Chapter 3
High-Yield Production of Biohydrogen from Carbohydrates and Water Based on In Vitro Synthetic (Enzymatic) Pathways

Jae-Eung Kim and Yi-Heng Percival Zhang

Abstract Distributed production of green and low-cost hydrogen from renewable energy sources is necessary to develop the hydrogen economy. Carbohydrates, such as cellulose, hemicellulose, starch, sucrose, glucose, and xylose, are abundant renewable bioresources and can provide the source of hydrogen. In this chapter, in vitro synthetic (enzymatic) pathways that overcome the limiting yields of hydrogen-producing microorganisms are discussed. These in vitro synthetic pathways produce hydrogen with theoretical yields from polymeric and monomeric hexoses or xylose with water of 2 mol of hydrogen per carbon molecule of carbohydrate. In the past years, hydrogen production rate of in vitro synthetic enzymatic pathways has been improved to 150 mmol/L/h by 750-fold through systematic optimization. All of the thermostable enzymes used in the pathways have been recombinantly produced in *E. coli*, and some of them are immobilized for enhanced stability and simple recycling. Redox enzymes are being engineered to work on low-cost and highly stable biomimetic coenzymes. It is expected that low-cost green hydrogen can be produced at $2.00/kg hydrogen in small-sized atmospheric pressure bioreactors in the future.

Keywords Carbohydrate • Hydrogen production • Synthetic enzymatic pathway • In vitro synthetic biosystem • Innovative biomanufacturing • Systems biocatalysis

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3.1 Introduction

3.1.1 Hydrogen

Dihydrogen gas is a colorless, odorless, but very flammable diatomic molecule. Later on throughout this chapter, hydrogen is used to mean dihydrogen gas for convenience. Hydrogen atoms widely exist in natural inorganic and organic matters, such as water, hydrocarbons, and carbohydrates. Hydrogen has been proposed as a future alternative fuel to reduce our demand on traditional fossil fuel-based energy consumption due to its clean energy property, enhanced energy conversion efficiency, and high specific energy density (J/kg) [7, 9, 54]. The combustion or electrochemical conversion of hydrogen produces only water as a final by-product. Vehicles equipped with hydrogen fuel cells are far more energy efficient than traditional internal combustion engine-based vehicles [53]. For example, Toyota will start producing a large number of affordable hydrogen fuel cell vehicles at selling prices of $~50,000 in 2016. Hydrogen also has a higher specific mass energy density than any other fuel sources including gasoline and diesel. However, the low volumetric energy density of hydrogen is currently the biggest issue for its practical use. Thus, the development of new types of green hydrogen production and storage technology remains as challenges.

3.1.2 Hydrogen Production Approaches

Currently most hydrogen is produced from natural gas or coals through a reforming process or gasification followed by water shifting, respectively. These processes are not environment-friendly, releasing CO_2, and the resources are not sustainable. Therefore, the use of hydrogen produced in such ways would have little impact on reducing our demand on traditional fossil fuel-based energy sources [43]. Alternatively, hydrogen can be generated by splitting water molecules with high-temperature thermal energy sources or electricity. Direct splitting of water molecules requires high-temperature thermal energy over 2,000 °C [43]. Sulfur-based thermochemical decomposition of water with heterogeneous metal oxide catalysts can take place at much lower temperatures than direct thermal decomposition, but it still requires at least 750 °C or higher [14]. The thermal energy required for water splitting can be derived from solar energy. Water splitting by solar energy to generate hydrogen, however, is a very slow and inefficient process due to photocatalysts using limited range of visible light and low insolation flux (e.g., ~200 W/m^2) [30, 58]. Most photocatalytic water-splitting processes have shown their production rate less than 1 mmol H_2/L/h [23]. Much higher rates of hydrogen production can be achieved with electricity. A stationary electrolyzer with current technology has been reported to be able to generate hydrogen from water at the rate of about 40 mol/L/h [22]. In spite of such high hydrogen production rates and purity

of hydrogen generation by water electrolysis, its practical applicability is limited due to its high production cost (e.g., ~0.05 US dollars per kWh of electricity). Water electrolysis cannot be free from environmental issues as long as the electricity for electrolyzers is generated from coal or natural gas-powered electric generators [17].

Solar energy and electricity can also be applied to microorganisms to produce hydrogen through photo-fermentation and microbial electrolysis cells (MECs), respectively. Dark fermentation has a theoretical maximum hydrogen production yield of 4 mol of hydrogen per mole of glucose (~33 % efficiency) because of the Thauer limit [42]. Practical efficiency of dark fermentation would be lower than the Thauer limit [16, 21]. Microbial electrolysis cells can achieve much higher efficiencies of about 80 %, but high costs of apparatus and slow production rates (~5.4 mmol H_2/L/h) are the biggest challenges for large-scale hydrogen production from MECs [17, 28]. Overall, microbial fermentation is not an efficient way to produce hydrogen because of the microbial basal metabolism that competes with hydrogen production and eventually reducing the overall product yield.

