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Biofuel production by *in vitro* synthetic enzymatic pathway biotransformation

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Cell-free synthetic pathway biotransformation (SyPaB) is the implementation of complicated biochemical reactions by *in vitro* assembling a number of enzymes or their complexes and coenzymes. Assembly of numerous enzymes without cellular membrane, gene regulation, or undesired pathway can circumvent some of the obstacles to modifying living microorganisms. Several synthetic pathways for the production of liquid biofuels — alcohols and hydrocarbon precursors (polyols) as well as gaseous biofuel — hydrogen have been presented. The present constraints to SyPaB include the lack of stable enzymes as Lego-like building blocks, the different optimal reaction conditions for individual enzyme, and the use of costly labile coenzymes. It is expected that high-yield SyPaB will be an important platform for producing low-cost biofuels and biochemicals.

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Introduction

Sustainable development of humankind needs the production of renewable energy at affordable costs. Current transportation accounts for approximately 20% of global energy consumption. Owing to some special requirements for the transportation sector, such as high power density and high-energy storage capacity in a small volume, potential solutions for the transportation sector are limited. Biomass, the most abundant renewable bioresource, is the only low-cost renewable resource that can be used for the production of large amounts of transportation fuels and renewable materials (e.g., paper and polylactic acid). Natural terrestrial plants collect nonpoint intermittent low-energy-flux solar energy and store it in the form of low-cost chemical energy biomass that can be stored, distributed, and utilized easily and economically.

Starting from biomass sugars, numerous biocatalytic or catalytic approaches have been proposed and investigated for the production of various biofuels, such as ethanol [1], high-chain alcohols [2], alkanes [3], fatty acids or their esters [4,5], hydrogen [6••,7••], methane [8], and hydrocarbons [9]. Liquid biofuels work well with current infrastructure and internal combustion engines, representing a short-term or middle-term solution to the transportation sector. In future, hydrogen fuel cell-motor system represents a long-term solution for the transportation sector because hydrogen fuel cells have higher energy efficiencies and produce less pollution than internal combustion engines. Hydrogen can also be produced from diverse primary energy sources [10•].

Cell-free synthetic pathway biotransformation (SyPaB) is the *in vitro* assembly of a number of (purified) enzymes and coenzymes for mediating complicated biochemical reactions [11••,12•].

Cell-free SyPaB, as one direction of synthetic biology, has a number of potential advantages over fermentations mediated by intact microbes. These advantages include high product yield, fast reaction rate, high product titer, broad range of reaction conditions, and simplified process control [11••]. The development cycle of SyPaB involves five parts: first, pathway reconstruction, second, enzyme selection, third, enzyme engineering, fourth, enzyme production, and fifth, process engineering [11••]. Furthermore, it is vital to construct *in vitro* enzyme complexes for minimizing degradation of labile metabolites and facilitating metabolite channeling for some cascade enzymatic reactions [13].

Living entities, such as microbial cells, can be engineered to produce desired products by *in vivo* synthetic biology technology. Achievement of high product yield through this means, however, requires labor-intensive and time-consuming optimization of complex cellular networks [5,14–16]. By contrast, high product yield can be achieved more efficiently with *in vitro* systems when system components are available [6••,7••].

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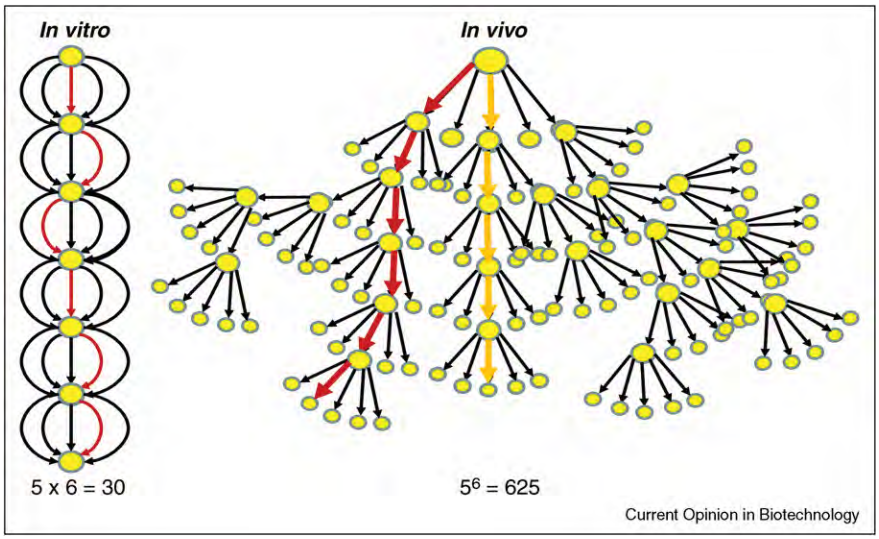
Figure 1



