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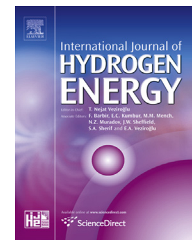

Development of a synthetic pathway to convert glucose to hydrogen using cell free extracts

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ABSTRACT

Sustainable production of biochemicals from cellulosic biomass is an attractive alternative to chemical production from fossil fuels. We describe the further development of a three protein synthetic pathway to convert NADPH and H^+ to hydrogen using a ferredoxin-NADP$^+$ reductase, a ferredoxin, and the [FeFe] hydrogenase from *Clostridium pasteurianum* at a rate greater than 14 mmol H_2 L^{-1} hr^{-1} using natural enzymes. We also demonstrate the feasibility of coupling this pathway to a cell-free extract to convert glucose to hydrogen in a potentially cost effective manner. Both of these accomplishments serve as the basis for further engineering to optimize both the yield and productivity of a low-cost cell-free process for the production of highly reduced biochemicals.
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Introduction

Hydrogen is an important commodity chemical with more than 50 million metric tons produced worldwide each year with a value of approximately $100 billion [1,2]. The majority of this hydrogen is produced through high temperature steam reformation of natural gas and is used directly in petroleum refining, the production of ammonia for fertilizer, or for other industrial processes such as metallurgical treatment [3]. An alternative to steam reformation that does not depend on fossil fuels and requires less energy input would be attractive as a sustainable method to ensure future hydrogen supply. One such method could be the use of cellulosic biomass to produce hydrogen using metabolically engineered organisms. The DOE estimates that a billion tons of biomass feedstock could be available in various forms for processing into biochemicals [4]. In particular, a biomass to hydrogen process could be advantageous by converting locally grown agricultural residuals to hydrogen and then immediately converting that hydrogen into ammonia for fertilizer. This fertilizer could then be delivered back to the agricultural areas from which

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the feedstock originated, thereby minimizing transportation for both feedstock and product.

Enzymatic depolymerization of cellulose and hemicellulose from pretreated agricultural residuals such as corn stover [5] releases the constituent sugars, which consist primarily of glucose and xylose as well as smaller amounts of other five-carbon sugars. Engineered organisms could then convert these sugars into biochemicals through sustainable processes [6] operating at low temperature and pressure relative to existing petrochemical processes.

Previous attempts to produce hydrogen with engineered organisms have focused on engineering cells to produce hydrogen *in vivo*. Wells et al. inserted a [NiFe] hydrogenase and associated maturation factors into an *Escherichia coli* BL21 cell line and produced hydrogen at a rate of 0.7 μmole H_2 L^{-1} hr^{-1} [7]. Subdubi et al. used a similar approach, expressing the HydA hydrogenase from *Clostridium butyricum* in *E. coli* to produce hydrogen at a rate of 0.625 mmol L^{-1} hr^{-1} [8]. Yoshida et al. produced hydrogen in *E. coli* using native formate hydrogen lyase and pyruvate formate lysae by knocking out competing pathways. By artificially concentrating their cell culture to 94.3 g dry cell weight/L, or approximately an OD610 of 230, they were able to produce hydrogen at a rate of 793 mmol L^{-1} hr^{-1} [9]. However, all these approaches were limited in overall yield of glucose to hydrogen: the theoretical maximum conversion *in vivo* is only 2 mol of hydrogen per mole of glucose using the formate-dependent pathway in facultative anaerobes such as *E. coli* and 4 mol of hydrogen per mole of glucose in certain obligate anaerobes [9]. In practice, yields of only 1.82–3.2 mol of hydrogen per mole of glucose have been reported [7–9] due to the need to maintain carbon and energy flow to other metabolic pathways as well. These yields are prohibitively low for bioprocesses that will be dominated primarily by feedstock cost.