3.1.3 In Vitro (Cell-Free) Enzymatic Pathways for Water Splitting

In vitro synthetic biosystems for water splitting can produce high-purity (i.e., zero CO production) hydrogen with high yields and rates [59]. In vitro synthetic biosystems are a new cell-free platform that assembles a number of (purified) enzymes and cofactors into different in vitro synthetic (enzymatic) pathways for implantation of various desired biochemical reactions [2, 10, 59, 63]. The optimal reaction condition for numerous enzymes can be found by examining different buffers, such as HEPES, Tris and PBS, a broad range of pH, and cofactor concentrations, and also multi-metal ions, such as Mg^{2+} and Mn^{2+}, to meet trade-off needs of different enzymes. If all enzymes are thermostable, high reaction temperature could be chosen as an optimal condition. These synthetic pathways can utilize different carbohydrates and ambient thermal energy as energy inputs to overcome thermodynamically unfavorable water-splitting reactions to produce hydrogen at mild conditions (below 100 °C). Near theoretical yields of 12 mol of hydrogen per mole of glucose unit consumed have been achieved [50, 60]. More important feature of these synthetic pathways is that they are able to produce hydrogen by absorbing low-temperature waste heat [63]. As the result, endothermic water-splitting reactions can generate more output of chemical energy in the form of hydrogen than input of chemical energy from carbohydrates and water (i.e., 122 % energy efficiency in terms of higher heating values). It is possible to achieve the energy efficiency over 100 % because the water-splitting reaction is a very unique entropy-driven chemical reaction [60]. The in vitro synthetic biosystem for water splitting is more advantageous for hydrogen production than microbial fermentation and photocatalytic water-splitting systems, because of the absence of cellular

membranes and microbial complexity that lower mass transfer and increase biocatalyst density [37]. The highest hydrogen production rate achieved by an in vitro synthetic pathway is about 150 mmol/L/h [38], while microbial fermentation has been reported to produce hydrogen with the rate of 1.96 mol/L/h [52].

3.2 Design of In Vitro Synthetic Enzymatic Pathways

In vitro synthetic pathways for water splitting powered by carbohydrates are reconstituted nonnatural catabolic pathways consisting of more than ten enzymes in four modules: (1) generation of phosphorylated sugars from poly- or monosaccharides without ATP, (2) NADPH generation via the oxidative pentose phosphate pathway (PPP), (3) hydrogen generation, and (4) G6P regeneration via the non-oxidative PPP and gluconeogenesis (Fig. 3.1 and Table 3.1). From the first module, poly- or monosaccharides are converted to their phosphorylated sugar units by phosphorylases or kinases without the use of ATP. For example, the phosphorylation of starch to glucose 1-phosphate units is catalyzed by starch phosphorylase, and different sugar substrates are catalyzed by different enzymes, cellodextrins and cellobiose by cellodextrin and cellobiose phosphorylases, sucrose by sucrose phosphorylase, glucose by polyphosphate glucokinase, and xylulose by polyphosphate xylulokinase. All poly- and oligosaccharides are phosphorylated to be glucose 1-phosphate, which is converted to glucose 6-phosphate by phosphoglucomutase, and then enter the second module reactions to generate NADPH. Xylose takes a different pathway to that catalyzed by xylose isomerase and polyphosphate xylulokinase to produce xylulose 5-phosphate. During the NADPH generation, glucose 6-phosphate (six-carbon sugar) enters the oxidative pentose phosphate pathway and becomes ribulose 5-phosphate (five-carbon sugar) by generating 2 mol of NADPH, releasing 1 mol of CO_2, and absorbing 1 mol of water molecule. When 1 mol of six-carbon sugar is completely consumed, 6 mol of CO_2 are released with the generation of 12 mol of NADPH. Each mole of NADPH is equivalent to 1 mol of hydrogen production catalyzed by NADPH-dependent hydrogenase. Thus, in the hydrogen generation module, 12 mol of hydrogen can be produced when 1 mol of six-carbon sugar is consumed for water splitting. In the non-oxidative pentose phosphate pathway, ribulose 5-phosphates are converted to fructose 6-phosphates and glyceraldehyde 3-phosphates via a carbon skeleton rearrangement. During the G6P regeneration module, a pair of glyceraldehyde 3-phosphates and dihydroxyacetone phosphate is combined to form a fructose 6-phosphate by multiple enzymes via the gluconeogenesis pathway. At this step, one extra mole of water molecule is absorbed by fructose 1,6-bisphosphatase. Finally, all fructose 6-phosphates are catalyzed to regenerate equal moles of glucose 6-phosphate which enter back to the NADPH generation module by completing the cofactor-balanced synthetic pathway for water splitting. The overall