Comparison of complexity of *in vitro* and *in vivo* synthetic biological system containing six enzymatic steps and five choices for each layer. The red lines represent the optimized pathways that can produce the highest yield for desired products.

Figure 1 compares biocatalysis complexity of *in vitro* and *in vivo* platforms. Taking a relatively simple pathway involving six cascade biochemical reactions where each step has five choices (genes or enzymes), *in vitro* systems would have 30 combinations since each enzymatic step can be easily exchanged by another enzyme. *In vivo* systems may have $5^6 = 625$ combinations because each enzymatic step is linked with others. Furthermore, *in vivo* systems are complicated by the possibility that reaction rate at each step in the enzymatic pathway involves regulation at the level of gene transcription, mRNA stability, and translation. They may also be regulated by processes involved in delivery of the gene product to the site of function in the cell as well as protein–protein and protein–cofactor interactions.

In this article, we present the advances in SyPaB for biofuel production, discuss SyPaB advantages and limitations and offer suggestions for R&D priorities in the future.

Production of biofuels

Synthetic pathway assembly is usually designed based on natural metabolic pathways with necessary modifications with ATP and coenzyme balanced. Since biofuels are low-value products, it is economically prohibitive to produce them using expensive feedstock.

Alcohols

A high octane liquid fuel blend of ethanol can be produced through microbial anaerobic fermentation. The Nobel Prize winner Eduard Buchner discovered ethanol fermentation by a cell-free yeast extract in 1897 [17]. Net ATP accumulation (i.e., 2 ATP produced per glucose), however, prevents complete conversion of glucose to ethanol (Eqn (1)) [18]:

$$C_6H_{12}O_6(aq) + 2ADP + 2P_i \rightarrow 2C_2H_6O(aq) + 2CO_2(gas) + 2ATP \quad (1)$$

Welch and Scopes [18] demonstrated the feasibility of high-yield ethanol production by a reconstituted yeast glycolytic enzyme system containing ATPase, which facilitates complete conversion of 180 g/L glucose to 90 g/L ethanol within eight hours with 99% of the theoretical yield. Alternatively, substitution of arsenate for inorganic phosphate can constrain ATP synthesis and prevent ATP accumulation in this reaction [19]. The enzymes that require phosphate can use arsenate because arsenate is structurally and chemically similar to phosphate. Since organic compounds of arsenate are less stable than the analogue phosphate compounds, they decompose rapidly by spontaneous hydrolysis, resulting in dissipation of high-energy phosphate bonds [19].

Another fuel that can be produced biologically is butanol. Butanol has a higher energy density and more hydrophobicity than ethanol, but it cannot be produced at as high a yield and titer as ethanol [20]. Cell-free SyPaB butanol production may have high product yield, produce high product titer, simplify process control, and have no risk of bacteriophage contamination [11••]. Because two moles of ATP are produced and accumulate per mole of glucose in the natural butanol pathway (Eqn (2)), substitution of

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arsenate for inorganic phosphate in the enzyme mixture or addition of ATPase to degrade the ATP can prevent ATP accumulation in this reaction [19]:

$$C_6H_{12}O_6(aq) + 2ADP + 2P_i \rightarrow C_4H_{10}O(aq) + 2CO_2(gas) + H_2O + 2ATP \quad (2)$$

Hydrogen

Carbon-neutral hydrogen gas is the preferred energy carrier for the future transportation sector. Hydrogen can be produced from biomass sugars through numerous methods employing chemical catalysis, biocatalysis, or a combination of both. However, most of these technologies suffer from low hydrogen yield (far below the theoretical yield = 12 H_2 per glucose), undesired products, and/or severe reaction conditions [10•].