Alternatively, other researchers have produced hydrogen *in vitro* using purified enzymes. Woodward et al. [10] first demonstrated this possibility by combining the enzymes of the pentose phosphate pathway with the [NiFe] hydrogenase from *Pyrococcus furiosus*. This enzyme can directly convert NADPH reduced by the pentose phosphate pathway to hydrogen. Woodward achieved a yield of 11.6 mol of H_2 per mole of glucose, over 90% of theoretical conversion. However, this was produced at a very low overall rate. In another example, Zhang et al. achieved a yield of 8.4 mol of hydrogen per mole of glucose at a significantly higher rate of 0.75 mmol L^{-1} hr^{-1} using the same system of purified enzymes with the *Pyrococcus furiosus* hydrogenase [11]. In addition, Zhang et al. later demonstrated that this *in vitro* pathway could also convert xylose to hydrogen after xylose isomerase and xylulokinase were included to generate xylose-5-phosphate, which could then enter the pentose phosphate pathway [12]. From this *in vitro* cocktail, Zhang and coworkers achieved 95% conversion of xylose to hydrogen at a rate of 1.95 mmol L^{-1} hr^{-1}. The ability to incorporate these five carbon sugars is particularly important in maximizing the yield of biochemicals from cellulosic biomass as a significant portion of the feedstock is composed of five carbon sugars. Further work by the Zhang group was also able to establish rates as high as 157 mmol H_2/L/hr using 100 mM Glucose 6 Phosphate and increased concentrations of their purified enzymes, including the hydrogenase which was increased 5.6 fold [13]. These rates are encouraging however, as they demonstrate the potential for *in vitro* bioconversion productivities similar to those of bioethanol plants While *in vitro* approaches can reach significantly higher yields compared to *in vivo* production, the processes described above have their own challenges in scale up due to the cost of producing and purifying 13–15 enzymes. Selection of thermostable enzymes that can be purified through simple heat treatment methods is one option to isolate multiple proteins without requiring costly resin based chromatography methods [14]. However, finding the right combination of heat stable, highly active enzymes, that can all be expressed in a small number of host cell lines still has its own challenges and currently still requires several separate purification methods [15].

Our proposed process seeks to combine the benefits of low cost *in vivo* enzyme production with high yield *in vitro* hydrogen production. We seek to accomplish this by engineering an *E. coli* cell extract in which a three protein synthetic pathway is coupled to the pentose phosphate pathway. The use of unpurified *E. coli* extracts eliminates the costly processing required to separate and purify individual components of the pathway as well as avoids potential challenges with heterologous expression of a completely synthetic pathway, while *in vitro* conversion potentially eliminates the loss of energy to other metabolic pathways thereby improving the overall yield.

We seek to complete this pathway with a [FeFe] hydrogenase from *Clostridium pasteurianum*, *CpI*. While use of CpI requires both ferredoxin NADPH reductase (FNR) and ferredoxin (Fd), CpI has higher reported turnover rates than the hydrogenases [16] previously used at the temperatures required for using *E. coli* extracts. In addition, CpI has been expressed heterologously in *E. coli* at high yields and only requires three proteins for maturation, as opposed 9 required to heterologously express the [NiFe] hydrogenase in addition to the four different subunits that must be coexpressed [17,18]. So while it may be possible to use other hydrogenases such as those from *Pyrococcus furiosus* or other [NiFe] hydrogenases, in order to achieve the simplest process of generating an *E. coli* cell extract without need for purification and have the highest potential rate of hydrogen production, we selected CpI as our hydrogenase.

Our pathway is shown in Fig. 1 and is composed of 14 proteins from the glycolytic and pentose phosphate pathways as well as our synthetic pathway of Ferredoxin $NADP^+$ reductase, Ferredoxin, and Hydrogenase. Glucose is first phosphorylated to glucose-6-phosphate, which is then oxidized through the pentose phosphate pathway to reduce two molecules of $NADP^+$ with the release of a single molecule of CO_2. The resulting 5-carbon sugar is then recycled through the non-oxidative pentose phosphate pathway. Six 5-carbon sugar units are converted back into five 6-carbon glucose-6-phosphate molecules, which can then reenter through the oxidative pentose phosphate pathway. The complete breakdown of a single glucose unit requires six cycles through this pathway, thereby reducing a total of 12 $NADP^+$ molecules. Our three-protein synthetic pathway (Ferredoxin-$NADP^+$ reductase, Ferredoxin, and Hydrogenase) potentially can utilize all of the resultant NADPH molecules to produce hydrogen

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