Biocatalysis

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High-Yield Production of Dihydrogen from Xylose by Using a Synthetic Enzyme Cascade in a Cell-Free System**

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Approximately 50 million metric tons of dihydrogen are produced annually from nonrenewable natural gas, petroleum, and coal.[1] H_2 production from water remains costly.[2] Technologies for generating H_2 from less costly biomass, such as microbial fermentation,[3] enzymatic decomposition,[4] gasification,[5] steam reforming,[6] and aqueous phase reforming,[7] suffer from low product yields.

The production of H_2 from relatively evenly distributed renewable biomass resources would address challenges pertaining to 1) sustainable H_2 production without net greenhouse gas emissions, 2) the availability of small decentralized H_2 production systems, and 3) a lack of H_2 distribution infrastructure.[7,8]

Xylose ($C_5H_{10}O_5$) is the most abundant pentose in our planet. It is a major building block of hemicellulose, one of the main constituents of lignocellulosic biomass. Xylose comprises ca. 20–30% weight of plant matter. Because it is not economically feasible to separate xylose from other biomass sugars, the co-utilization of pentose and hexose is essential to the success of the production of biocommodities from biomass. In principle, one mole of xylose when combined with H_2O can generate 10 mole of H_2 according to $C_5H_{10}O_5 + 5H_2O \rightarrow 10H_2 + 5CO_2$. However, by using microorganisms, one mole of xylose can theoretically generate at most 3.33 mole of H_2 when acetate is the major product.[9] A number of microorganisms or consortia can utilize xylose and glucose for the production of H_2,[3a,10] but they cannot do so in high yields, because H_2 is released as a way of keeping the redox balance when acetate, ethanol, or butyrate are major products.[8] In practice, the H_2 yields obtained from xylose are much lower than this yield (i.e., 3.33 mole of H_2 per mole of xylose) regardless of whether a single microorganism,[3c] coculture,[3a] or genetically modified microorganism[3b] is utilized. By using chemical catalysis[5,7] H_2 can be produced in higher yields than with microbial fermentation, but they are still 50% lower than the maximum yield owing to lower chemical selectivity. In addition, this endothermic chemical reaction requires high-temperature heat input through partial oxidation of xylose or biomass, thereby resulting in low product yields.[5]

Enzymatic H_2 production in vitro from a glucose was first achieved by Woodward and co-workers using glucose dehydrogenase and hydrogenase.[11] Enzymatic H_2 generation based on xylose was also reported, but the yield was very low, only 0.53 moles of H_2 per mole of xylose.[12] To efficiently release all of the chemical energy stored in glucose, glucose-6-phosphate (G6P) and eleven enzymes were combined and these generated almost the theoretical yield (11.6/12.0) of H_2 from G6P.[4] However, the use of the expensive G6P substrate precluded this as a method to produce H_2 at low costs.[13] To solve this problem, Zhang and his collaborators utilized the chemical bond energy stored in the glycosidic linkage in oligo- and polysaccharides to generate low-cost hexose phosphates by adding a small amount of recyclable phosphate ions and the appropriate phosphorylase.[14] As a result, nearly 12 moles of H_2 can be produced directly from starch or cellulosic materials without costly ATP or G6P.[14]

To maximize H_2 production from xylose, we designed an in vitro synthetic pathway (Figure 1), in which xylose is isomerized to xylulose by xylose isomerase followed by phosphorylation mediated by xylulokinase at a cost of one mole of ATP per mole of xylose. Xylulose-5-phosphate is produced and then oxidized by enzymes of the pentose phosphate pathway and glycolysis/gluconeogenesis to generate 10 NADPH and 5 CO_2. The NADPH-specific hydrogenase then converts NADPH to H_2. As a result, one mole of xylose