These cell-free synthetic enzymatic pathways generate nearly 12 moles of hydrogen per mole of glucose unit of starch or cellulosic materials [6••,7••]:

$$C_6H_{10}O_5(aq) + 7H_2O(l) \rightarrow 12H_2(g) + 6CO_2(g) \quad (3)$$

These synthetic pathways contain: first, a chain-shortening phosphorylation reaction for producing glucose-1-phosphate (G-1-P) catalyzed by glucan phosphorylase; second, conversion of G-1-P to glucose-6-phosphate (G-6-P) catalyzed by phosphoglucomutase; third, a pentose phosphate pathway containing 10 enzymes for producing 12 NADPH per G-6-P, and fourth hydrogen generation from NADPH catalyzed by hydrogenase. For the first time, these endothermic entropy-driven biochemical reactions achieve a chemical energy output (hydrogen)/input (carbohydrate) = 1.22 by absorbing ambient-temperature waste heat [6••,7••]. Since glucan phosphorylases are responsible for generating G-1-P in these pathways, the yield of G-1-P is $(n-1)/n$, where n is the degree of polymerization of oligosaccharides or polysaccharides. Therefore, it is vital to identify the enzymes that can hydrolyze long chain insoluble cellulose for the generation of soluble G-6-P without use of costly ATP.

Polyols — alkane precursors

Long chain alkanes made from biomass sugars are a sulfur-free liquid fuel that can be used for jet planes and heavy-duty vehicles [21]. Recently, we have developed a new approach to produce alkane precursors — polyols (e.g., xylitol and sorbitol) from biomass sugars by SyPaB (submitted). For the production of alkanes, aqueous-phase reforming (APR), chemical reforming occurring in aqueous phase under modest temperature and high pressure conditions, can convert water-soluble polyols to water-immiscible alkanes [3,22,23]. This combination of biocatalysis and chemical catalysis has several advantages: a very high-energy retraining efficiency from sugars to alkanes (95%, overall; 99.6%, SyPaB; 95%, APR), high yield and low cost for xylitol and sorbitol generation, tolerance to the dilute acid-pretreated biomass hydrolysate that inhibits microorganism growth and metabolisms, modest reaction conditions, and low product separation costs.

This new pathway contains 12 enzymes similar to the cellobiose ($C_{12}H_{22}O_{11}$)-to-hydrogen enzyme cocktail [7••] but without hydrogenase. The latter is replaced by an aldose reductase (EC 1.1.1.21) [24], whereby 1 mole of cellobiose can produce 12 NADPH and one glucose (Eqn (4)):

$$C_{12}H_{22}O_{11} + 12NADP^+ + 7H_2O \rightarrow C_6H_{12}O_6 + 12NADPH + 12H^+ + 6CO_2 \quad (4)$$

The overall reactions for xylitol (xylitol) and sorbitol (sorbitol) synthesis from xylose and glucose plus a small amount of cellobiose are

$$12C_5H_{10}O_5 + C_6H_{10}O_5 + 7H_2O \rightarrow 12C_5H_{12}O_5 + 6CO_2 \quad (5)$$

$$12C_6H_{12}O_6 + C_6H_{10}O_5 + 7H_2O \rightarrow 12C_6H_{14}O_6 + 6CO_2 \quad (6)$$

The conversion of xylitol and sorbitol to alkanes by APR is a weakly exothermic process [21,22,25]:

$$19C_6H_{14}O_6 \rightarrow 13C_6H_{14} + 36CO_2 + 42H_2O \quad (7)$$

The products of alkanes have only 30% of the mass of polyols while retaining approximately 95% of the combustion energy in the reactants [23,26]. In addition, separation of water-insoluble alkanes with water is far less costly than that of alcohols and water. Longer chain alkanes from C7 to C15 can be produced by combining the aqueous-phase dehydration/hydrogenation process with a C–C bond-forming aqueous-phase aldol condensation step [21].

