Efficient, Highly Productive Hydrogen Production from Glucose
Annual Report 5/1/12 to 4/30/13

Investigators
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Abstract
Our objective is to develop cost-effective technology for the production of hydrogen from glucose, xylose, and other by products of cellulosic biomass. Our plan is to couple the pentose phosphate pathway with a three protein synthetic pathway composed of ferredoxin NADPH reductase (FNR), ferredoxin, and a clostridial hydrogenase. We plan to produce these enzymes *in vivo* and then lyse the cells to generate a cell-free extract, which can then be used directly to produce hydrogen. The objective of this project is to develop technology with high hydrogen productivities (100kJ/l-hr) and conversion yields (>80%) from glucose.

Previous work has demonstrated the feasibility of using an unpurified cell extract to enable glucose conversion into hydrogen, which has been further confirmed. However, the major challenge is to increase the electron flux from NADPH to hydrogen by increasing the turnover number for the FNR step in the electron pathway [1]. Further evaluation of plant FNRs revealed a root FNR that expressed well in *E. coli* and produced up to 3-fold higher hydrogen volumetric productivity. Meanwhile, use of the FNRs in fusion FNR-hydrogenase protein has demonstrated the potential for up to 5-fold improvement in rates. Simultaneously, the binding site of FNR for ferredoxin can be mutated for higher affinity and electron transfer between the two proteins in the hydrogen producing pathway.

With several improvements in the synthetic pathway, we have now begun to focus on the cell-free metabolic engineering. The new approach based on cell-free extract will eliminate protein purification cost, but we need to overcome the potential complexity and inefficiency due to loss of our feedstock (glucose/xylose) to other competing reactions. These reactions are often found in pathways essential for the cell's survival and growth, which makes it difficult to knockout or down-regulate the deleterious enzymes. This problem can be addressed by establishing two distinct process phases: the earlier phase dedicated for cell growth and protein expression *in vivo*, and the latter hydrogen production phase during which competing pathways can be inactivated following cell lysis to generate more efficient cell-free extracts. By combining electron pathway improvement with the silencing of competing pathways we can move 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, while xylose and other five carbon sugars will also be phosphorylated by enzymes such as xylulose kinase after

undergoing isomerization. Both types 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 a highly activate hydrogenase. Protons are also generated by the pentose phosphate pathway and the electron transfer pathways to supply all of the required substrates to produce hydrogen.

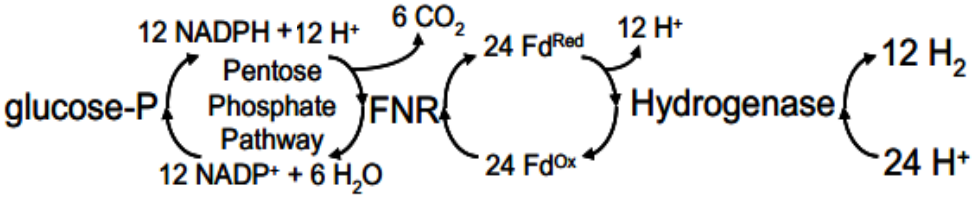

Figure 1: The pathway for conversion of chemical energy from glucose to hydrogen using the pentose phosphate pathway, ferredoxin $NADP^+$ reductase (FNR), a ferredoxin, and an [Fe-Fe] hydrogenase from *Clostridium pasteurianum* (CpI).

Potentially, each carbon atom in glucose can be converted to CO_2 through the pentose phosphate pathway while transferring its four electrons to two $NADP^+$ molecules. FNR (Ferredoxin $NADP^+$ Reductase), an FAD containing enzyme, will then transfer the electrons from NADPH to ferredoxin, which subsequently transfers those electrons to a clostridial hydrogenase. The [Fe-Fe] hydrogenase, CpI, from *C. pasteurianum* was chosen for this pathway due to its known high rates of hydrogen production. Twelve hydrogen gas molecules become the theoretical yield from a single glucose molecule that enters the synthetic pathway. Previous work in the lab has already demonstrated a 30-fold higher volumetric productivity for the NADPH to hydrogen pathway than for the published alternative technologies [2].

