

# Efficient Cell-Free Hydrogen Production from Glucose: Extension Annual Report 8/1/10 to 4/30/11

## Investigators

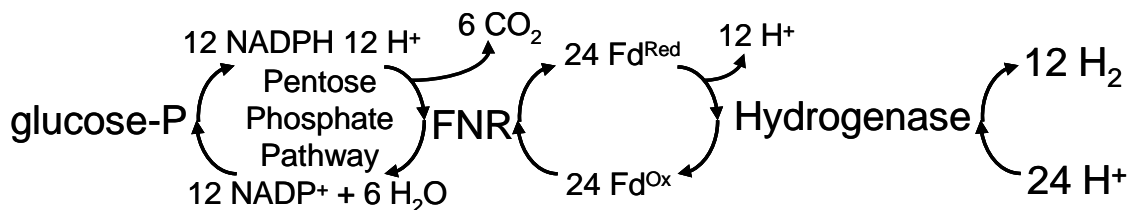
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## Abstract

Our long term objective is to develop efficient and cost-effective technology for the production of hydrogen from glucose and cellulosic hydrolysates. 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. The objective of this exploratory grant extension is to further develop feasibility data that will motivate sufficient funding to then develop a process that will in turn motivate industrial deployment. The previous project demonstrated a maximum electron pathway flux for H<sub>2</sub> production rates of 2.3 kJ/L-hr. More recent work has tripled that to 7 kJ/L-hr. More importantly, recent work with new pathway components has indicated that more fundamental knowledge of pathway performance is required and that key pathway components (the enzymes and ferredoxin) will benefit from protein evolution. We are now developing methods to enable the rapid and precise characterization of individual pathway modules; i.e., a) the transfer of electrons from NADPH to ferredoxin and b) from ferredoxin to the hydrogenase. In parallel, we are developing a numerical simulation of the pathway to guide further development, and we are beginning to evolve FNR, the key enzyme in the first module, to more effectively interact with the two electron carrying ferredoxin that interacts most effectively with our hydrogenase.

## 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 glucokinase.



**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*.

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 which will be controlled by a very slow addition of oxygen to the hydrogen reactor. Each carbon atom in glucose will be converted to CO<sub>2</sub> by the pentose phosphate pathway while transferring four electrons to two NADP<sup>+</sup> molecules. FNR (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 keep 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 14 billion gallons of ethanol were produced from corn in 2010. The volumetric 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. In the last years update, 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 that are a small fraction of activities measured for the individual reactions.

## Results

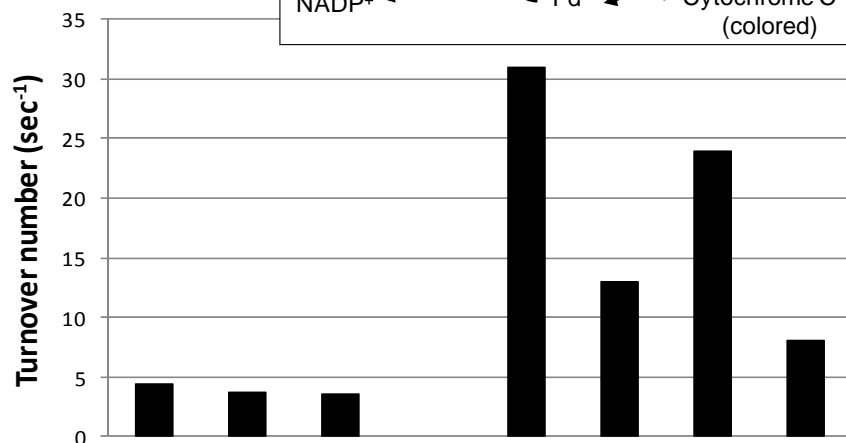
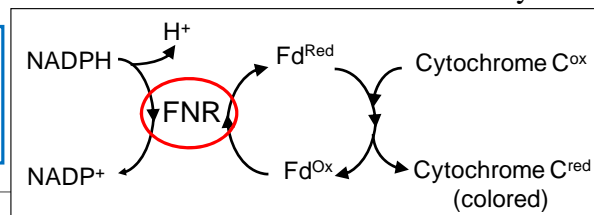
The first phase of this new project focused on evaluating FNRs and ferredoxins that might enable faster electron flux from NADPH to hydrogen (see Figure 1). We evaluated