Weight-based total turn-over number

A major difference between microbial fermentation and SyPaB is that microbes can duplicate themselves whereas enzymes cannot. Because SyPaB requires enzyme production by microbial fermentation, purification, and stabilization of enzymes as well as the addition of costly coenzymes, it is plausible that SyPaB is more costly than microbial fermentation. Therefore, we suggest that comparison of microbial fermentation and SyPaB must be based on weight-based total turn-over number (TTN_W) in terms of kg of product per kg of biocatalyst:

$$TTN_W = \frac{\text{product}}{\text{biocatalyst}} \quad (8)$$

As shown in Figure 2, self-duplicating living entity-based biotransformations have very low values of TTN_W

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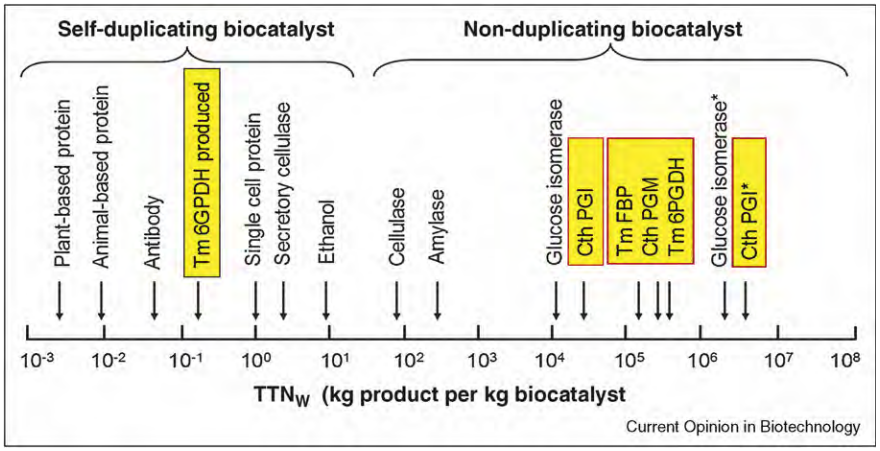
Figure 2



Comparison of biocatalysis mediated by self-duplicating living biocatalysts (e.g., microorganism) and non-duplicating biocatalysts (e.g., enzyme) in terms of weight-based total turnover number (TTN_W). The enzymes in the yellow box have been obtained in our laboratory during the past several years. Cth PGM, *C. thermocellum* phosphoglucomutase; Cth PGI, *C. thermocellum* phosphoglucose isomerase [29]; TM 6PGDH, *T. maritima* 6-phosphogluconate dehydrogenase [28]; TM FBPase. *T. maritima* fructose-1,6-bisphosphatase [13]. * denotes immobilized enzyme.

(kg product per kg biocatalyst). A typical ethanol batch fermentation has TTN_W values of 3–6. However, when yeast cells are recycled (i.e., less cell mass is generated and more ethanol is produced), this operation leads to higher TTN_W values (e.g., 10–20) [27]. Typical TTN_W values for intracellular recombinant protein production by microbial fermentations reactions range from 0.005 to 0.25 (i.e., ~1–50% of cellular protein is target recombinant protein and total cellular proteins account for ~50% of cellular weight). For example, a recombinant protein *Thermotoga maritime* 6-phosphogluconate dehydrogenase (Tm 6PGDH) accounts for approximately 30% of the *Escherichia coli* cellular protein [28], resulting in a TTN_W value of ~0.15 (Figure 2). Production of secretory proteins such as cellulase or protease has $TTN_W > 1$, that is, more cost-effective protein production. Biotransformations mediated by enzymes usually have far higher TTN_W values than by living entities (Figure 2). Three thermoenzymes from our laboratory exhibit very high TTN_W values: 415,000, *Clostridium thermocellum* phosphoglucomutase [29]; 420,000, Tm 6PGDH [28]; and ~200,000, *T. maritime* fructose-1,6-bisphosphatase [13]. Much higher TTN_W values of these thermostable enzymes are expected after immobilization, like those of immobilized thermophilic glucose isomerase [30]. Recently, we have obtained TTN_w values of more than 5,000,000 for immobilized *C. thermocellum* phosphoglucose isomerase (unpublished).