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* [3]. We are continuing to study the requirements for hydrogenase maturation in *E.coli* to further increase enzyme yields and specific activities. 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). Further, by including xylose as the starting substrate in the pathway, in addition to the glucose feedstock, we will be able to use ~70% of cellulosic biomass degradation products for conversion to hydrogen [4]. The synthetic pathway has been demonstrated feasible, even though the pathway flux rates were significantly lower than expected considering the measured activities of the individual enzymes. Evaluating different FNRs and ferredoxins produced a 3-fold increase in volumetric rates, and further developments have increased the productivity another 3 fold. In addition, the application of the fusion FNR-hydrogenase protein has demonstrated the ability to improve the turnover number up to 5 times faster than the individual proteins mixed together. Taken together, these results further point to the feasibility of our approach.

Results

Demonstration of Glucose to Hydrogen using unpurified Cell-free Extract

In order to demonstrate that an unpurified extract could be used to generate hydrogen through the pathway we proposed, we tested cell extract combined with purified FNR, Fd, and CpI hydrogenase with glucose as the substrate.

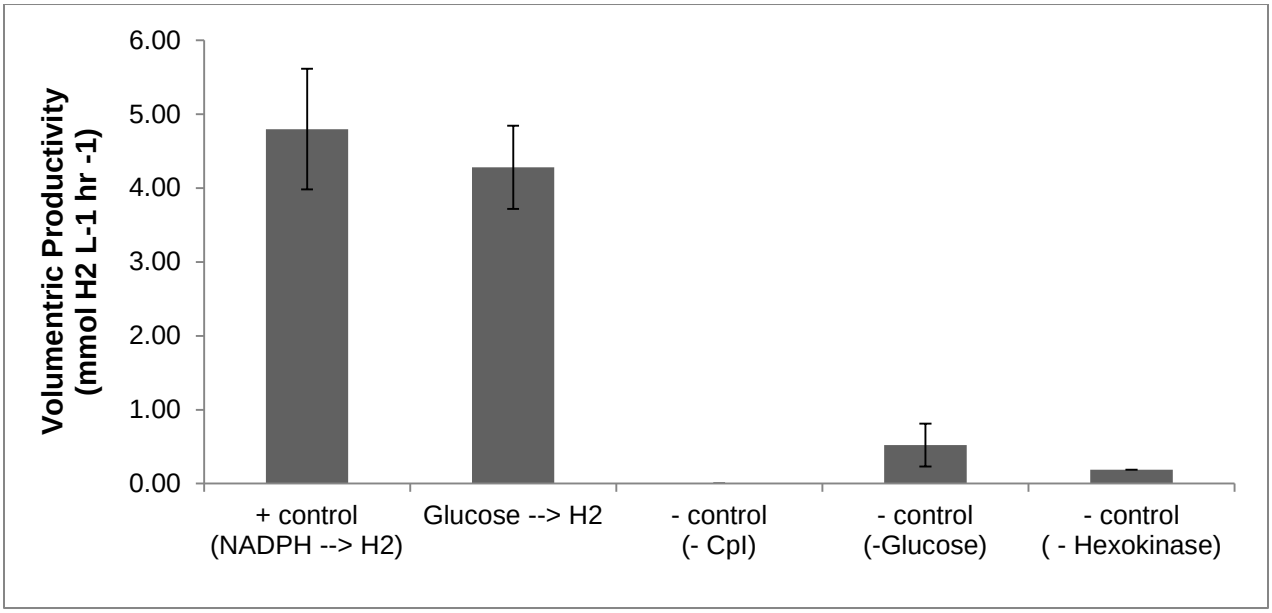

Figure 2: Hydrogen production measured by gas chromatography in a sealed gas-tight vial stirred and heated at 37^0C. Purified FNR, Fd, and CpI were added with either NADPH or glucose to the cell-free extract.

Previously we had demonstrated that by adding NADPH to the purified enzyme system we could generate hydrogen at volumetric productivities significantly higher than previous published reports. However in order to utilize glucose from cellulosic biomass, the available electrons in this substrate must be transferred to $NADP^+$ using the pentose phosphate pathway. While previous reports have shown that this could be accomplished by a mixture of 10-13 individually purified enzymes, we have now demonstrated the potential for significant cost reduction by eliminating the need to purify or isolate these proteins. Instead we use a cell extract, or unpurified lysed cells, to accomplish the same feat at hydrogen productivities above previous reports.