a commercially available FNR purified from spinach. We also cloned, produced, and purified an *Anabaena* FNR as well as the ferredoxins from *E.coli* and from *Clostridium pasteurianum*. The latter ferredoxin is particularly interesting as it carries two electrons while all the other ferredoxins which have been evaluated only carry one. Also, the *C.p.* ferredoxin is the one that has evolved to couple efficiently with the [Fe-Fe]hydrogenase we are using (which also comes from *C.p.*).

Figure 2 shows the characterization results for the FNRs from *E.coli* and *Anabaena*. Both enzymes were produced *in vivo* in *E.coli* as well as in cell-free reactions. The enzymes were then purified and evaluated using the assay diagrammed in the inset in Fig. 2. The FNR transfers the electrons from NADPH to a ferredoxin. Oxidized cytochrome C then accepts the electrons from the ferredoxin and becomes colored to indicate the rate of electron transfer. Figure 2 indicates the observed rates for both FNRs when they are

delivering electrons to either the ferredoxin from *Synechocystis* or that from *C.p.* Clearly the *Anabaena* FNR supports much higher turnover numbers, with the cell-free produced enzyme apparently being more effectively folded and

**Figure 2:**  
Characterization of Different FNR's

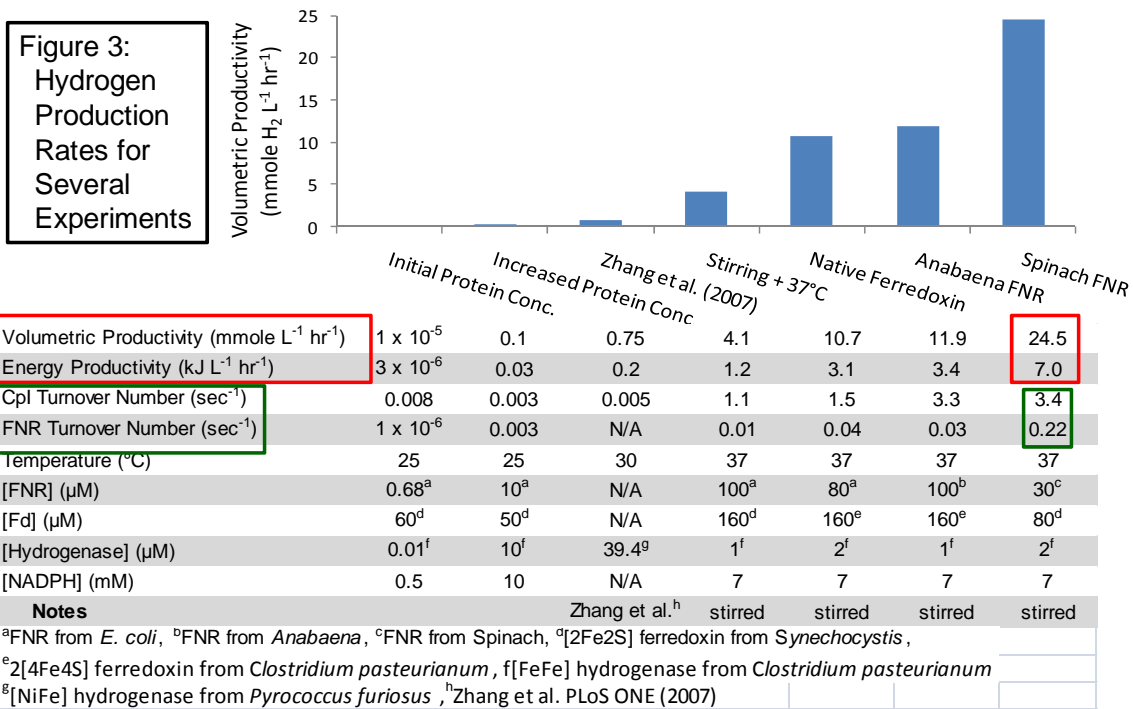


Expression	Cell-free	In Vivo	Cell-free	In Vivo	Cell-free	In Vivo	Cell-free	In Vivo
FNR	E. Coli				Anabaena			
Ferredoxin	SynFd	SynFd	CpFd	CpFd	SynFd	SynFd	CpFd	CpFd