Carbohydrate allocation to biofuels and biocatalysts

Carbohydrate is used as both a carbon source to support microorganism growth for producing biofuel or producing enzymes and an energy source for biofuel production at the same time. We refer to the allocation of carbohydrate between biocatalysts (enzymes or microorganisms) and biofuels. When a significant amount of carbohydrate feedstock is consumed for the synthesis of cell mass, it lowers biofuel practical yields. Since the carbohydrate costs account for more than 50% of the final prices of biofuels [11••,31], it is vital to estimate carbohydrate allocation (percentage) between the desired biofuel and biocatalysts (enzymes or microorganisms).

Figure 3 shows typical values of carbohydrate allocation to biocatalysts ($A_{X/S}$): ~50%, aerobic fermentations [32]; ~20%, microaerobic fermentations [33]; ~10%, anaerobic fermentations [34,35]; and ~2–5%, resting-cell biotransformation [32]. These data suggest that it is not economically feasible to produce biofuels through aerobic fermentations because of the high carbohydrate allocation to microorganism. Because carbohydrate has nearly constant costs ($0.18/kg carbohydrate = $11.1/GJ) [10•,36], it is important to evaluate any biofuel ($20–40/GJ) produced from carbohydrate based on its mass yield and energy-retaining efficiency [37].

In SyPaB, carbohydrate allocation to the enzymes depends on TTN_W and recombinant protein yield based on carbohydrate ($Y_{P/S}$, gram of protein per gram of carbohydrate consumed) (Figure 3b). Higher $Y_{P/S}$ results in lower carbohydrate allocation to enzyme synthesis, that is, more carbohydrate is allocated to the formation of desired biofuel. When all enzymes are reaching a threshold TTN_W value of ~100,000, ~99% of carbohydrate will be allocated to the desired product regardless of $Y_{P/S}$ values from 0.01 to 0.2.

Cost analysis for hydrogen production

Production costs for hydrogen are based on three major cost components — carbohydrate, enzymes, and coen-

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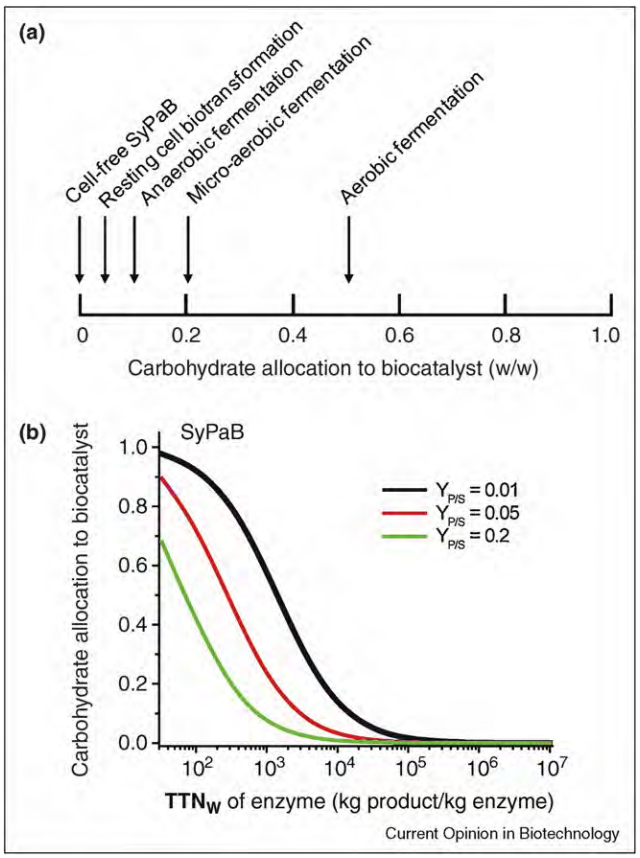
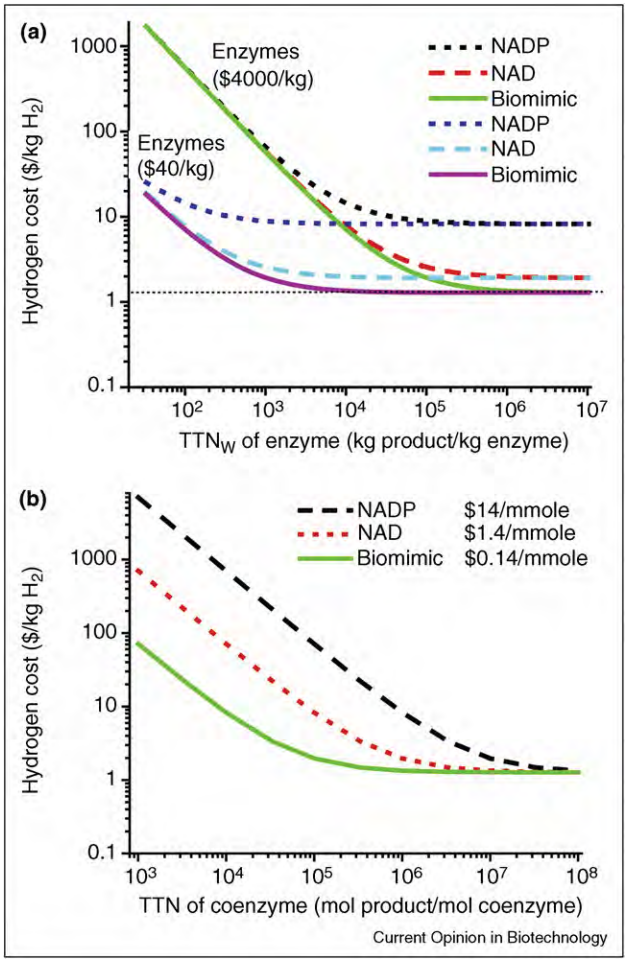
Figure 3