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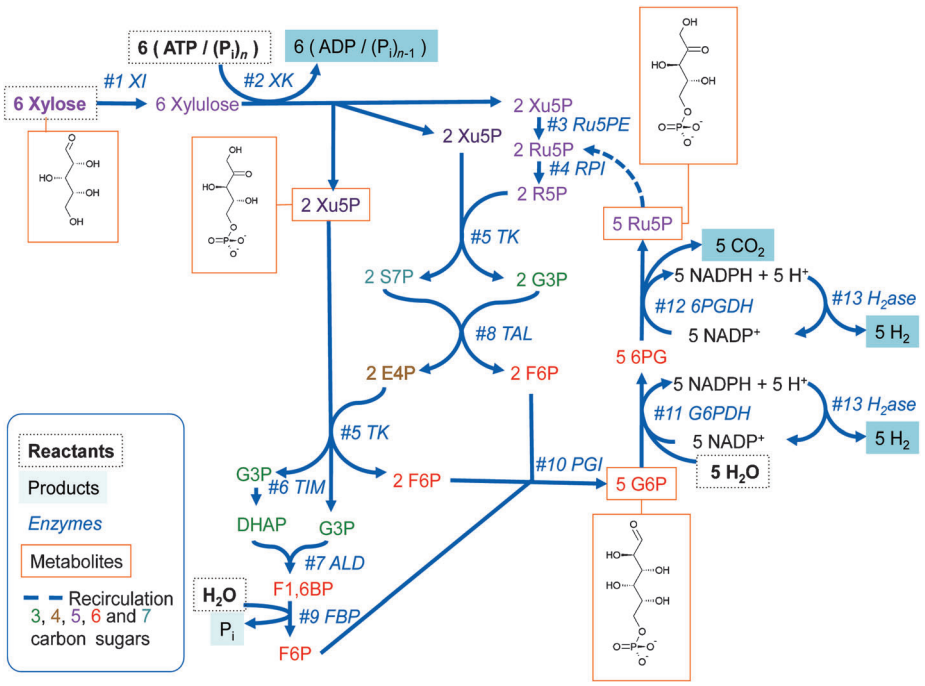

Figure 1. Synthetic enzymatic pathway for H_2 production from xylose. The metabolites are: Xu5P (xylulose 5-phosphate); Ru5P (ribulose 5-phosphate); R5P (ribose 5-phosphate); G3P (glyceraldehyde 3-phosphate); DHAP (dihydroxacetone phosphate); S7P (sedoheptulose 7-phosphate); F1,6BP (fructose 1,6-biphosphate); F6P (fructose 6-phosphate); E4P (erythrose 4-phosphate); G6P (glucose 6-phosphate); 6PG (6-phosphogluconate); P_i (inorganic phosphate); and $(P)_n$ (polyphosphate with a degree of polymerization of *n*). The enzymes are listed in Table S1 in the Supporting Information. The enzymes are #1 XI (xylose isomerase); #2 XK (xylulokinase); #3 Ru5PE (ribulose-5-phosphate 3-epimerase); #4 RPI (ribose 5-phosphate isomerase); #5 TK (transketolase); #6 TIM (triose phosphate isomerase); #7 ALD (aldolase); #8 TAL (transaldolase); #9 FBP (fructose biphosphatase); #10 PGI (phosphoglucose isomerase); # 11 G6PDH (glucose-6-phosphate dehydrogenase); #12 6PGDH (6-phosphogluconate dehydrogenase); and #13 H_2ase (hydrogenase).

plus six moles of water can generate 10 moles of H_2 and 5 moles of carbon dioxide at a cost of one ATP to ADP and phosphate [Eq. (1)]. In Equation (1), 5 molecules of water are

$$C_5H_{10}O_5 + 6\,H_2O + ATP \rightarrow 10\,H_2 + 5\,CO_2 + ADP + P_i \qquad (1)$$

consumed for xylose oxidation, and 1 molecule of water is eventually consumed for the hydrolysis of the phosphate bond of ATP. This synthetic metabolic pathway cannot work in microorganisms, because ATP must be supplied as the energy source and the overall reaction [Eq. (1)] is endothermic ($\Delta H° = +479.6\ kJ\,mol^{-1}$) but may occur owing to an entropy gain from the aqueous phase to gas phase ($\Delta G° = -71.4\ kJ\,mol^{-1}$).

To investigate the viability of this approach, eleven of the thirteen enzymes were obtained in their recombinant forms after expression in *E. coli* BL21 (DE3) and purified to homogeneity (Table S1 and Figure S1 in the Supporting Information). Most of the enzymes were purified by using their His-tag on nickel-charged resins. Two enzymes containing a cellulose-binding module tag were purified through the high-affinity adsorption on a cellulosic material followed by intein self-cleavage. Four of them were purified by inducing precipitation of *E. coli* cellular proteins at 80 °C (heat precipitation) for 20 min; the supernatants of the heat-treated cell lysates were used to prepare the enzyme cocktail directly. The details of recombinant enzyme sources, expression conditions, and purification methods are presented in Table S1. The other two enzymes were xylose isomerase, which was commercially available (Sigma–Aldrich), and the NADPH-dependent hydrogenase, which was obtained after overexpression in its native host, the hyperthermophilic archaeon *Pyrococcus furiosus*.[15] To validate the synthetic pathway design, the thirteen enzymes (Table S1) were put into a bioreactor at 50 °C. H_2 was generated from xylose (2 mM) as expected, and the maximum rate of H_2 generation was 1.95 mmol of H_2 per liter per hour after the first hour (Figure 2). The integrated H_2 yield after 20 hours was 95 % or 9.5 moles of H_2 per mole of xylose.