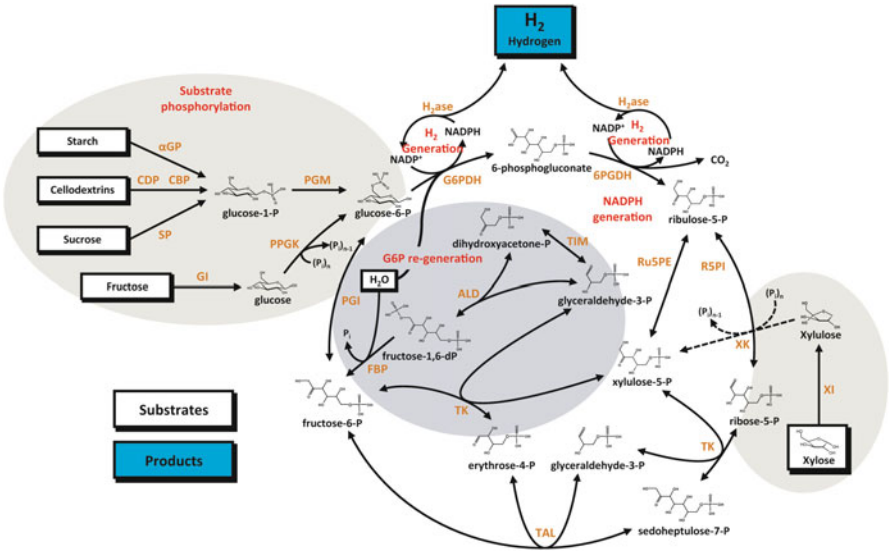


Fig. 3.1 ATP-free cofactor-balanced pathway for hydrogen generation from various types of carbohydrates with water

Table 3.1 Net reactions of each module and the overall reaction of in vitro synthetic biosystem for water splitting

Modules	Net reactions
Module #1	$C_6H_{10}O_5 + H_3PO_4\ (= P_i) \rightarrow$ glucose-6-P
Module #2 (oxidative phase) × 6	6 × (glucose-6-P + 2 $NADP^+$ + $\mathbf{H_2O}$ → ribulose-5-P + 2 NADPH + 2 H^+ + CO_2)
Module #2 (non-oxidative phase) × 2	2 × (3 ribulose-5-P → 2 fructose-6-P + glyceraldehyde-3-P)
Module #3 × 12	12 × (NADPH + H^+ → $NADP^+$ + H_2)
Module #4	2 glyceraldehyde-3-P + $\mathbf{H_2O}$ → fructose-6-P + P_i
Module #4 × 5	5 × (fructose-6-P → glucose-6-P)
Final reaction equation:	$C_6H_{10}O_5 + \mathbf{7H_2O} \rightarrow 12H_2 + 6CO_2$

reaction of this in vitro synthetic pathway for water splitting can be summarized by the equation (Eq. 3.1) as shown in Table 3.1. All of the enzymes used for this hydrogen-producing pathway are listed in Table 3.2.

$$CH_2O(aq) + H_2O(l) \rightarrow 2H_2(g) + CO_2(g) \tag{3.1}$$

Table 3.2 Enzymes used for in vitro synthetic biosystem for water splitting

Modules	E.C. #	Enzyme names (abbreviations)	Reactions
Substrate phosphorylation	2.4.1.1	Glycogen phosphorylase (αGP)	$Glycogen_{(n)} + P_i \rightarrow glucose\text{-}1\text{-}P + glycogen_{(n-1)}$
	2.4.1.49	Cellodextrin phosphorylase (CDP)	$Cellodextrin_{(n)} + P_i \rightarrow glucose\text{-}1\text{-}P + cellodextrin_{(n-1)}$
	2.4.1.20	Cellobiose phosphorylase (CBP)	$Cellobiose + P_i \rightarrow glucose\text{-}1\text{-}P + glucose$
	2.4.1.7	Sucrose phosphorylase (SP)	$Sucrose + P_i \rightarrow glucose\text{-}1\text{-}P + fructose$
	5.3.1.5	Glucose isomerase (GI)	$Fructose \rightarrow glucose$
	2.7.1.63	Polyphosphate glucokinase (PPGK)	$Glucose + (P_i)_n \rightarrow glucose\text{-}1\text{-}P + (P_i)_{n-1}$
	5.4.2.2	Phosphoglucomutase (PGM)	$Glucose\text{-}1\text{-}P \rightarrow glucose\text{-}6\text{-}P$
	5.3.1.5	Xylose isomerase (XI)	$Xylose \rightarrow xylulose$
	2.7.1.17	Polyphosphate xylulokinase (PPXK)	$Xylulose + (P_i)_n \rightarrow xylulose\text{-}5\text{-}P + (P_i)_{n-1}$
NADPH generation	1.1.1.49	Glucose-6-phosphate dehydrogenase (G6PDH)	$Glucose\text{-}6\text{-}P + NADP^+ + H_2O \rightarrow 6\text{-}phosphogluconate + NAHPH + H^+$
	1.1.1.44	6-phosphogluconic dehydrogenase (6PGDH)	$6\text{-}phosphogluconate + NADP^+ \rightarrow ribulose\text{-}5\text{-}P + NADPH + CO_2$
	5.3.1.6	Ribose 5-phosphate isomerase (RPI)	$Ribulose\text{-}5\text{-}P \rightarrow ribose\text{-}5\text{-}P$
	5.1.3.1	Ribulose-5-phosphate 3-epimerase (RPE)	$Ribulose\text{-}5\text{-}P \rightarrow xylulose\text{-}5\text{-}P$
	2.2.1.1	Transketolase (TK)	$Xylulose\text{-}5\text{-}P + ribose\text{-}5\text{-}P \rightarrow sedoheptulose\text{-}7\text{-}P + glyceraldehyde\text{-}3\text{-}P$
	2.2.1.1	Transketolase (TK)	$Xylulose\text{-}5\text{-}P + erythrose\text{-}4\text{-}P \rightarrow fructose\text{-}6\text{-}P + glyceraldehyde\text{-}3\text{-}P$
	2.2.1.2	Transaldolase (TAL)	$Sedoheptulose\text{-}7\text{-}P + glyceraldehyde\text{-}3\text{-}P \rightarrow fructose\text{-}6\text{-}P + erythrose\text{-}4\text{-}P$
Hydrogenation	1.12.1.3	Hydrogenase (H_2ase)	$NADPH + H^+ \rightarrow NADP^+ + H_2$
G6P regeneration	5.3.1.1	Triose-phosphate isomerase (TIM)	$Glyceraldehyde\text{-}3\text{-}P \rightarrow dihydroxyacetone\ phosphate$
	4.1.2.13	Aldolase (ALD)	$Glyceraldehyde\text{-}3\text{-}P + dihydroxyacetone\ phosphate \rightarrow fructose\text{-}1,6\text{-}bisphosphate$
	3.1.3.11	Fructose-1,6-bisphosphatase (FBP)	$Fructose\text{-}1,6\text{-}bisphosphate + H_2O \rightarrow fructose\text{-}6\text{-}P + P_i$
	5.3.1.9	Phosphoglucose isomerase	$Fructose\text{-}6\text{-}P \rightarrow glucose\text{-}6\text{-}P$