Carbohydrate allocation to biocatalyst in aerobic fermentation, microaerobic fermentation, and anaerobic fermentation, resting-cell biotransformation, and SyPaB **(a)**. The effects of TTN_W and protein yield based on sugar ($Y_{P/S}$, g protein per g glucose) on carbohydrate allocation to biofuel through SyPaB **(b)**. The simulated curves are based on the assumptions: first, a 14-enzyme mixture and second, the values of $Y_{P/S}$ (recombinant enzyme production through aerobic fermentation) ranging from 0.01 (a low expression level) to 0.2 (a high expression level).

Figure 4



Estimated production costs of hydrogen by SyPaB based on enzymes' TTN_W and their production **(a)**, when TTN of the cofactor = 1,000,000. Estimated production costs of hydrogen by SyPaB based on costs of NADP, NAD, and biomimetic cofactors **(b)**, when TTN_W = 100,000 and enzyme = $400/kg. The above calculations are based on three inputs: first, $0.18/kg carbohydrate, second, 14-enzyme SyPaB, and third, coenzymes.

zyme (NAD). Figure 4a shows the effects of the costs of enzyme ($40 or $4000/kg enzyme) and NAD on hydrogen production costs. The cost decreases rapidly with increasing TTN_W, and then levels off when all enzymes regardless of their production costs have TTN_W values of more than ~100,000. When all enzymes exhibit TTN_W values of more than 200,000, their cost accounts for a small fraction in the total cost of hydrogen production (i.e., less than 10 cents per kg of hydrogen). Enzyme stability is a key determinant of enzyme cost.

The costs of cofactors (NADP ~=10 × NAD ~= 100 × biomimic) also have a significant impact on the final costs of hydrogen production. Cofactor costs for oxidoreductase enzyme reactions have been reduced by replacing the natural cofactor (NADP) with the less costly NAD [38,39]. Typical biomimic NAD coenzymes include N-benzyl-1,4 dihydrononicotinamide and its derivatives [40•,41,42]. Although they have been used in conjunction with native enzymes, they may also be used with engineered oxidoreducatases such as cytochrome P450 [43••]. Figure 4a shows that it is important to create artificial oxidoreductases that can utilize economically advantageous and more stable biomimetic cofactors.