To avoid using costly ATP, we sought to find an alternative method to generate xylulose-5-phosphate. Polyphosphate is a less costly phosphate donor than ATP. Because it is abundant in volcanic condensates and deep-oceanic steam vents, ancient organisms are thought to have utilized polyphosphate instead of ATP in their metabolisms.[16] We hypothesized that xylulokinase from an ancestral thermophilic microorganism *Thermotoga maritima* might utilize

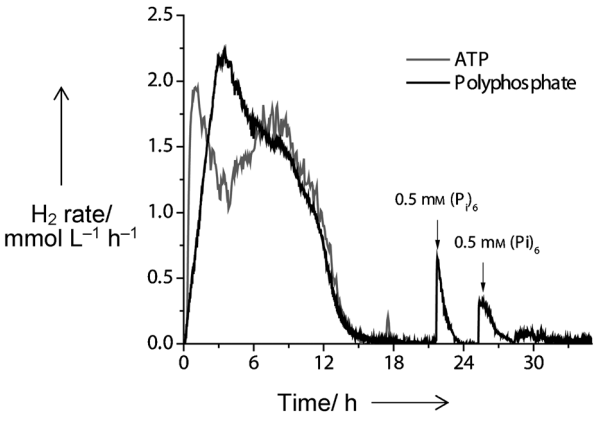

Figure 2. The profile of H_2 generation from xylose (2 mM) with ATP (3 mM) or polyphosphate (4 mM) at 50 °C. The reaction buffer was HEPES (100 mM, pH 7.5) containing $NADP^+$ (4 mM), thiamine pyrophosphate (0.5 mM), $MgCl_2$ (10 mM), and $MnCl_2$ (0.5 mM), along with the thirteen enzymes (Table S1). Sodium $(Pi)_6$ (0.5 mM final concentration) was added twice as shown at the end of the reaction.

polyphosphate to activate xylulose, particularly since polyphosphate regulates the Embden–Meyerhof pathway in this organism[17] and a number of enzymes are found to have some promiscuous activities.[18] The gene encoding xylulokinase (TM1762) was cloned and expressed in *E. coli* BL21(DE3). As hypothesized, the resulting enzyme was able to phosphorylate xylulose by using either ATP or polyphosphate. The specific activities when using ATP or polyphosphate at 50 °C were 30.4 and 12.0 U mg^{-1}, respectively. Hence, when ATP is replaced with polyphosphate in the synthetic pathway, the theoretical yield of 10 moles of H_2 should be generated per mole of xylose and water [Eq. (2)]. Similar to Equation (1),

$$C_5H_{10}O_5 + 6\,H_2O + (P_i)_n \rightarrow 10\,H_2 + 5\,CO_2 + (P_i)_{n-1} + P_i \qquad (2)$$

5 molecules of water are consumed for xylose oxidation and 1 molecule of water is eventually consumed for the hydrolysis of each phosphate bond of polyphosphate.

To investigate the feasibility of using polyphosphate rather than ATP to drive H_2 production, the thirteen enzymes were mixed with xylose (2 mM) and polyphosphate (4 mM). Since *T. maritima* xylulokinase is less active on polyphosphate than on ATP, we increased its concentration to 4 mM. The maximum rate of H_2 generation was 2.23 mmol H_2 L^{-1} h^{-1} at 3.5 hours, and the overall H_2 yield was 90 % after the first 20 h (Figure 2). The addition of polyphosphate (0.5 mM) at hours 21 and 25.5 enhanced more utilization of xylose, thereby resulting in an overall H_2 yield of 96 % (that is, 9.6 moles of H_2 per mole of xylose). The above experiments based on ATP and polyphosphate were repeated at least three times. The standard deviations of the final H_2 yields and maximum rates of H_2 generation were less than 4 % and 6 %, respectively.