Root FNRs and Evolution of Volumetric Productivity

Previous work had demonstrated significant improvements in volumetric productivity up to 11.2 mmol H_2 L^{-1} hr^{-1} using FNRs from bacterial enzymes with a clostridial ferredoxin. However the bacterial FNR enzymes have evolved to consume hydrogen and generate NADPH, and therefore operate physiologically in the opposite direction of our desired pathway.

Consequently, we wanted to investigate other FNRs, specifically those used in plant roots that have evolved to consume NADPH and reduce other enzymes as part of the natural process of nutrient uptake and nitrate and sulfur metabolism in the roots. Three root FNRs

were therefore cloned and expressed in E.coli in addition to a root ferredoxin and were evaluated in our *in vitro* assays. Of these, only one root FNR could be produced in sufficient quantity to be evaluated in conjunction with the root ferredoxin. Fortunately, the root FNR tested was able to provide nearly 3-fold improvement to our hydrogen volumetric productivity, as shown in Figure 3.

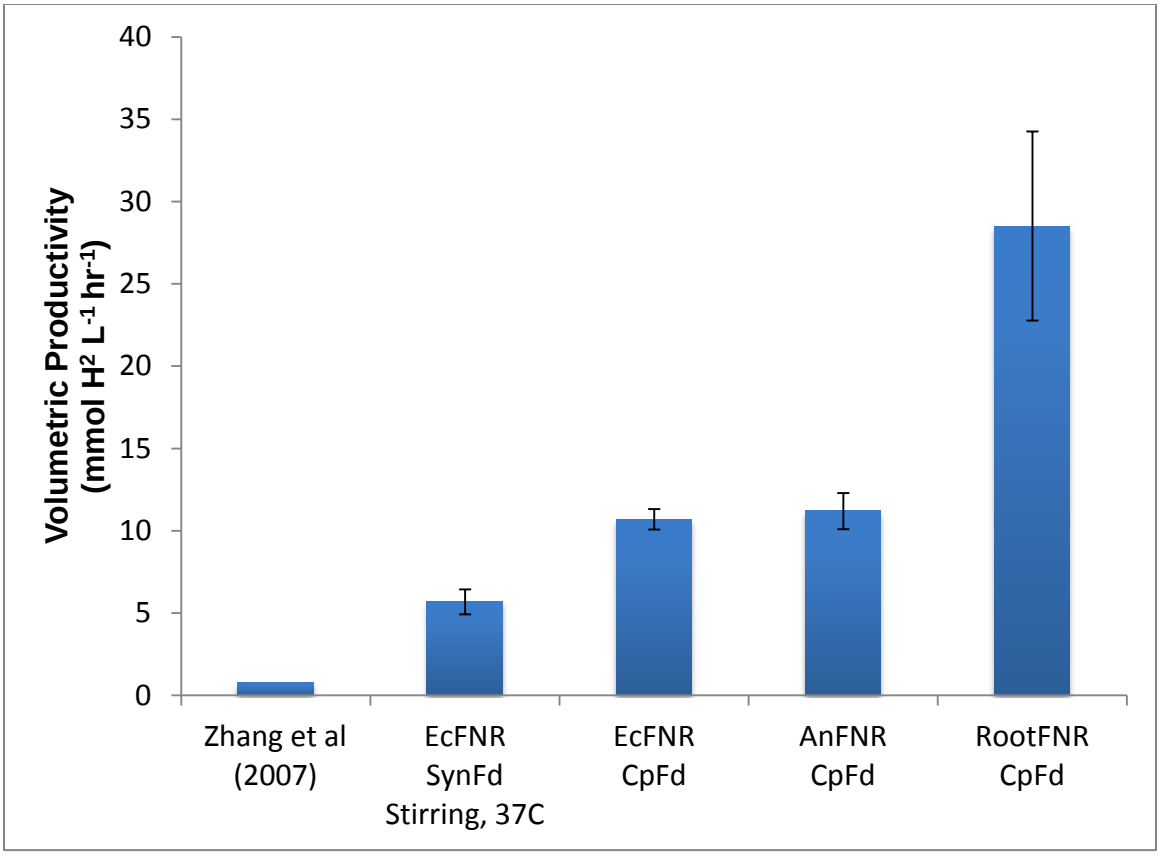

Figure 3: Hydrogen production measured by gas chromatography. The reaction was initiated by NADPH addition to the reactor containing 100μM FNR, 80-160μM Fd, 1-2μM hydrogenase. (EcFNR: Ecoli FNR, AnFNR: Anabaena FNR, SynFd: Synechocystis ferrodoxin, CpFd: Clostridial ferrodoxin)

Ongoing efforts to improve hydrogen production also include mutagenesis of the FNR binding site that recognizes ferredoxin to improve binding and electron transfer.

