Efficient, Highly Productive Hydrogen Production from Glucose
Annual Report 9/1/11 to 4/30/12

Investigators
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Abstract
Our long term objective is to develop efficient and cost-effective technology for the production of hydrogen from glucose, xylose, and other cellulosic hydrolysis products. We will use cell-free technology to provide precise control over metabolic fluxes while minimizing the toxic effects of cellulosic byproducts. Initial process calculations suggest the potential for high conversion efficiencies and high volumetric productivities by combining the pentose phosphate pathway with a relatively short electron transfer pathway from NADPH to an [FeFe] hydrogenase. The objective of this project is to develop technology with high hydrogen productivities (100kJ/l-hr) and conversion yields (>80%) from glucose. The major initial challenge is to increase the electron flux from NADPH to hydrogen by increasing the turnover number for the FNR (ferredoxin $NADP^+$ reductase) step in the electron pathway to about $20sec^{-1}$ from previously observed values of less than $0.1sec^{-1}$. We also needed to demonstrate the conversion of glucose into hydrogen to show the feasibility of using cell extracts. This latter objective was met, at least at this point in the project, by showing that equivalent hydrogen production rates were obtained using either NADPH or glucose as the entry substrate. We also made a significant breakthrough toward increasing the electron flux rates from NADPH to the hydrogenase by producing a fusion protein in which the FNR and hydrogenase are physically linked by a polypeptide chain. The hydrogenase segment was only partially activated, but based on the concentration of the fully active enzyme pairs, the calculated FNR turnover number for the fusion protein increased to about $32sec^{-1}$. This is an increase of approximately 250-fold and exceeds our activity target. A provisional patent application was filed to provide important IP protection. We will next confirm and extend this observation by learning to produce fully active fusion protein. Because of this advance, we have decided to focus on improvement and evolution of the fusion protein instead of seeking to understand the previous limitation. This will allow us to move more quickly toward a fully integrated and scalable production process.

Introduction and Background

Hydrogen is already an important chemical feedstock and has the potential to be an important fuel. We are seeking to develop efficient, cost-effective technology to convert cellulosic biomass (cellulose hydrolysates) into hydrogen. The proposed chemical energy conversion pathway is outlined in Figure 1 with glucose 6-phosphate as the substrate. The glucose will be phosphorylated using ATP and glucokinase. Xylose and other five carbon sugars will also be phosphorylated, and both sets of substrates can then enter the pentose phosphate pathway to produce NADPH as the electron source for hydrogen production. An FNR enzyme and the electron carrier, ferredoxin (Fd), then deliver the electrons to the hydrogenase. Protons are also generated by the pathway to supply all of the required substrates to produce hydrogen.

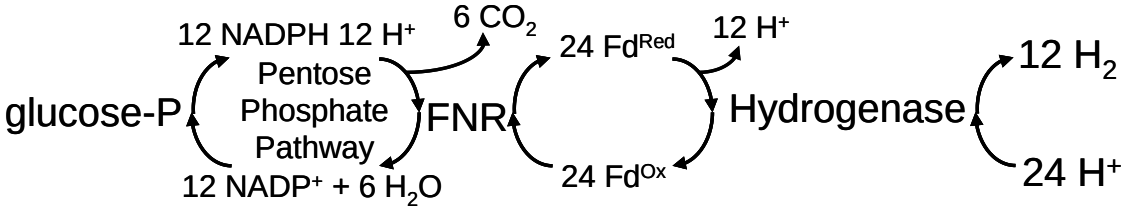

Figure 1: The pathway for conversion of chemical energy from glucose to hydrogen using the pentose phosphate pathway, ferredoxin $NADP^+$ reductase (FNR), a ferredoxin, and an [Fe-Fe] hydrogenase such as the CpI enzyme from *Clostridium pasteurianum* (*C.p.*).