3.3 Examples of Hydrogen Production from Carbohydrates

3.3.1 Hydrogen Production from Starch and Cellodextrins

In vitro enzymatic pathways to produce hydrogen from carbohydrates were first demonstrated by Woodward and colleagues using glucose, xylose, or sucrose with only a couple of enzymes [47, 48]. These simple enzymatic pathways could achieve less than 10 % of the theoretical yields of sugars, due to only one NADPH generation per hexose or pentose. To complete the oxidation, the oxidative pentose phosphate cycle was coupled with hydrogenase to produce hydrogen from glucose 6-phosphate, resulting in about 96 % of the theoretical yield [49]. High cost of glucose 6-phosphate prevents its practical application. These in vitro synthetic pathways were further improved by Zhang and collaborators to demonstrate hydrogen production from different types of carbohydrates: starch [60], cellulosic materials [50], xylose [31], and sucrose [32]. The first hydrogen production from starch by an in vitro synthetic enzymatic pathway proved its feasibility to produce low-cost hydrogen from inexpensive starch without ATP by achieving a high production yield surpassing the theoretical production yield of dark fermentation [60]. Most enzymes used for this proof-of-principle experiment producing hydrogen from starch were off-the-shelf enzymes, and the enzymatic pathway exhibited a production rate of 0.4 mmol/L/h with 43 % yield (5.2 mol H_2/mol glucose consumed) (Table 3.3). A couple of years later, the hydrogen production rates and yields were enhanced to 0.5 mmol/L/h with 93 % yield and 3.9 mmol/L/h with 68 % yield when cellobiose or cellodextrins were used as a substrate, respectively, and through minor optimizations including increased substrate concentration, reaction temperature, and more rate-limiting enzyme loadings [50] (Table 3.3). The hydrogen production rate using cellodextrins was greatly improved by increasing hydrogenase loading and substrate concentration to 8 mM. However, low-yield hydrogen production was observed due to the incomplete reaction. Cellobiose and cellodextrins were prepared as hydrolytic products of cellulose through incomplete enzymatic or mixed acid hydrolysis, respectively.

Table 3.3 Comparison of hydrogen production rates and yields from different carbohydrates

Substrate	Conc. (mM)	Reaction temperature (°C)	H_2 production rates (mmol H_2/L/h)	Yield (%)	Ref.
Starch	1	30	0.4	43	[60]
Cellobiose	2	32	0.5	93	[50]
Cellodextrins (cellopentaose)	8	32	3.9	68	[50]
Xylose	2	50	2.2	96	[31]
Sucrose	2	37	3.0	97	[32]
G6P	100	60	150	–	[38]
Biomass sugar	3.19	40	2.3	100	[36]
G6P	100	50	54	–	[36]

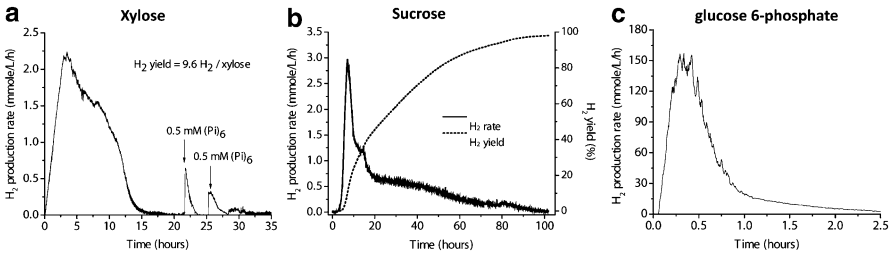


Fig. 3.2 Hydrogen production profiles from xylose (**a**), sucrose (**b**), and glucose 6-phosphate (**c**). (Reproduced with permission from [31, 32, 36])

