Efficient Cell-Free Hydrogen Production from Glucose: Extension

Exploratory Project Final Report 8/1/10 to 5/31/11

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, 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 exploratory grant extension was to further develop feasibility data that would motivate sufficient funding for process development that would then motivate industrial deployment. The previous project demonstrated a maximum electron pathway flux for H_2 production rates of 2.3kJ/L-hr. This project tripled that to 7kJ/L-hr. Still, functional characterization of individual enzymes indicates that the pathway electron flux is more than 100-fold lower than its predicted value. Effective activation and coupling of the enzymes would enable fuel value hydrogen production rates (on a volumetric basis) 10-fold higher than current fuel ethanol technologies. These feasibility data and analytical calculations prompted the preparation of a proposal for a full GCEP-funded research project to characterize and eliminate the limitations and to demonstrate high conversion efficiencies and production rates. The full project was funded after favorable reviews.

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. Xylose and other five carbon sugars can also 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.

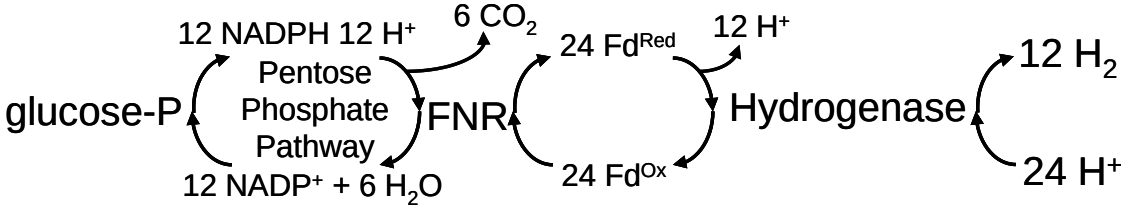


Figure 1: The pathway for conversion of chemical energy from glucose to hydrogen using the pentose phosphate pathway, ferredoxin $NADP^+$ reductase (FNR), a ferredoxin, and an [Fe-Fe] hydrogenase such as the CpI enzyme from *Clostridium pasteurianum* (*C.p.*).

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 which will be controlled by a very slow addition of oxygen to the cell-free bioreactor. 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 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 for hydrogen production than that of the [Fe-Fe] hydrogenase. As we designed our process, a key concern (in addition to the low rates) was the low thermodynamic driving force between NADPH and hydrogen. To counter-act this, we realized that we would need to keep the NADPH concentration relatively high and the hydrogen partial pressure 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. 14 billion gallons of ethanol were produced from corn in 2010. The volumetric productivity estimate of about 40 kJ/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 issues for the proposed technology were 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 (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. This extension project was therefore designed to evaluate a variety of different FNRs and ferredoxins to determine if the low pathway flux rates were caused by incompatibilities between proteins that had evolved to function in different organisms.

Results

The first phase of this project focused on evaluating FNRs and ferredoxins that might enable faster electron flux from NADPH to hydrogen (see Figure 1). We 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 only carry one. Also, the *C.p.* ferredoxin has evolved to couple efficiently with the [Fe-Fe] hydrogenase we are using (also from *C.p.*). We also evaluated a commercially available FNR purified from spinach.

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. 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 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. Note that the *Anabaena* FNR has a turnover number of 30 sec^{-1} with SynFd.