As originally proposed, the ATP will derive from a slow rate of oxidative phosphorylation using electrons from one of the NADPH molecules generated from glucose-P. The rate of this reaction will be controlled by a very slow addition of oxygen to the cell-free bioreactor to avoid using more NADPH than necessary. 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 (ferredoxin $NADP^+$ reductase) is an FAD containing enzyme that transfers the electrons from NADPH to a ferredoxin. Figure 1 shows a ferredoxin that is a single electron carrier. Thus, 24 transfers would be required to produce 12 molecules of hydrogen. The required number of protons for the hydrogen will be generated by earlier steps in the pathway suggesting that the pH will be relatively stable. Previous work by our lab has already demonstrated a 30-fold higher volumetric productivity for the NADPH to H_2 pathway than for recently published alternative technologies (Previous project report; Zhang et al., 2007).

To judge economic feasibility for our proposed technology, we elected to use current corn to ethanol technology as our comparison benchmark. 14 billion gallons of ethanol were produced from corn in 2010. The volumetric productivity estimate of about 40kJ/L-hr 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. We estimate that our proposed process could achieve higher conversion efficiencies; about 90% not considering H_2 recovery and compression.

Key initial issues for the proposed technology included the feasibility for efficiently producing the complex [Fe-Fe] hydrogenase and the feasibility of obtaining commercially viable volumetric rates of hydrogen production. Previously, we reported data showing the ability to produce high specific concentrations of the hydrogenase in *E.coli* (Kuchenreuther et al., 2010), and we also described calculations suggesting that 10 times greater fuel value productivities than for ethanol production could be expected based on key enzyme turnover numbers (specific rates). In addition, the first phase of the exploratory project indicated feasibility for the proposed pathway, even though the pathway flux rates were significantly lower than expected based upon measured activities of the individual enzymes. Evaluating different FNRs and ferredoxins produced a 3-fold increase in volumetric rates, but still fell far short of our overall objective. This project now aims to increase productivities by nearly 100-fold and to demonstrate effective hydrogen production using cell extracts rather than purified enzymes.

Results

Demonstrating hydrogen production from glucose. Previous work had focused on the NADPH to hydrogen pathway as the most likely bottleneck for the conversion of glucose to hydrogen, but it was also important to demonstrate that this was the case. The data in Figure 2 show the results of this evaluation. As expected, the same rate of hydrogen production resulted when the NADPH was supplied from glucose using the cell extract enzymes compared to a purified enzyme system in which NADPH was added to the system and regenerated from glucose 6-P using glucose dehydrogenase (the last two columns). In the cell extract case, ATP was generated from phosphoenolpyruvate using the pyruvate kinase in the extract, the ATP was accepted by the enzyme hexokinase to phosphorylate the glucose, and the cell extract enzymes then supplied the NADPH. Without hydrogenase addition, no hydrogen was detected, and without the addition of either glucose or hexokinase, very little hydrogen was produced. At least in this experiment, the cell extract enzymes supplied the NADPH as fast as the electron transport pathway could deliver the reducing equivalents to the hydrogenase.

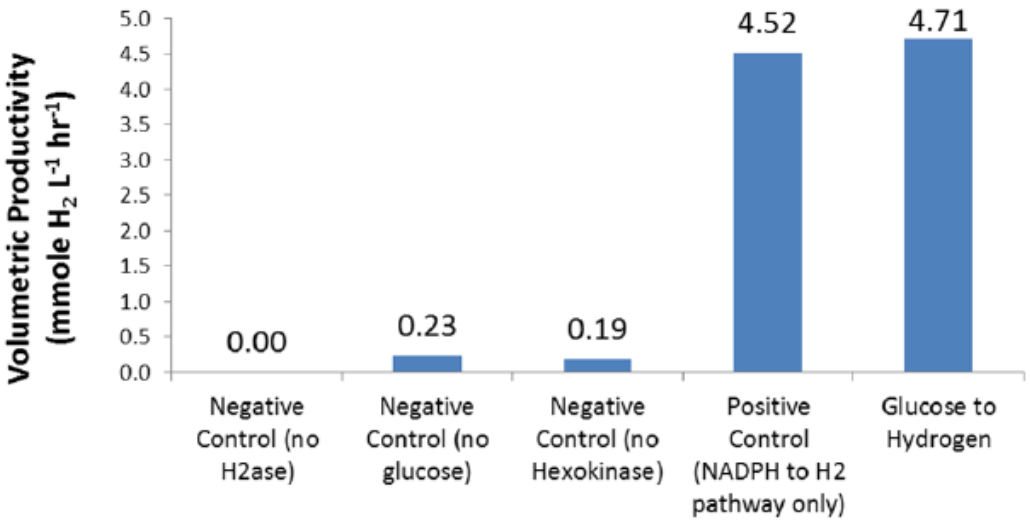

Figure 2: Data showing that the cell extract supplies NADPH for hydrogen production as fast as the electron transport pathway can accept it.