3.3.2 Hydrogen Production from Xylose

Hemicellulose is another major component of lignocellulosic biomass besides cellulose. Xylose is the most abundant pentose and the major component of hemicellulose. Xylose composes about 20–30 % of lignocellulosic biomass by weight [31]. Thus, it is essential to use not only cellulosic materials but also the major portion of hemicellulosic materials to produce low-cost hydrogen from renewable carbohydrates. One mole of xylose can theoretically produce 10 mol of hydrogen by splitting 5 mol of water molecule when completely oxidized to carbon dioxide (Eq. 3.2). Since xylose is a five-carbon sugar, the in vitro synthetic pathway was modified by replacing hexose-phosphorylating enzymes with xylose isomerase and polyphosphate xylulokinase (Fig. 3.1). The hydrogen production from xylose exhibited the production rate of 2.2 mmol/L/h with 96 % yield (Table 3.3). Such high-yield hydrogen production was achieved with the addition of extra polyphosphate driving the reaction to completion (Fig. 3.2a), and the relatively high production rate was achieved with increased reaction temperature to 50 °C:

$$C_5H_{10}O_5(aq) + 5H_2O(l) \rightarrow 10H_2(g) + 5CO_2(g) \tag{3.2}$$

3.3.3 Hydrogen Production from Sucrose

Sucrose, also known as table sugar, is one of the cheapest carbohydrates because of its simple production process and abundance of cultivated sugar crops: sugarcane and sugar beets. Sucrose is a disaccharide composed of glucose and fructose. Therefore, two additional enzymes are required to convert fructose to equal moles of glucose 6-phosphate. An in vitro synthetic enzymatic pathway consisting of total 15 enzymes was designed to catalyze water-splitting process producing hydrogen powered by sucrose [32]. The hydrogen production from sucrose achieved the production rate of 3.0 mmol/L/h with 97 % yield (Fig. 3.2b). When

increased sucrose concentration, the maximum hydrogen production rate was as high as 9.7 mmol/L/h [32].

3.3.4 Hydrogen Production from Biomass Sugars

The complete conversion of glucose and xylose from plant biomass to hydrogen and carbon dioxide has been achieved via an in vitro synthetic enzymatic pathway. Pretreated biomass was hydrolyzed to glucose and xylose by using a commercial cellulase. Glucose and xylose were simultaneously converted to hydrogen with the theoretical yield of 2 mol of hydrogen per each carbon molecule [36]. A genetic algorithm was used to find the best fitting parameters of a nonlinear kinetic model with experimental data. Global sensitivity analysis was used to identify the key enzymes that have the greatest impact on reaction rate and yield. After optimization of enzyme loadings using computational modeling and data analysis methods, the hydrogen production rate could be increased to 32 mmol/L/h. The production rate was further enhanced to 54 mmol/L/h by increasing reaction temperature, substrate, and enzyme concentrations. The production of hydrogen from locally produced biomass is a promising means to achieve global green hydrogen production.

3.3.5 High-Rate Hydrogen Production from Glucose 6-Phosphate

High-yield hydrogen production from these different carbohydrates has opened up a new way to produce low-cost hydrogen from renewable biomass. In the past years, the hydrogen production rate has been increased to 150 mmol/L/h by 750-fold when glucose 6-phosaphte is used as a substrate by an in vitro synthetic enzymatic pathway (Fig. 3.2c) [38, 49]. All enzymes used in the pathway have been replaced with recombinant thermostable enzymes produced in *E. coli*. Some of these enzymes are immobilized to enhance their stability. Hydrogen production rates and yields from different carbohydrates are summarized in Table 3.3.

3.4 Technical Obstacles to Low-Cost H_2 Production

To achieve low-cost hydrogen production from carbohydrates, a few obstacles have to be overcome. The ultimate hydrogen production costs are strongly related to the following factors: costs of substrate, enzyme cost, cofactor cost, and product-related downstream processing cost especially for product separation and purification [61]. It has been shown that the in vitro synthetic biosystem can produce

hydrogen from various types of carbohydrates in near theoretical yields. Therefore, the use of inexpensive, abundant carbohydrate sources, such as cellulosic and hemicellulosic materials, to produce hydrogen can solve one of the major obstacles. Gaseous products can be easily separated and purified from the aqueous phase enzymatic reaction, leaving the other two obstacles unsolved. Hydrogen production rate is also an important factor, because it determines potential implementation of low-cost hydrogen production techniques from carbohydrates mainly related to capital investment.

3.4.1 Enzyme Cost and Stability

Enzyme costs are highly related to their production costs, turnover number (TTN), and stability. The current production costs for enzymes produced as recombinant proteins in *E. coli* at lab scales are high, for example, $~1,000,000 per kg of dry protein, but it is expected that their production costs will decrease to the level of industrial bulk enzyme production costs of around $5–100 per kg of dry protein [57]. Increasing TTNs can decrease enzyme costs exponentially through increasing enzyme efficiency [55]. It is estimated that TTNs over 10^8–10^9 are required to reduce enzyme costs low enough for industrial-scale use [11, 12, 51]. High TTNs can be achieved by using thermostable enzymes from thermophilic microbes, or through enzyme immobilization [33, 44, 54].

