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In vitro metabolic engineering of hydrogen production at theoretical yield from sucrose

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ABSTRACT

Hydrogen is one of the most important industrial chemicals and will be arguably the best fuel in the future. Hydrogen production from less costly renewable sugars can provide affordable hydrogen, decrease reliance on fossil fuels, and achieve nearly zero net greenhouse gas emissions, but current chemical and biological means suffer from low hydrogen yields and/or severe reaction conditions. An in vitro synthetic enzymatic pathway comprised of 15 enzymes was designed to split water powered by sucrose to hydrogen. Hydrogen and carbon dioxide were spontaneously generated from sucrose or glucose and water mediated by enzyme cocktails containing up to15 enzymes under mild reaction conditions (i.e. 37 °C and 1able sugars can provide affordable hydrogen, decrease r mol of dihydrogen per mole of sucrose, i.e., 96.7% of the theoretical yield (i.e., 12l $_2$ per hexose). In a fed-batch reaction, increasing substrate concentration led to 3.3-fold enhancement in reaction rate to 9.74 mmol of H_2/L/h. These proof-of-concept results suggest that catabolic water splitting powered by sugars catalyzed by enzyme cocktails could be an appealing green hydrogen production approach.

1. Introduction

Concerns about the depletion of fossil fuels and accumulation of greenhouse gases motivate the use of renewable energy sources and enhanced energy utilization efficiencies. Hydrogen is widely believed to be one of the best future energy carriers and energy storage compounds, especially in the hypothetical hydrogen economy, mainly because of higher energy conversion efficiencies through fuel cells and fewer pollutants generated in end users. Full development of the hydrogen economy requires breakthroughs in hydrogen production, storage, transportation and distribution (Armaroli and Balzani, 2011; Zhang, 2009). Also, hydrogen, which is mainly produced from natural gas, is one of the most important chemical commodities used for making fertilizers and refining liquid transportation fuels (Armaroli and Balzani, 2011; Navarro et al., 2007). The future of energy chains depends on innovative breakthroughs in the design of cheap, sustainable, and efficient systems for the harvesting, conversion, and storage of renewable energy sources, such as solar energy and carbohydrates (Artero et al., 2011).

Sunlight-driven water splitting for the production of hydrogen through artificial photosynthesis can be implemented by using natural photosynthetic systems, namely hydrogenases and photosystem II (Barber and Tran, 2013; Ducat et al., 2011; Wang et al., 2012; Wells et al., 2011); artificial photosynthetic systems based on photosensitizers/semiconductors/photocatalysts (Artero et al., 2011; Mubeen et al., 2013); and their hybrids (Iwuchukwu et al., 2009). Because solar energy is intermittent, broad wavelength electromagnetic radiation with an average energy concentration of ~170 W/m^2, great challenges result from solar energy harvesting, high-efficiency conversion under different strength insolations, and gaseous product collection from large-surface solar energy harvesting systems (Barber and Tran, 2013). Therefore, technologies for water splitting powered by direct solar energy are still far from practical applications (Armaroli and Balzani, 2011; Artero et al., 2011; Esswein and Nocera, 2007).

In vitro metabolic engineering or cell-free metabolic engineering has been used to understand complicated cellular metabolisms (Hodgman and Jewett, 2012; Jung and Stephanopoulos, 2004; Zhang, 2010). Recently, it is under investigation for its manufacturing potentials (Hodgman and Jewett, 2012; Rollin et al., 2013; Swartz, 2013), such as the synthesis of special proteins (Goerke et al., 2008; Hodgman and Jewett, 2012) and high-value polysaccharides (Xu et al.,

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2011), as well as the production of biofuels and bioelectricity (Guterl et al., 2012; Krutsakorn et al., 2013; Martín del Campo et al., 2013; Zhu et al., 2014), biochemicals (Bujara et al., 2011; Korman et al., 2014; Wang et al., 2011), and even potential food/feed (You et al., 2013). in vitro metabolic engineering feature several compelling biomanufacturing advantages, such as high product yields without the formation of by-products or the synthesis of cell mass; fast reaction rates without cell membrane (Hodgman and Jewett, 2012; Zhu et al., 2014); the tolerance of toxic products or substrates (Guterl et al., 2012; Wang et al., 2011); broad reaction conditions such as high temperature, low pH, the presence of organic solvents or ionic liquids (Panke et al., 2004); easy product separation; the implementation of non-natural reactions, for example, enzymatic transformation of cellulose to starch (You et al., 2013); among others.

Sucrose is the primary product of plant photosynthesis and then converted to other plant components. Sucrose is a disaccharide composed of glucose linked to fructose via an ether bond between C1 on the glucosyl subunit and C2 on the fructosyl unit. It is the most abundant disaccharide and approximately 168 million metric tons was produced from sugarcane, sugar beet, sorghum, and so on, in 2011 (Qi et al., 2014). Although its price varied greatly by several folds in the past 10 years (Qi et al., 2014), sucrose is among the cheapest fermentable sugars. When feedstock cost is considered only, hydrogen production from sucrose might be more cost competitive than from starch. For example, Brazil produces the lowest-cost ethanol compared to ethanol produced from starch and lignocellulosic biomass sugars.