Figure 4b presents the effects of cofactor costs and their total turn-over number (TTN) on hydrogen production costs. Increasing TTN of coenzymes drastically decreased hydrogen production costs. It is worth pointing out that cost saving from low-cost biomimic would be negligible when it can be recycled more than ~500,000. Since the TTN for NAD has been reported to be more

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than 1,000,000 in synthesis of chiral compounds [44], it is expected that more stable biomimic cofactors can be recycled at the same level or higher.

With further development of engineered oxidoreductases that can use biomimetic NAD factors and stable enzymes as building blocks of SyPaB, we estimate that hydrogen production costs may decrease to ~$1.50 per kg of hydrogen (Figure 4), where carbohydrate accounts for ~80% of its production costs, which is similar to any mature biofuel production process, such as biodiesel production from vegetable oil.

Conclusions

The economical analyses clearly suggest that development of stable enzymes or their complexes as building blocks for SyPaB, efficient recycling of the coenzymes, and use of low cost and more stable biomimetic coenzyme analogues, would efficiently enable the economically viable production of low-value biofuels or their precursors. The SyPaB development may follow that of computers. Making the first several computers with relatively low performance was extremely costly. After intensive efforts in developing computers' components, standardization, and mass production, it becomes easier to assemble a customized high-performance computer at a very low cost. Now it is time to discover and develop more stable enzymes as Lego-like building blocks [13,28,29] and utilize less costly and more stable cofactor biomimics [43••]. Cell-free SyPaB can be applied to produce not only low-value biofuels but also high-value products, such as fine chemicals, non-natural carbohydrates, proteins, and glycoproteins [45–47]. It is worth pointing out that SyPaB's largest application would be for biofuel production mostly because of a very high product yield.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, Hogsett DA, Lynd LR: **Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield**. *Proc Natl Acad Sci* 2008, **105**:13769-13774.

2. Atsumi S, Hanai T, Liao JC: **Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels**. *Nature* 2008, **451**:86-89.

3. Huber GW, Chheda JN, Barrett CJ, Dumesic JA: **Production of liquid alkanes by aqueous-phase processing of biomass-derived carbohydrates**. *Science* 2005, **308**:1446-1450.

4. Kalscheuer R, Stolting T, Steinbuchel A: **Microdiesel: *Escherichia coli* engineered for fuel production**. *Microbiology* 2006, **152**:2529-2536.

5. Lu X, Vora H, Khosla C: **Overproduction of free fatty acids in *E. coli*: implications for biodiesel production**. *Metab Eng* 2008, **10**:333-339.

6. •• Zhang Y-HP, Evans BR, Mielenz JR, Hopkins RC, Adams MWW: **High-yield hydrogen production from starch and water by a synthetic enzymatic pathway**. *PLoS One* 2007, **2**:e456.
This paper is the first example of SyPaB that can produce more than 4 moles of hydrogen per mole of glucose, breaking the Thauer limit for hydrogen production via dark anaerobic fermentation.

7. •• Ye X, Wang Y, Hopkins RC, Adams MWW, Evans BR, Mielenz JR, Zhang Y-HP: **Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails**. *ChemSusChem* 2009, **2**:149-152.
This paper demonstrates the production of nearly 12 moles of hydrogen per mole of glucose unit of cellulosic materials, and a great increase in enzymatic hydrogen production rates.

8. Kobayashi F, Take H, Asada C, Nakamura Y: **Methane production from steam-exploded bamboo**. *J Biosci Bioeng* 2004, **97**:426-428.

9. Bond JQ, Alonso DM, Wang D, West RM, Dumesic JA: **Integrated catalytic conversion of {gamma}-valerolactone to liquid alkenes for transportation fuels**. *Science* 2010, **327**:1110-1114.

10. • Zhang Y-HP: **A sweet out-of-the-box solution to the hydrogen economy: is the sugar-powered car science fiction?** *Energy Environ Sci* 2009, **2**:272-282.
This perspective present potential applications based on SyPaB-mediated sugar-to-hydrogen production.