The use of thermostable enzymes can decrease their production costs by decreasing enzyme purification costs. Thermostable enzymes cloned from thermophilic microbes, such as *Thermotoga maritima*, can be stable at 60–70 °C, which is higher than the temperature where most of the other enzymes cloned from mesophilic hosts are stable. Therefore, purification procedures retaining only soluble thermostable enzymes after treating at high temperature for relatively short times (10–30 min) make it a simple and cost-effective way to purify target enzymes from cell lysates. Simple enzyme purification will eventually lower the overall production costs.

Enzyme immobilization is a technique in a relatively mature stage, and various immobilization techniques have been introduced and used to improve TTNs, enzyme stability, and catalytic efficiency [24, 33, 41]. Among many techniques, the cellulose-binding module (CBM) tagged protein immobilization combines enzyme purification and immobilization into one step [18]. This simple one-step enzyme purification and immobilization technique showed about an 80-fold enhanced half-life time of phosphoglucose isomerase (PGI) when the enzyme with CBM tag was immobilized on regenerated amorphous cellulose (RAC) (Fig. 3.3) [33]. Enzyme immobilization also enables enzymes to be recyclable. Enzymes immobilized on magnetic nanoparticles (MNPs) have shown to improve enzymatic reactions rates and also be able to recycle simply by using a magnetic force [34]. Green fluorescent protein (CBM-TGC) was used to demonstrate simply selective recycling of CBM-tagged GFP immobilized on Avicel-containing MNPs

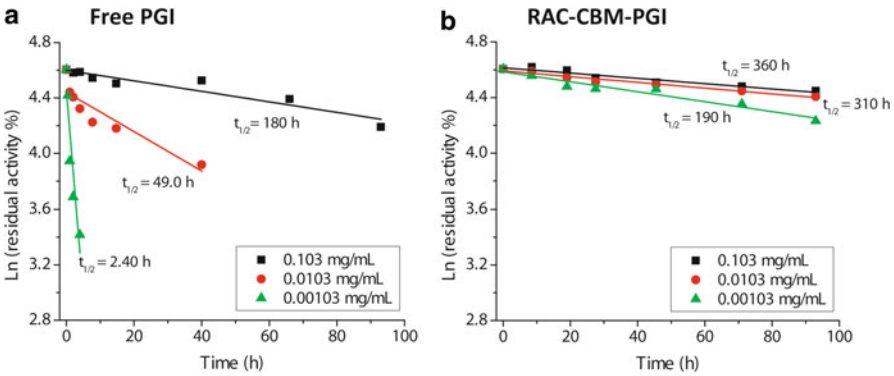


Fig. 3.3 Comparison of thermal stability between free PGI (**a**) and immobilized PGI (**b**) in different concentrations (Reproduced with permission from Myung et al. [33])

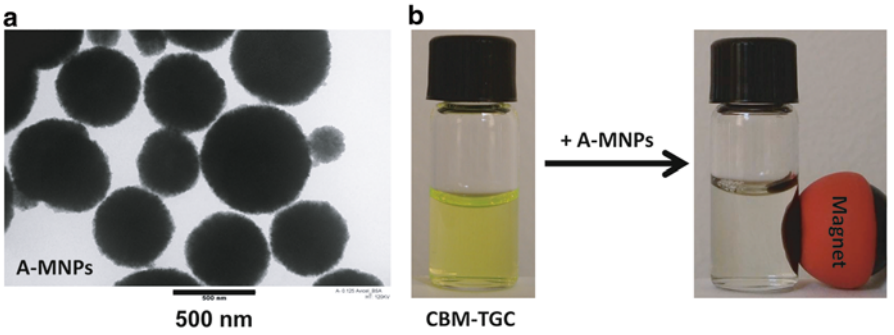


Fig. 3.4 Selective recycling of enzymes immobilized on Avicel-containing magnetic nanoparticles (A-MNPs) (**a**). The simple process of collecting CBM-tagged green fluorescent proteins (CBM-TGC) adsorbed on A-MNPs by a magnetic force (**b**) (Reproduced with permission from Myung et al. [34])

(A-MNPs) (Fig. 3.4). As the result of selective recycling, enzyme-related costs can be greatly reduced.

Protein protection additives, such as ligands and salts, have been studied as a simple approach to enhance enzyme storage and reaction stability. DMSO and glycerol are popular additives as cryoprotectants, stabilizing proteins during multi-freezing-and-thawing cycles. Various polyethylene glycols have been used as thermoprotectants. Thermostability of trypsin was increased from 49 to 93 °C without deteriorating its catalytic properties in the presence of glycol chitosan [13]. Amines, polyethylene glycol, and glycerol as additives improved catalase storage stability as well as its enzymatic performance in high temperature and alkaline pH [6]. These additives may, however, be potential inhibitors to the reaction system [19]. Therefore, additional dialysis or ultrafiltration may be required before the reactions.