Hence, in our previous work it was demonstrated that nearly theoretical yields of H_2 (that is, 12 moles per mole of glucose) can be produced directly from cellulose, one of the major components of plant biomass, by a synthetic enzyme pathway.[14] We now show that the other major component of biomass, xylose, can similarly be converted to H_2 by a synthetic pathway. The co-utilization of hexoses and pentoses in lignocellulosic biomass is essential to produce economically viable biofuels, because it is economically prohibited to separate pentoses from hexoses. The synthetic pathway presented here results in nearly theoretical yields of H_2 from xylose for the first time. Additionally, this entropy-driven endothermic reaction leads to a net increase in the chemical energy by absorbing heat from the surrounding environment (that is, the combustion energy of 10 moles of H_2 is larger than that of one mole of xylose). This is another example for the conversion of waste heat into high-quality chemical energy, such as H_2. The key to this pathway is our discovery of the first polyphosphate-dependent xylulokinase, which does not require the use costly ATP. The released phosphate ions can be recycled by dehydration of phosphoric acid at an elevated temperature or through biological linkage by polyphosphate-accumulating microorganisms in wastewater treatment processes.[19]

In vitro synthetic biology is evolving from fundamental research tools to a new biomanufacturing platform.[13] In contrast to in vivo synthetic biology projects that involve complex regulation, self-duplication, and undesired branched pathways, in vitro synthetic biology is based on purified stable enzymes, where enzyme production and pathway assembly are separated in space and time. Cell-free biosystems have unique advantages over living microorganisms in industrial applications. These include high product yield,[14] fast reaction rate,[20] great engineering flexibility for in vitro assembly,[21] easy access and control,[22] tolerance of toxic compounds[23] and products,[24] and shifting unfavorable reaction equilibrium.[25] With the development of more thermostable enzymes as pathway building blocks, the maturation of protein engineering methods,[26] and engineered redox enzymes that can work with low-cost stable biomimetic coenzymes,[27] cell-free biosystems could become an innovative biomanufacturing platform with characteristic high yields and potentially low production costs.

Experimental Section

All chemicals were reagent-grade or higher and purchased from Sigma–Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA). Xylose isomerase from *Streptomyces murinus* (G4166) was purchased from Sigma–Aldrich. *P. furiosus* hydrogenase SH1 with an N-terminal His_9-tag on subunit PF0891 was obtained as described earlier.[15] The 11 recombinant thermoenzymes were produced in *E. coli* BL21 (DE3) in the Luria–Bertani (LB) medium and purified to homogeneity (Figure S1 and Table S1) by using their His-tag, cellulose-binding module, or heat precipitation at 80 °C for 20 min.

The experiments were carried out in a continuous-flow system, which was purged with ultrapure nitrogen (Airgas).[12,14] H_2 evolution was detected with a tin oxide thermal conductivity sensor (Figaro TGS 822, Osaka, Japan) that was previously calibrated with in-line flow-controllers and ultrapure hydrogen. The working volume of the reactor was kept constant by humidifying ultrapure nitrogen. Data collection was conducted by Ni-module NI USB-6210 (National Instruments Corp., Austin TX, USA) and analyzed by LabView SignalExpress.

The reaction buffer was HEPES (100 mM, pH 7.5) contained $NADP^+$ (4 mM), thiamine pyrophosphate (0.5 mM), $MgCl_2$ (10 mM), and $MnCl_2$ (0.5 mM). The concentrations of xylose, ATP, and sodium hexametaphosphate were 2 mM, 3 mM, and 4 mM, respectively. The working volume of the bioreactor was 1 mL. Immobilized XI (28 mg) was placed in the bioreactor, and then the buffer containing substrates and cofactor and the mixture of enzymes were added in sequence.

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[1] R. M. Navarro, M. A. Pena, J. L. G. Fierro, *Chem. Rev.* **2007**, *107*, 3952 – 3991.
[2] A. J. Esswein, D. G. Nocera, *Chem. Rev.* **2007**, *107*, 4022 – 4047.
[3] a) X. Zhang, X. Ye, K. T. Finneran, J. L. Zilles, E. Morgenroth, *Biotechnol. Bioeng.* **2013**, *110*, 164 – 172; b) T. Maeda, V. Sanchez-Torres, T. K. Wood, *Microb. Biotechnol.* **2012**, *5*, 214 – 225; c) T. A. Ngo, T. H. Nguyen, H. T. V. Bui, *Renewable Energy* **2012**, *37*, 174 – 179.
[4] J. Woodward, M. Orr, K. Cordray, E. Greenbaum, *Nature* **2000**, *405*, 1014 – 1015.
[5] D. Castello, L. Fiori, *Bioresour. Technol.* **2011**, *102*, 7574 – 7582.