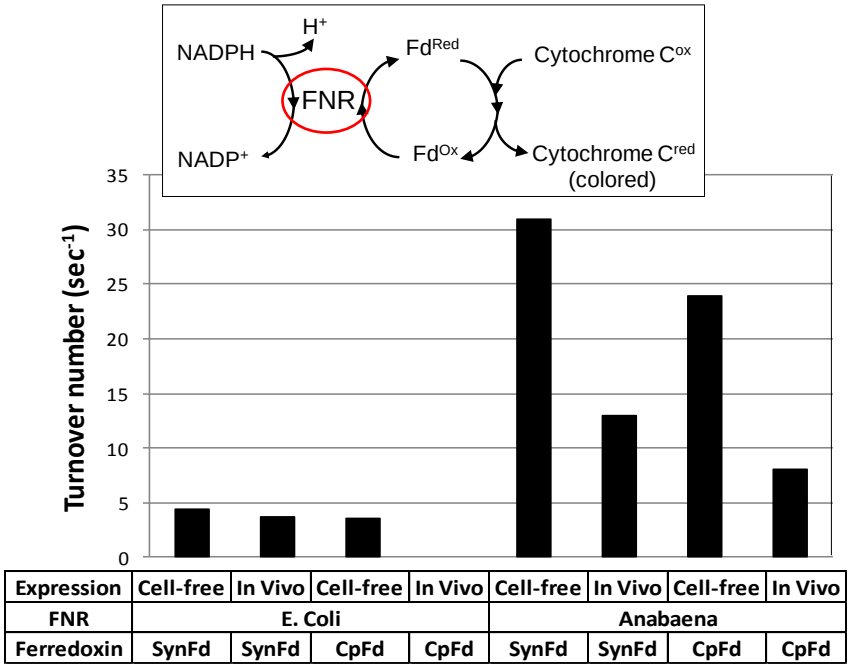
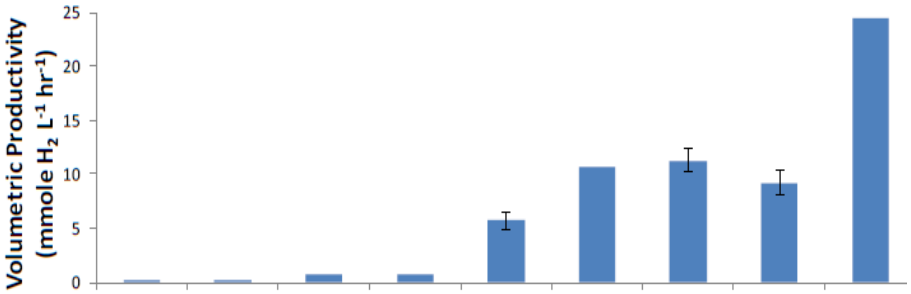


Figure 2: Characterization of the *E.coli* and *Anabaena* FNRs produced either *in vivo* by *E.coli* or by cell-free synthesis when coupled to ferredoxins either from *Synechocystis* (SynFd) or from *Clostridium pasteurianum* (CpFd). The upper inset indicates the assay format.

Figure 3 summarizes several key experiments evaluating the flux rates for the electron pathway from NADPH to hydrogen. The published rates from several recent

reports of competing technologies are also given. Most relevant is the work from the Zhang lab that used purified proteins in cell-free reactions. Clearly, our pathway is capable of much higher volumetric productivities than the other technologies. Earlier work with our system indicated that we needed to consider mass transfer limitations as stirring and increasing the concentrations of the proteins both substantially increased reaction rates. This motivated the use of the high protein concentrations indicated.



	Oncel et al. (2009)	Wells et al. (2011)	Subudhi et al. (2011)	Zhang et al. (2007)	EcFNR SynFd	EcFNR CpFd	AnFNR CpFd	AnFNR AnFd	SpFNR SynFd
Volumetric Productivity (mmole L^{-1} hr^{-1})	0.038	0.042	0.625	0.75	5.68±0.76	10.7	11.2±1.1	9.2±1.1	24.5
Energy Productivity (kJ L^{-1} hr^{-1})	0.010	0.011	0.163	0.2	1.48±0.20	3.1	2.9±0.3	2.4±0.3	7.0
Hydrogenase Turnover Number (sec^{-1})	ND	ND	ND	0.005	1.58±0.21	1.5	3.1±0.3	2.6±0.3	3.4
FNR Turnover Number (sec^{-1})	NA	NA	NA	N/A	0.02±0.002	0.04	0.03±0.003	0.09±0.01	0.22
Temperature (°C)	27	37	37	30	37	37	37	37	37
[FNR] (μM)	NA	NA	NA	N/A	30[a]	80[a]	100[b]	30[b]	30[c]
[Fd] (μM)	NA	NA	NA	N/A	160[d]	160[e]	160[e]	80[f]	80[d]
[Hydrogenase] (μM)	ND[g]	ND[h]	ND[i]	39.4[j]	1[k]	2[k]	1[k]	1[k]	2[k]
[NADPH] (mM)	NA	NA	NA	N/A	7	7	7	7	7
Notes	Photosyn[l]	Gluc Ferm[m]	Gluc Ferm[n]	*In vitro*[o]	stirred[p]	stirred	stirred[q]	stirred[r]	stirred

[a]FNR from *E. coli*, [b]FNR from *Anabaena*, [c]FNR from Spinach, [d][2Fe2S] Fd from *Synechocystis*, [e]2[4Fe4S] Fd from *Clostridium pasteurianum*, [f][2Fe2S] Fd from *Anabaena*, [g][FeFe] H_2ase from *Chlamydomonas reinhardtii*, [h][NiFe] H_2ase from *Synechocystis* sp. PCC6803, [i][FeFe] H2ase from *C. butyricum*, [j][NiFe] H_2ase from *Pyrococcus furiosus*, [k][FeFe] H_2ase from *C. pasteurianum*, [l]Photosynthetic H_2 production by *C. reinhardtii*, Oncel *et al*. (2009), [m]H_2 production in *E. coli* by glucose fermentation, Wells *et al*. (2011), [n]H_2 production in *E. coli* by glucose fermentation, Subudhi et al. (2011), [o]*In vitro* H_2 production from glucose, Zhang *et al*. (2007), [p]*In vitro* H_2 production from NADPH, Smith *et al*. (2011) n=3, [q]n=3, [r]n=2

