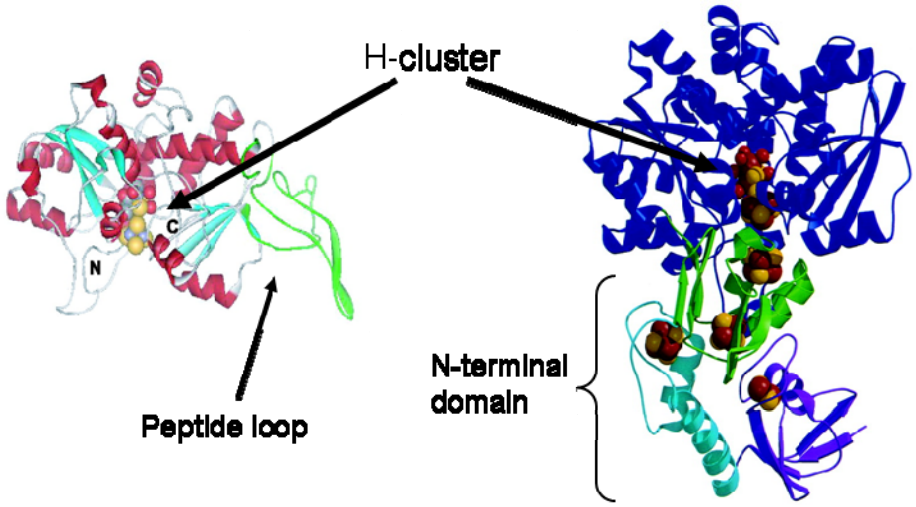
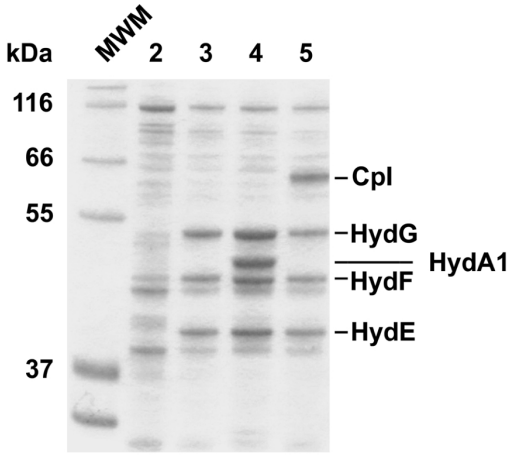


Figure 2: Estimated structure of *C.r* HydA1 (left) and the X-ray crystal structure of *C.p.* CpI (right).

Figure 3 shows a protein separation gel with samples from a control organism (lane 2), an organism engineered to express only the maturase proteins (lane 3), an organism engineered to express the maturases plus the HydA1 hydrogenase (lane 4), and an organism engineered to express the maturases plus the CpI hydrogenase (lane 5). As can be seen, the products accumulate to much higher levels than any of the native proteins.

Figure 3: This image of a PAGE protein gel shows the soluble protein fractions from cells engineered to produce the three maturase enzymes, Hyd E, Hyd F, and HydG as well as either the HydA1 hydrogenase or the CpI hydrogenase. The appropriate bands are labeled. The products are the major protein bands suggesting very efficient expression even though they were expressed under anaerobic conditions. (MWM = molecular weight markers)



Characterization of the hydrogenases produced from this process showed that they were fully active. Even though the fermentation protocols were designed for low cell densities, they produced at least 10-fold more active hydrogenase per liter than had ever before been reported. This process will need to be developed for high cell density and larger volumes, but certainly appears to have the potential to provide cell extracts with very high concentrations of active hydrogenase.

Feasibility of high volumetric productivities of hydrogen from glucose. To establish the feasibility of hydrogen production from glucose (and xylose) using cell extracts, we decided to first use purified enzymes so that experiments could be better controlled. We

have cloned and expressed the single electron carrying ferredoxins from *E.coli* and from a photosynthetic organism, *Synechocystis.* Both of these carriers are functional, but the latter ferredoxin couples more readily with the CpI hydrogenase.

For the FNR activity, we cloned and expressed the FNR from *E.coli*. We found that the enzyme was more active when expressed in cell-free reactions, most likely because the FAD cofactor could be provided at higher concentrations. This agrees with our previous experience in expressing glutathione reductase, another FAD containing enzyme. Early attempts to improve activity for the *in vivo*-expressed enzyme by adding riboflavin to the culture medium were not effective, but this will be repeated with higher concentrations. In the meantime, we have been able to test the pathway with FNR produced by cell-free reactions. The enzyme has a turnover rate of about 2 electrons per second and is soluble and stable when taken to high concentrations.

After a series of experiments, we have demonstrated hydrogen production rates from glucose 6-P and NADPH with CpI hydrogenase turnover numbers of 1.5 sec^{-1}. Using only ferredoxin and CpI we have observed CpI turnover numbers of approximately 20^{-1}. These observations suggest that we can achieve a turnover number of at least 5 per second for the hydrogenase with an optimized enzyme pathway.

Progress and Implications

We believe that the progress shown here indicates the feasibility for a large scale, highly attractive glucose to hydrogen conversion technology. Optimized overall efficiencies of >80% for transferring the energy value in glucose into hydrogen are reasonable. Given that distillation is not required for separation, we expect that energy conversion efficiencies for this process will be higher than can be achieved for ethanol.

We believe we can develop the process to economically provide a hydrogenase concentration of at least 86 μM. The following calculation indicates the potential for the hydrogen fuel value volumetric productivity.

$$\left(\frac{86\ \mu\text{mole } H_2\text{ase}}{\text{L}}\right)\left(\frac{5\ \mu\text{moles } H_2}{\text{mole } H_2\text{ase} \cdot \text{sec}}\right)\left(\frac{3600\ \text{sec}}{\text{hr}}\right)\left(\frac{\text{mole}}{10^6\ \mu\text{mole}}\right)\left(\frac{2\ \text{g } H_2}{1\ \text{mole } H_2}\right)\left(\frac{0.13\ \text{MJ}}{\text{g } H_2}\right) = 0.40\ \text{MJ/L-hr}$$

For comparison, the estimated volumetric fuel value productivity for a current ethanol plant is:

$$\left(\frac{100\ \text{g EtOH}}{\text{L}}\right)\left(\frac{1}{60\ \text{hr}}\right)\left(\frac{0.025\ \text{MJ}}{\text{g EtOH}}\right) = 0.04\ \text{MJ/L-hr}$$

Thus, the proposed technology has the potential to deliver somewhat higher conversion efficiencies and dramatically higher productivities than the ethanol technology currently being used. In the envisioned plant design, a single set of fermentors for cell growth and enzyme production would support several hydrogen reactors. This consideration justifies the comparison in productivities being based on the volumetric productivity of the fuel producing reactor in each case.

Future Plans

Based on these results and this analysis, we would like to apply for a fully funded GCEP project.

Publications

None to date

References

Zhang, Y. H. P., B. R. Evans, et al. (2007). "High-Yield Hydrogen Production from Starch and Water by a Synthetic Enzymatic Pathway." PLoS ONE **2**(5): e456.

Woodward, J., M. Orr, et al. (2000). "Biotechnology: Enzymatic production of biohydrogen." Nature **405**(6790): 1014-1015.

Contacts

James R. Swartz: jswartz@stanford.edu
Phillip Smith: psmith2@stanford.edu
Jon Kuchenreuther: jonny17@stanford.edu
Alyssa Bingham: abingham@stanford.edu