[6] L. He, D. Chen, *ChemSusChem* **2012**, *5*, 587 – 595.
[7] R. D. Cortright, R. R. Davda, J. A. Dumesic, *Nature* **2002**, *418*, 964 – 967.
[8] Y.-H. P. Zhang, *Energy Environ. Sci.* **2009**, *2*, 272 – 282.
[9] P. Kongjan, B. Min, I. Angelidaki, *Water Res.* **2009**, *43*, 1414 – 1424.
[10] S.-Y. Wu, C.-Y. Lin, K.-S. Lee, C.-H. Hung, J.-S. Chang, P.-J. Lin, F.-Y. Chang, *Energy Fuels* **2008**, *22*, 113 – 119.
[11] J. Woodward, S. M. Mattingly, M. Danson, D. Hough, N. Ward, M. Adams, *Nat. Biotechnol.* **1996**, *14*, 872 – 874.
[12] J. Woodward, K. A. Cordray, R. B. Edmonston, M. Blanco-Rivera, S. M. Mattingly, B. R. Evans, *Energy Fuels* **2000**, *14*, 197 – 201.
[13] Y.-H. P. Zhang, S. Myung, C. You, Z. G. Zhu, J. Rollin, *J. Mater. Chem.* **2011**, *21*, 18877 – 18886.
[14] a) X. Ye, Y. Wang, R. C. Hopkins, M. W. W. Adams, B. R. Evans, J. R. Mielenz, Y.-H. P. Zhang, *ChemSusChem* **2009**, *2*, 149 – 152; b) Y.-H. P. Zhang, B. R. Evans, J. R. Mielenz, R. C. Hopkins, M. W. W. Adams, *PLoS One* **2007**, *2*, e456.
[15] S. K. Chandrayan, P. M. McTernan, R. C. Hopkins, J. S. Sun, F. E. Jenney, M. W. W. Adams, *J. Biol. Chem.* **2012**, *287*, 3257 – 3264.
[16] H. H. Liao, S. Myung, Y.-H. P. Zhang, *Appl. Microbiol. Biotechnol.* **2012**, *93*, 1109 – 1117.
[17] Y.-H. R. Ding, R. S. Ronimus, H. W. Morgan, *J. Bacteriol.* **2001**, *183*, 791 – 794.
[18] H. Nam, N. E. Lewis, J. A. Lerman, D.-H. Lee, R. L. Chang, D. Kim, B. O. Palsson, *Science* **2012**, *337*, 1101 – 1104.
[19] A. Kuroda, K. Nomura, R. Ohtomo, J. Kato, T. Ikeda, N. Takiguchi, H. Ohtake, A. Kornberg, *Science* **2001**, *293*, 705 – 708.
[20] A. Zebda, C. Gondran, A. Le Goff, M. Holzinger, P. Cinquin, S. Cosnier, *Nat. Commun.* **2011**, *2*, 370.
[21] M. C. Jewett, K. A. Calhoun, A. Voloshin, J. J. Wuu, J. R. Swartz, *Mol. Syst. Biol.* **2008**, *4*, 57.
[22] a) E. D. Carlson, R. Gan, C. E. Hodgman, M. C. Jewett, *Biotechnol. Adv.* **2012**, *30*, 1185 – 1194; b) M. Bujara, M. Schümperli, R. Pellaux, M. Heinemann, S. Panke, *Nat. Chem. Biol.* **2011**, *7*, 271 – 277.
[23] Y. Wang, W. Huang, N. Sathitsuksanoh, Z. Zhu, Y.-H. P. Zhang, *Chem. Biol.* **2011**, *18*, 372 – 380.
[24] J.-K. Guterl, D. Garbe, J. Carsten, F. Steffler, B. Sommer, S. Reiße, A. Philipp, M. Haack, B. Rühmann, U. Kettling, T. Brück, V. Sieber, *ChemSusChem* **2012**, *5*, 2165 – 2172.
[25] A. Moradian, S. A. Benner, *J. Am. Chem. Soc.* **1992**, *114*, 6980 – 6987.
[26] U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185 – 194.
[27] J. D. Ryan, R. H. Fish, D. S. Clark, *ChemBioChem* **2008**, *9*, 2579 – 2582.