Evaluation of FNA-hydrogenase fusion proteins for production of hydrogen from NADPH. Initial attempts to determine the reason for the unexpectedly low enzyme turnover number in the NADPH to hydrogen pathway did not provide the required insights. We therefore decided to evaluate a different approach, the use of fusion proteins to bring the enzymes in the pathway closer together. This would be expected to diminish any rate limitations due to protein diffusion, but might also effect allosteric regulation of the enzymes, if any.

Figure 3 shows the design of the fusion proteins. The CpI hydrogenase was expressed at the N-terminal end of the fusion with a polypeptide linker extending from its C-terminus. The linker then continued into the N-terminus of the *Anabaena* FNR enzyme. This configuration was required since the C-terminal tyrosine of FNR must be available to participate in active site catalysis.

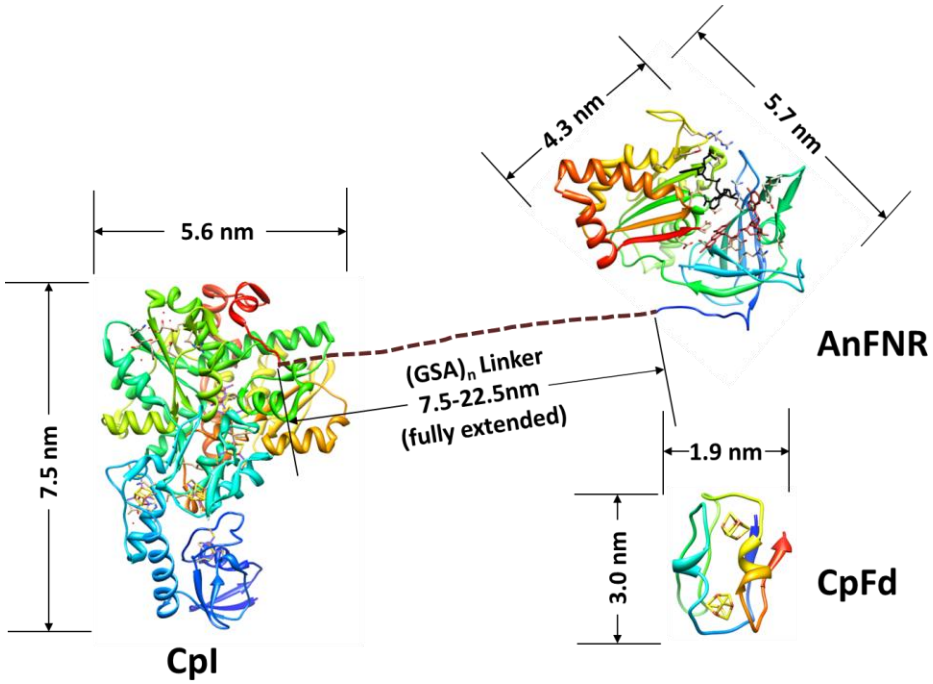

Figure 3: Diagram depicting the configuration and scale of the FNR-hydrogenase fusion protein and the CpFd (*Clostridium pasteurianum* ferredoxin) used to shuttle the electrons from FNR to the hydrogenase.

The genes were constructed to express fusion proteins with 5, 8, 12, and 15 repeats of a GSA tripeptide as the linker sequence. They were also constructed with the T7 promoter and T7 terminator already attached to enable convenient protein expression in cell-free reactions. The resultant expression products (unpurified) were then evaluated for both hydrogenase and FNR activities. ^{14}C-leucine incorporation provided a measure of fusion protein production. All of the fusion proteins expressed well, accumulating to about 200μg/ml soluble concentrations. The fusion proteins on average had about 7% of the expected hydrogenase activity, about the same as normally observed when the hydrogenase is expressed at these levels. The cell free reaction was enriched with FAD (the cofactor for FNR) and the FNR portion of the fusion proteins had about 70% of the expected activity. Based on previous observations with unconnected enzymes, we would not expect the reduced ferredoxin produced by one fusion protein to be used at high rates by the hydrogenase of another fusion protein, so we therefore assumed that only those fusion proteins with an active hydrogenase enzyme could contribute to hydrogen production.