activated, probably because of the increased supply of the FAD cofactor in the cell-free synthesis reactions. Both FNRs can transfer electrons to both ferredoxins with an apparent preference for the *Synechocystis* ferredoxin.

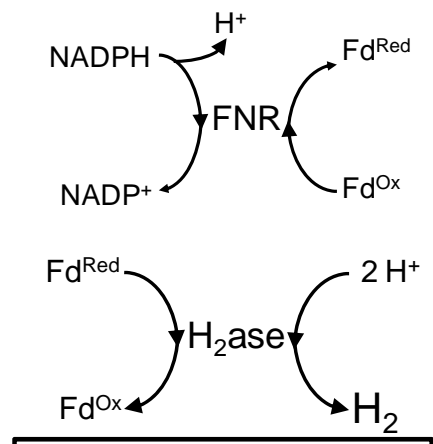
Figure 3 summarizes several key experiments evaluating the flux rates for the electron pathway from NADPH to hydrogen. The published rates from the work of the Zhang lab are given for comparison. Several interesting and important points emerge. The first is that we need to consider mass transfer limitations as stirring and increasing the concentrations of the proteins both substantially increased reaction rates. Second, we observed that the use of the two electron carrying ferredoxin from *C.p.* (the “native ferredoxin”) increased the hydrogen production rate. However, the increase was much less than would have been predicted based on its ability to transfer electrons to the *C.p.* hydrogenase. When dithionite is used to reduce the ferredoxin during the hydrogenase

assay, the observed turnover number for the *Synechocystis* ferredoxin is about 45 sec<sup>-1</sup> while the turnover number for the *C.p.* ferredoxin is about 2500 sec<sup>-1</sup>. This raises the possibility that the *C.p.* ferredoxin does not couple well with the FNR from *E.coli*.



However, using the FNR from *Anabaena* produced no significant benefit even though that enzyme has a potentially higher catalytic rate than the *E.coli* FNR used in the previous experiment. Perhaps the *C.p.* ferredoxin docks even more poorly with the *Anabaena* FNR. However, this is not suggested in Figure 2. Note also that the FNR turnover numbers are about 0.04 sec<sup>-1</sup> in the pathway even though the rates shown in Figure 2 are greater than 20. Finally, the last column shows our highest observed rates of hydrogen production which were obtained using the FNR from spinach. Although the observed FNR turnover number was 0.22 sec<sup>-1</sup>, this is substantially lower than the published activity of over 200 sec<sup>-1</sup>. Unfortunately, initial attempts to produce the spinach FNR were unsuccessful.

It is now clear that we do not understand the principles that govern the performance of this electron transfer pathway. We are now beginning to characterize the specific reactions shown in Figure 4. In both cases, we will use a pH indicator to measure the rate of the reactions since the top reaction will cause the pH to decrease while the bottom reaction will increase the pH. This information will then be used to prepare a numerical simulation of the pathway.



**Figure 4: Characterization Assays**

## Future Plans

Based on these results and this analysis, we have applied for a fully funded GCEP project. This project has also been presented to Exxon Mobil and to General Electric to solicit guidance. We are now proceeding to carefully analyze the performance of the pathway components as shown in Figure 4. We will also produce and test the ferredoxin from *Anabaena* to see if this provides better performance, and we are beginning to evolve the *Anabaena* FNR to dock more productively with the *C.p.* ferredoxin.

## Publications

Kuchenreuther JM et al. (2010) High-yield expression of heterologous [FeFe] hydrogenases in *Escherichia coli*. PlosOne **5**(11): e15491.

## 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.

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