3.4.2 Enzymatic Reaction Rates

Reaction rate is an important factor for determining potential applications of the enzymatic reaction and capital investment. Currently, the fastest hydrogen production rate achieved by an in vitro synthetic enzymatic pathway is about 150 mmol/L/h (=0.3 g H_2/L/h) [38]. This current rate is the fastest enzymatic hydrogen production from sugars and fast enough for distributed hydrogen generators, but it is slower than the need of on-demand hydrogen production for hydrogen fuel cell vehicles. It is anticipated that the production rate can be improved to 20 g H_2/L/h because microbial fermentation, which is usually slower than cell-free enzymatic reactions, has already achieved a production rate of over 20 g H_2/L/h [5, 54, 62]. Approaches made to increase enzymatic hydrogen production rate include elevated reaction temperature, optimized enzyme ratio, high substrate concentration, high enzyme loading, and substrate channeling among cascade enzymes. The substrate channeling was done by co-immobilizing multiple cascade enzymes, so that the local enzyme concentration is increased and susceptible intermediates have less time exposed to reaction solutions [20]. The cluster of multi-enzymes held by co-immobilizing scaffoldins results in increased reaction rates. Three important cascade enzymes, TIM, ALD, and FBP, in the enzymatic hydrogen production were expressed with dockerin domain which can self-assemble with mini-scaffoldin for co-immobilization on A-MNPs (Fig. 3.5a) [34]. In comparison with non-immobilized enzymes, the co-immobilized enzyme complex results in about 4.6 times increased reaction rate (Fig. 3.5b).

3.4.3 Cofactor Cost and Stability

Cofactors are chemical compounds required for enzymatic reactions, such as ATP and NAD(P)H. The issues from cofactor costs and stability have been addressed by various approaches including cofactor recycling systems [25, 46] and use of low-cost stable biomimetic cofactors [1, 29]. Although the regeneration of natural cofactors through recycling systems is economically beneficial to most current enzymatic reactions, the most farsighted solution would be to replace native cofactors with low-cost stable biomimetic cofactors. The structures of natural cofactors and biomimetic cofactors are shown in Fig. 3.6 with their estimated prices (USD, 2015). By sharing the nicotinamide moiety as a universal binding site for electron carriers, these natural and biomimetic cofactors vary in the chemical structures bound to the nitrogen atom in pyridine (Fig. 3.6). The alternative natural cofactors and biomimetic cofactors with simpler structures than NADP or NAD are estimated to have lower costs. However, most wild-type redox enzymes have no activities with such biomimetic cofactors. A number of studies have been done to change the cofactor specificity or preference through cofactor engineering. Cofactor engineering has three major types of approaches: rational design, directed

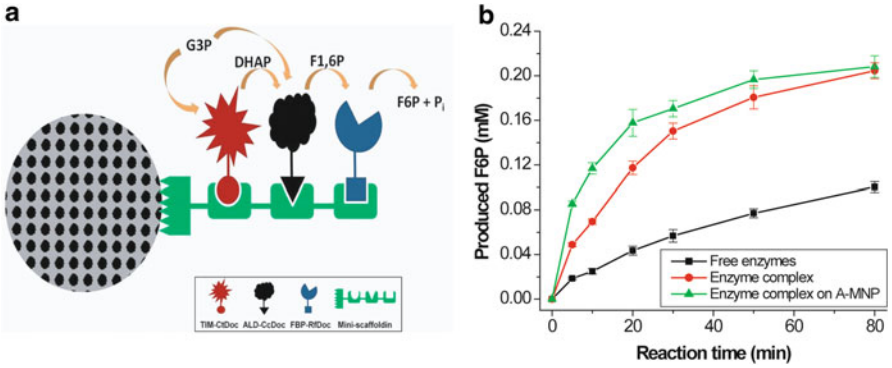


Fig. 3.5 The cascade enzymatic reaction among co-immobilized enzymes on MNPs for substrate channeling (**a**). Comparison of the reaction rates among free enzymes, non-immobilized enzyme complex, and the enzyme complex immobilized on A-MNP (**b**) (Reproduced with permission from Myung et al. [34])

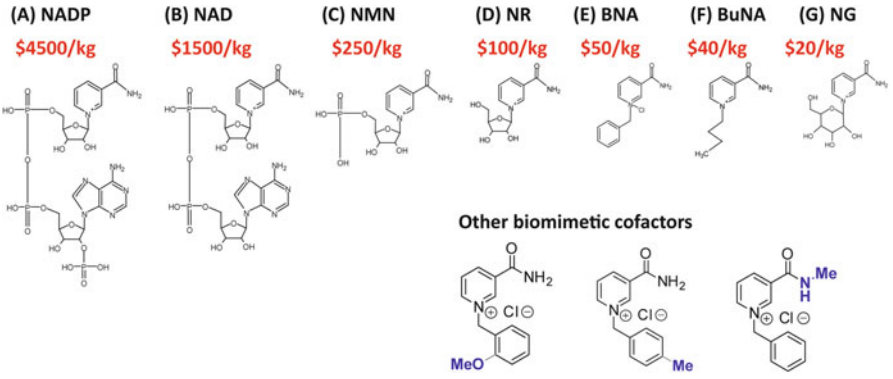


Fig. 3.6 Structures and estimated prices of natural cofactors (**A**, **B**), alternative natural cofactors (**C**, **D**), and biomimetic cofactors (**E**–**G**)