The results from evaluating the fusion proteins are shown in Figure 4. The positive control was a reaction with unconnected enzymes added at higher effective concentrations. The calculated turnover numbers (rates) for the FNR (either free or as part of the fusion protein) are shown below the graph. This evaluation suggests that the fusion protein design now allows the FNR to operate at or above the highest rates that we have measured for the FNR when using the cytochrome C assay that actively keeps the ferredoxin predominantly in its oxidized state. Although we must still confirm our assumptions and must also improve the activation of both members of the fusion proteins,

these observations are very exciting. They suggest that the fusion proteins can provide the specific rates needed to meet our volumetric productivity objectives. A provisional patent application has been filed to protect this breakthrough.

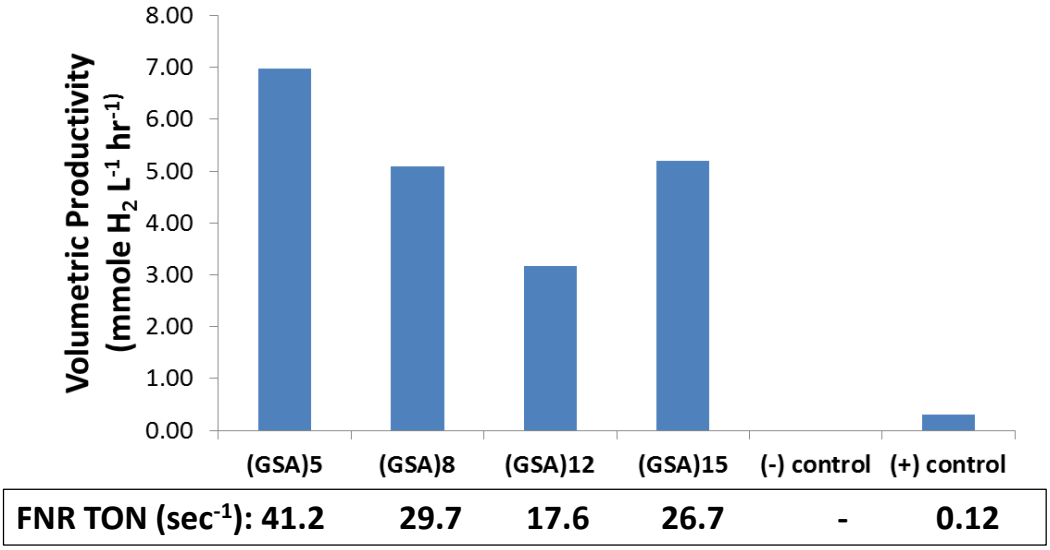

Figure 4: Data indicating a major breakthrough in achieving high enzymatic rates for our NADPH to hydrogen electron transfer pathway. The experiments were conducted with unpurified cell-free reaction products to which 50μM CpFd had been added.

Conclusions

Even though this project is relatively new (started in September), we have already demonstrated the use of cell extracts to accept glucose for producing the NADPH that couples to the use of purified FNR, Fd, and hydrogenase for hydrogen production. Our new fusion protein design also appears to be a major breakthrough toward our goal of very high volumetric hydrogen productivities.

Publications and Patents

1. Smith, P.R. and J.R. Swartz, A CpI hydrogenase-FNR fusion protein offers greatly improved hydrogen production rates through a synthetic enzyme pathway, Provisional U.S. Patent Appl. 61/609,477, Filed 3/12/2012.

References

1. Kuchenreuther JM et al., High-yield expression of heterologous [FeFe] hydrogenases in *Escherichia coli*. PlosOne **5**(11): e15491, 2010.
2. Zhang, Y. H. P., B. R. Evans, et al., High-Yield Hydrogen Production from Starch and Water by a Synthetic Enzymatic Pathway, PLoS ONE **2**(5): e456, 2007.

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