Water splitting powered by sugars instead of insolation is promising due to potentially high reaction rates and easy product collection and separation. However, natural and metabolically engineered hydrogen-producing microorganisms cannot produce high-yield hydrogen from sugars due to the Thauer limit (i.e., 4 mol of hydrogen per mole of hexose) (Agapakis et al., 2010; Chou et al., 2008; Ducat et al., 2011; Maeda et al., 2012). To break the constraints of microorganisms, in vitro metabolic engineering can be used to implement complicated biological reactions by the in vitro assembly of numerous (purified) enzymes or cell extracts, a system that insulates biocatalyst preparation from product formation in space and time. Woodward and his co-workers demonstrated the production of 11.6 mol of hydrogen from 1ne m of glucose 6-phosphate (Woodward et al., 2000) (i.e., $12H_2$ can be produced from one glucose 6-phosphate and seven H_2O), but the high cost of the substrate prevented its potential application. Later, we proposed the utilization of the 1,4-glycosidic bond energy stored between two anhydroglucose units of polysaccharides (e.g., starch and cellodextrins) mediated by glucan phosphorylases plus recycled phosphate ions for producing glucose 6-phopshate without costly ATP (Ye et al., 2009; Zhang et al., 2007). As a result, nearly 12 mol of hydrogen was produced from each glucose unit of polysaccharides, where the theoretical yield of hydrogen was $12H_2$ per hexose (Ye et al., 2009; Zhang et al., 2007). However, 1ne m of glucose unit per mole of polysaccharides cannot be utilized. When the degree of polymerization of polysaccharides and oligosaccharides is small, a significant fraction of hexose cannot be utilized for hydrogen production.

In this study, a novel in vitro non-natural enzymatic pathway comprised of 15 enzymes was designed to convert sucrose, glucose or fructose to high-yield hydrogen without the use of costly ATP. Also, a fed-batch reaction was run for enhancing hydrogen generation rates at high substrate concentrations.

2. Materials and methods

2.1. Chemicals and strains

All chemicals were reagent grade, purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Avicel PH105, microcrystalline cellulose, was purchased from FMC (Philadelphia, PA). The genomic DNA sample of *Thermus thermophilus* HB27 was purchased from the American Type Culture Collection (Manassas, VA). *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) containing a protein expression plasmid was used to produce the recombinant protein. Luria–Bertani (LB) medium was used for *E. coli* cell growth and recombinant protein expression supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin. Oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA) and Fisher Scientific. Xylose isomerase (G4166) from *Streptomyces murinus* and sucrose phosphorylase (S0937) from *Leuconostoc mesenteroides* were purchased from Sigma. Recombinant hydrogenase SH1 was produced and purified from *Pyrococcus furiosus* (Chandrayan et al., 2012).

2.2. Plasmid construction

Two new plasmids were prepared for producing *T. thermophilus* HB27 fructose-bisphosphate aldolase (TtcALD) and transketolase (TtcTK) in *E. coli* BL21 (DE3). Plasmids pET20b-ttc-ald and pET20b-ttc-tk were constructed by the newly developed restriction enzyme-free, ligase-free and sequence-independent simple cloning method (You et al., 2012b). The other plasmids were described elsewhere (Martín del Campo et al., 2013; Wang et al., 2011).

The 918-bp DNA fragment containing the open reading frame (ORF) of the fructose-bisphosphate aldolase (Ttc1414) was amplified by PCR from the genomic DNA of *T. thermophilus* HB27 using a pair of primers (forward primer:5′-TAACT TTAAG AAGGA GATAT ACATA TGCTG GTAAC GGGTC TAGAG ATCT-3′; reverse primer: 5′-AGTGG TGGTG GTGGT GGTGC TCGAG AGCCC GCCCC ACGGA GCCGA AAAGC-3′). The vector backbone of pET20b was amplified by PCR using a pair of primers (forward primer: 5′-GCTTT TCGGC TCCGT GGGGC GGGCT CTCGA GCACC ACCAC CACCA CCACT-3′; reverse primer: 5′-AGATC TCTAG ACCCG TTACC AGCAT ATGTA TATCT CCTTC TTAAA GTTAA-3′). The PCR products were purified using the Zymo Research DNA Clean & Concentrator Kit (Irvine, CA). The insertion DNA fragment and vector backbone were assembled by prolonged overlap extension PCR (You et al., 2012b), and then the PCR product (DNA multimer) was directly transformed into *E. coli* TOP10 cells, yielding the desired plasmid.

The 1956-bp DNA fragment containing the ORF of the transketolase (Ttc1896) was amplified by PCR from the genomic DNA of *T. thermophilus HB27* using a pair of primers (forward primer: 5′-TTAAC TTTAA GAAGG AGATA TACAT ATGAA GGAGA CGCGG GACCT AGAGA-3′; reverse primer: 5′-GATCT CAGTG GTGGT GGTGG TGGTG CACCA GGGAG AGGAA GGCCT CCGCC-3′). The vector backbone of pET20b was amplified by PCR using a pair of primers (forward primer: 5′-GGCGG AGGCC TTCCT CTCCC TGGTG CACCA CCACC ACCAC CACTG AGATC-3′; reverse primer: 5′-TCTCT AGGTC CCGCG TCTCC TTCAT ATGTA TATCT CCTTC TTAAA GTTAA-3′). The insertion DNA fragment and vector backbone DNA fragment was assembled to the desired plasmid by using prolonged overlap extension PCR (You et al., 2012b).

2.3. Recombinant protein expression and purification

For the preparation of recombinant proteins: two hundred milliliters of LB culture containing 50 μg/mL of kanamycin or 100 μg/mL of ampicillin in 1-L Erlenmeyer flasks was incubated with a rotary shaking rate of 250 rpm at 37 °C. When the absorbance (A_{600}) reached *ca.* 0.6–1.2, recombinant protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (0.01–0.1 mM, final concentration). The culture was incubated at 37 °C for 4 h or at 18 °C for 20 h. The cells were harvested by centrifugation at 4 °C, washed twice by 50 mM of Tris–HCl buffer (pH 7.5), and re-suspended in a 15 mL of 30 mM

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