evolution, or swapping modules. Using these approaches, a number of studies have shown their redox enzymes with changed cofactor preferences from NADP to NAD [3, 8, 39], or from NAD to NADP [15, 45]. In 2012, Scott et al. discovered that their engineered alcohol dehydrogenase for broadened cofactor specificity and improved activity with NAD can utilize a minimal natural cofactor, NMN [4]. Fish et al. proposed the use of 1-benzyl-3-carbamoyl-pyridinium (BNA) as a biomimetic cofactor to replace NAD(P)H and discovered that two wild-type enzymes, horse liver alcohol dehydrogenase and monooxygenase, can actually utilize this cofactor [26, 27]. Clark collaborated with Fish and demonstrated that engineered P450 with two amino acid mutations can utilize BNA as a cofactor [40]. Recently, a large international collaborative group synthetized another biomimetic cofactor, 1-butyl-3-carbamoyl-pyridinium (BuNA), and demonstrated that wild-type enoate reductase can utilize it as a cofactor [35]. In most cases, such changes will decrease

apparent activities greatly. The best example may be the engineered P450. The mutant P450 (W1064S/R966D) exhibited its activity on biomimetics up to seven times of that of wild-type enzyme on NADH [40]. Cofactor engineering is in its early stage, but its success will greatly influence enzymatic synthesis of organic chemicals and in vitro synthetic biosystems for biocommodity production.

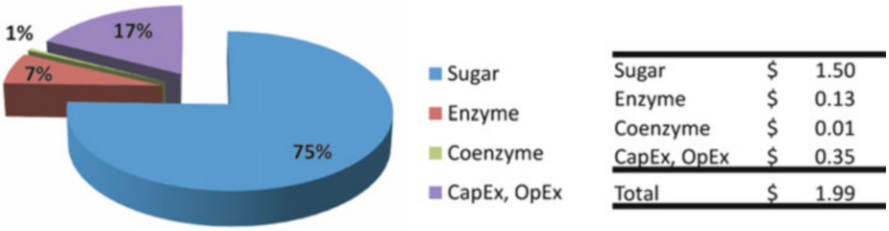
3.5 Conceptual Obstacles to Enzymatic H_2 Production

In vitro synthetic biosystems consist of numerous enzymes as building bricks, enzyme complexes as building modules, and/or (biomimetic) coenzymes. These many components are assembled into in vitro synthetic pathways for implementing complicated bioreactions. They emerge as an alternative solution for accomplishing desired biotransformation without concerns of cell proliferation, complicated cellular regulation, and side-product formation. In addition to the capability of achieving high product yields as the most important advantage, in vitro synthetic biosystems feature several other biomanufacturing advantages, such as fast reaction rates, easy product separation, open process control, broad reaction conditions, and tolerance to toxic substrates or products.

The largest obstacle to the enzymatic hydrogen production is conceptual change. Microbe-based fermentation has been used by human beings for more than 10,000 years. As a result, most biotechnologists believe that living microbes are the best biocatalysts because they can duplicate themselves. Indeed, the primary goal of living microbes is their proliferation while bioconversions are side effects. Consequently, the success of several examples is needed to convince biotechnologists of accepting a new paradigm of in vitro synthetic biosystems.

3.6 Conclusions and Future Outlook

Hydrogen is an important commodity chemical with a global market size of approximately 100 billion US dollars. High-yield hydrogen has been produced from carbohydrates and water catalyzed by in vitro synthetic enzymatic pathways. The overall hydrogen production costs are mainly proportional to carbohydrate cost [59]. Assuming a substrate cost contributes a major portion of hydrogen product cost, it is expected that hydrogen can be produced at costs less than $2.00/kg hydrogen when the technology is well developed [59] (Fig. 3.7). Carbohydrates are the most abundant renewable natural resources on earth, and they are estimated to cost about $1.50/kg accounting for 75 % of the prospective hydrogen production cost [59] (Fig. 3.7). The next biggest portion of the hydrogen production cost is from the initial capital investment and operating expense accounting for about 17 %. The sum of capital and operating expenses would be about $0.35/kg hydrogen according to the similar expenses based on anaerobic digestion. Enzyme and



Sugar	$	1.50
Enzyme	$	0.13
Coenzyme	$	0.01
CapEx, OpEx	$	0.35
Total	$	1.99

Fig. 3.7 Prospective H_2 production cost and its contribution factors: substrate costs, enzyme costs, cofactor costs, and initial capital expense with operating expense

cofactor costs account for 7 % and 1 % of the enzymatic hydrogen production cost, respectively, when their TTN values are more than 10^9 and 10^7, respectively.

High-yield hydrogen production from carbohydrates will open up several potential applications for the hydrogen economy, where hydrogen is an alternative transportation fuel or a short-term electricity storage carrier. The potential applications include from small-sized distributed hydrogen refueling stations (e.g., 1–2 kg per day for a single house, or 50–200 kg for village) to the most ambitious application, such as a sugar fuel cell vehicles [53, 56].

The potential global market size for hydrogen as a future energy carrier replacing gasoline and diesel could be trillions of dollars. Such great potential, along with a bright future featuring enhanced energy conversion efficiency, nearly zero pollutants, and zero greenhouse gas emissions, will motivate the world to solve the remaining obstacles within next decades.

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