Fusion FNR-hydrogenase Proteins and Further Improvement of FNR Turnover Number

Increased electron flux rates from NADPH to hydrogenase were previously obtained by a cell-free produced fusion protein CpI-AnFNR, in which the FNR from *Anabaena sp*. (AnFNR) and hydrogenase from *C. pasteurianum* (CpI) are physically linked by a polypeptide chain. Figure 4 shows the design of the fusion protein: CpI was expressed at the N-terminus of the fusion with a repeated Glycine-Serine-Alanine (GSA) linker extending from its C-terminus to link to the N-terminus of the AnFNR enzyme. This configuration was the only possible linkage order since the C-terminal Tyrosine of FNR must be available to participate in the active site catalysis. The fusion was hypothesized to diminish any rate limitations due to

protein diffusion. There is also the possibility that allosteric inhibition could be reduced or avoided. Based on previous observations with unconnected proteins, we assumed that only those fusion proteins with an active hydrogenase enzyme could contribute to hydrogen production.

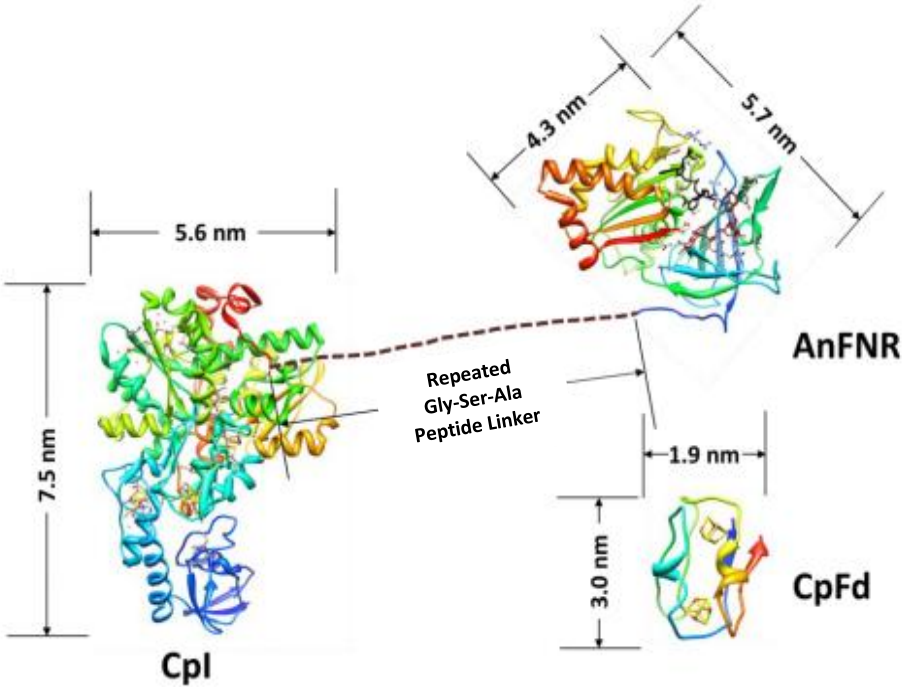

Figure 4: Diagram of the AnFNR-CpI hydrogenase fusion protein and the CpFd (*Clostridium pasteurianum* ferredoxin) used to shuttle electrons from FNR to the hydrogenase.