Figure 3: A comparison of the cell-free pathway performance versus state-of-the-art competing technologies. The table also indicates the measured turnover numbers of the hydrogenase and FNR enzymes using a variety of FNR's and ferredoxins with the CpI [FeFe] hydrogenase.

Using CpFd nearly doubled hydrogen production rates with the *E.coli* FNR (EcFNR), but, contrary to the results shown in Figure 2, the *Anabaena* FNR (AnFNR) did not produce higher rates. In addition, using the CpFd provided less benefit than predicted based on its faster ability to transfer electrons to the *C.p.* hydrogenase. When dithionite is used to reduce the ferredoxin during a hydrogenase activity assay, the observed turnover number for the *C.p.* hydrogenase (CpI) while accepting electrons from the *Synechocystis* ferredoxin is about 530 sec^{-1} while the turnover number with the *C.p.* ferredoxin is about 2500 sec^{-1}. This raises the possibility that the *C.p.* ferredoxin does not couple well with the FNR from *E.coli*. However, the measured turnover numbers for this pair are about the same as when the EcFNR is using its native ferredoxin.

The published turnover number for AnFNR is about 200 sec^{-1}, much faster than we observed with either the SynFd or the CpFd. We therefore cloned and expressed the ferredoxin from *Anabaena* (AnFd). However, this produced no benefit for hydrogen production, most likely because it is a one electron carrier and does not couple well with the CpI hydrogenase. Finally, we purchased an FNR extracted from spinach leaves. This provided the highest rate so far observed (24.5 mmoles H_2/l-hr), more than 30-fold higher than for other biological production systems. Unfortunately, initial attempts to produce the spinach FNR in *E.coli* were unsuccessful, and the enzyme produced by the cell-free system was not as active as the extracted enzyme.

However, the most important conclusion from all of this work is that the enzyme turnover numbers in the pathway are still much lower than observed in the assays for individual enzyme activities. Note that the FNR turnover numbers are more than 100-fold lower than shown in Figure 2. The same also applies to the FNR from spinach. Although the observed FNR turnover number in the pathway was 0.22 sec^{-1}, this is substantially lower than the published activity of over 200 sec^{-1}.

It is now clear that we do not understand the principles that govern the performance of this electron transfer pathway. In the final stages of the project, we began to develop methods to more carefully evaluate the performance of the enzymes relative to the concentrations of either reduced or oxidized ferredoxins. We also began to mutate the AnFNR gene so that we could search for mutations that allow it to couple better to the CpFd in the hydrogen production pathway. These and other measures were proposed for further work in a proposal submitted to GCEP in February of 2011. This proposal received favorable reviews and was funded.

Conclusions

Even though this pathway for hydrogen production from cellulosic hydrolysates has produced the highest hydrogen production rates so far reported, the rates are still much lower than needed for a commercially viable process. This is particularly puzzling since the individual enzymes, when tested in standard assays, exhibit rates that are commercially attractive if they could also be achieved in the pathway. The results from this exploratory project indicate that we either need significant new insights into the factors governing these enzyme activities or we need another breakthrough that avoids the limitations.

Publications and Patents

1. Kuchenreuther JM et al., High-yield expression of heterologous [FeFe] hydrogenases in *Escherichia coli*. PlosOne **5**(11): e15491, 2010.
2. Smith, P.R., A.S. Bingham, and J.R. Swartz, Generation of hydrogen from NADPH using an [FeFe] hydrogenase, International. J. of Hydrogen Energy, 37(3), 2977-83, 2012.
3. Smith, P.R. and J.R. Swartz, Efficient cell-free hydrogen production, U.S. Patent Appl. 13/246,542, Filed 9/27/2011.

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1. Oncel S. and F. Vardar-Sukan, Photo-bioproduction of hydrogen by *Chlamydomonas reinhardtii* using a semi-continuous process regime, International J. of Hydrogen Energy, 34(18): 7592-7602, 2009.

2. Subudhi S. and B. Lal, Fermentative hydrogen production in recombinant *Escherichia coli* harboring a [FeFe]-hydrogenase gene isolated from *Clostridium butyricum*, International J. of Hydrogen Energy, 36(12):14024-30, 2011.
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