Our last report showed that such a fusion protein was active when expressed by cell-free protein synthesis. We then confirmed and extended this observation by expressing the CpI-AnFNR fusion *in vivo* in an *E. coli* BL21 DE3 ΔiscR strain (with the gene for the iron-sulfur Isc repressor deleted to provide FeS centers at a faster rate). The fusion protein gene was first constructed with five repeats of a GSA tripeptide, $(GSA)_5$, as the linker sequence. The assembled gene was then inserted into pET21b plasmid vector, between the T7 promoter and T7 terminator for efficient protein expression upon IPTG induction. The three maturase proteins required to activate the hydrogenase portion of the fusion protein were co-expressed from a separate plasmid, also using the T7 promoter. We also varied the amino acid composition of the polypeptide linker to include Valine and Threonine residues, in addition to the Gly-Ser-Ala repeats, to produce a $(GSAVT)_2$ peptide linker. Further, we fused CpI hydrogenase to the root FNR (RootFNR) which had been shown to have higher electron transfer rates compared to that of AnFNR. The expressed fusion proteins were first analyzed for hydrogen-producing activities in the unpurified cell lysates using gas chromatography.

The results are shown in Figure 5 and are presented as the calculated turnover numbers (rates) for the FNR (either free or as part of the fusion protein). The control was a reaction with un-fused enzymes added at equimolar concentrations. The data confirm that the

fusion protein allows the FNR to operate at ~5-fold higher rates than the control reaction. However, no significant difference was seen in hydrogen-producing activities with different linkers.

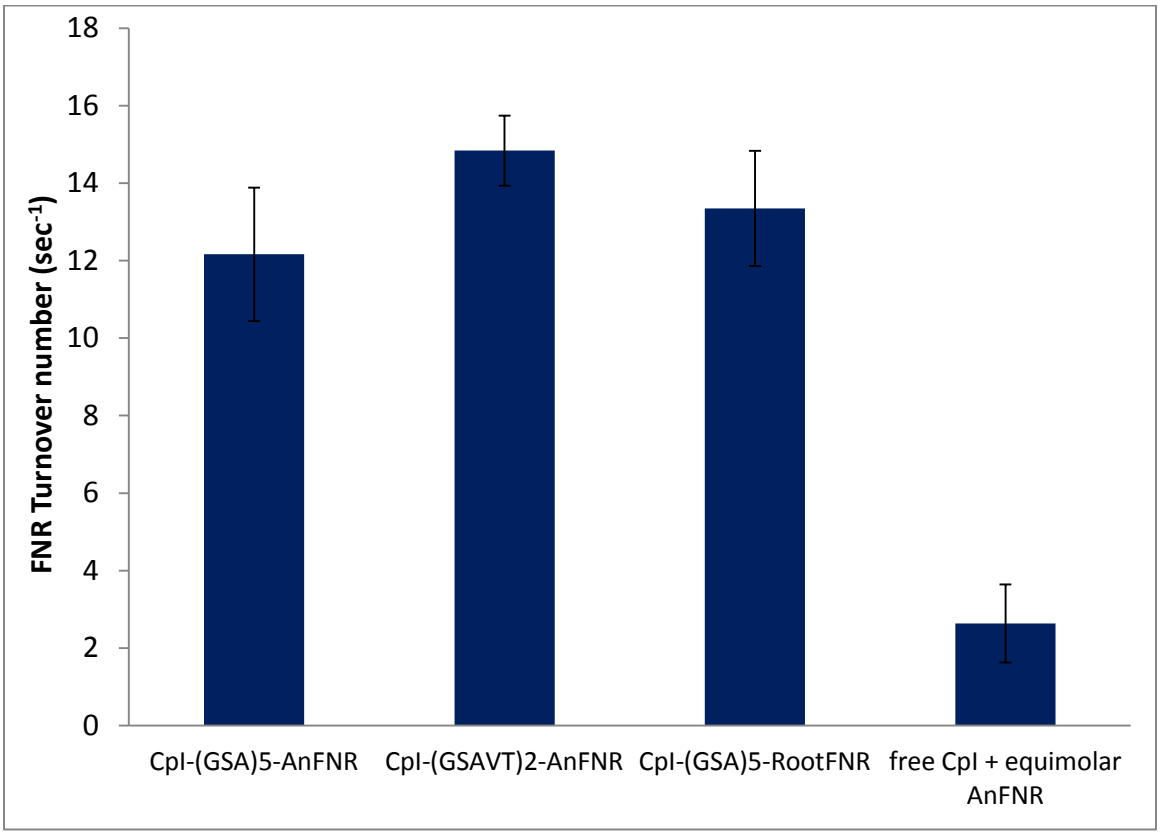

Figure 5: The performance of fusion FNR-hydrogenase proteins, as reflected by the FNR turnover number during hydrogen production from NADPH. The experiments were conducted with unpurified proteins in cell lysates to which 50μM CpFd had been added. For the control, equimolar AnFNR was added into the cell lysate containing un-fused CpI hydrogenase.

Attempts to purify the fusion proteins for more precise evaluation have been hampered by an apparent proteolytic cleavage that separates the FNR from the hydrogenase. Unfortunately, this occurs with both linker designs. Future work will focus on new linker designs as well as cell-strains that more effectively activate the hydrogenase.

Cell Free Metabolic Engineering – Inactivate Wasteful Side Reactions

Since the combination of our improved FNR with our fusion proteins support targeted productivities asuming effective expression of the fusion protein, we have now begun to focus on the engineering of the cell free extract to efficiently harvest the electrons and transfer them to $NADP^+$. While using a cell free extract is advantageous because of the elimination of purification cost, it also contributes potential complexity and inefficiency if our feedstock (glucose/xylose) is lost to other competing reactions either directly or by

metabolism of pathway intermediates. Common ways of addressing this problem *in vivo* include knocking out or down regulating competing pathways. However, the primary competition for converting glucose to hydrogen involves the normal energy generation and aerobic respiration pathways required for the cell to live. Fortunately, since we are producing a cell free extract, we only need these functions to operate while the cell is growing and producing the proteins we desire. When the cells have been lysed we are free to treat our cell free reaction and eliminate these competing pathways.

One method to accomplish this, pioneered by Greenlight Biosciences, is to insert a protease targeting site into key enzymes that are needed for growth but that reduce conversion efficiencies when the cell lysate is used. One such enzyme is glyceraldehyde 3 phosphate dehydrogenase (GAPDH) which converts glyceraldehyde 3 Phosphate (G3P) to 1,3 bisphosphoglycerate as part of glycolysis. Each cycle of the pentose phosphate pathway releases one molecule of CO_2 and generates 2 NADPH's. In order to completely oxidize glucose, the phosphorylated five carbon sugar intermediate that results from these oxidative steps must be recycled through conversion to a seven carbon sugar and a three carbon intermediate, glyceraldehyde 3 phosphate (G3P). Two G3P molecules can then be returned to glucose through gluconeogenesis, and the seven carbon intermediate is further processed. These reactions are essential for our process. However, the G3P can also be consumed through glycolysis by the activity of the GAPDH enzyme. Consequently, GAPDH is a key enzyme to inactivate in order to increase hydrogen production using our cell free extract.

We have successfully assembled genes for a library of 361 GAPDH mutants that could potentially be inactivated in the extract. We are currently utilizing cell free protein synthesis and a spectroscopic activity assay to screen for candidates that would retain activity during growth but could be inactivated in the cell lysate.

Simultaneously we are establishing a flow-through H_2 assay for continuous measurement of H_2 production in order to quantify our yields, evaluate further improvements, and evaluate enzyme stabilities. This assay will allow us to make real time measurements of H_2 production by utilizing a purge gas to carry away hydrogen as it is being produced, allowing our reaction to operate at maximum rate, as well as allowing us to determine the overall yield of our process to identify the next steps for further cell free metabolic improvements.

Conclusions

With our efforts, we have now been able to demonstrate volumetric hydrogen productivity 30 fold higher than the best previously published reports with potential for another five-fold or more improvement through utilization of our fusion proteins and future protein evolution. In addition, we have demonstrated the overall feasibility for a cost effective process that uses unpurified cell extracts and have begun to shift focus toward establishing both high productivity and high yield by optimizing our extracts through cell-free metabolic engineering.

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

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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.
3. Kuchenreuther, J. M. et al., High-yield expression of heterologous [FeFe] hydrogenases in *Escherichia coli*, *PLoS ONE* **5**(11): e15491, 2010.
4. Martin del Campo, J. S., J. Rollin, et al., High-Yield Production of Dihydrogen from Xylose by Using a Synthetic Enzyme Cascade in a Cell-Free System, *Angew. Chem. Int. Ed.* **52**: 4587–4590, 2013.

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