11. •• Zhang Y-HP: **Production of biocommodities and bioelectricity by cell-free synthetic enzymatic pathway biotransformations: challenges and opportunities**. *Biotechnol Bioeng* 2010, **105**:663-677.
This perspective proposes the design principles of cell-free SyPaB, presented several SyPaB examples, and discussed its R&D priorities.

12. • Zhang Y-HP: **Using extremophile enzymes to generate hydrogen for electricity**. *Microbe* 2009, **4**:560-565.
Discovery and utilization of thermoenzymes from extremophiles is highly operative.

13. Myung S, Wang YR, Zhang Y-HP: **Fructose-1,6-bisphosphatase from a hyper-thermophilic bacterium thermotoga maritime: characterization, metabolite stability and its implications**. *Process Biochem* 2010 doi: 10.1016/j.procbio.2010.1003.1017. [Epub.]

14. Steen E, Chan R, Prasad N, Myers S, Petzold C, Redding A, Ouellet M, Keasling J: **Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol**. *Microb Cell Fact* 2008, **7**:36.

15. Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC: **Metabolic engineering of *Escherichia coli* for 1-butanol production**. *Metab Eng* 2008, **10**:305-311.

16. Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, del Cardayre SB, Keasling JD: **Microbial production of fatty-acid-derived fuels and chemicals from plant biomass**. *Nature* 2010, **463**:559-562.

17. Buchner E: **Alkoholische Gahrung ohne Hefezellen (Vorlaufige Mittheilung)**. *Ber Chem Ges* 1897, **30**:117-124.

18. Welch P, Scopes RK: **Studies on cell-free metabolism: ethanol production by a yeast glycolytic system reconstituted from purified enzymes**. *J Biotechnol* 1985, **2**:257-273.

19. Nelson DL, Cox MM: *Lehninger Principles of Biochemistry (5th edition)*. New York: WH Freeman; 2008.

20. Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS: **Fermentative butanol production by clostridia**. *Biotechnol Bioeng* 2008, **101**:209-228.

21. Kunkes EL, Simonetti DA, West RM, Serrano-Ruiz JC, Gartner CA, Dumesic JA: **Catalytic conversion of biomass to monofunctional hydrocarbons and targeted liquid-fuel classes**. *Science* 2008, **322**:417-421.

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22. Chheda J, Huber G, Dumesic J: **Liquid-phase catalytic processing of biomass-derived oxygenated hydrocarbons to fuels and chemicals**. *Angew Chem Int Ed* 2007, **46**:7164-7183.

23. Huber GW, Cortright RD, Dumesic JA: **Renewable alkanes by aqueous-phase reforming of biomass-derived oxygenates**. *Angew Chem Int Ed* 2004, **43**:1549-1551.

24. Woodyer R, Simurdiak M, van der Donk WA, Zhao H: **Heterologous expression, purification, and characterization of a highly active xylose reductase from *Neurospora crassa***. *Appl Environ Microbiol* 2005, **71**:1642-1647.

25. Metzger JO: **Production of liquid hydrocarbons from biomass**. *Angew Chem Int Ed* 2006, **45**:696-698.

26. Carlson T, Vispute T, Huber GW: **Green gasoline by catalytic fast pyrolysis of solid biomass-derived compounds**. *ChemSusChem* 2008, **1**:397-400.

27. Wheals AE, Basso LC, Alves DMG, Amorim HV: **Fuel ethanol after 25 years**. *Trends Biotechnol* 1999, **17**:482-487.

28. Wang Y, Zhang Y-HP: **Overexpression and simple purification of the *Thermotoga maritima* 6-phosphogluconate dehydrogenase in *Escherichia coli* and its application for NADPH regeneration**. *Microb Cell Fact* 2009, **8**:30.

29. Wang Y, Zhang Y-HP: **A highly active phosphoglucomutase from *Clostridium thermocellum*: cloning, purification, characterization, and enhanced thermostability**. *J Appl Microbiol* 2010, **108**:39-46.

30. Vasic-Racki D: **History of industrial biotransformations—dreams and realities**. In *Industrial Biotransformations.* Edited by Liese A, Seebald S, Wandrey C. Wiley-VCH; 2006:1-37.

31. Lynd LR, Wyman CE, Gerngross TU: **Biocommodity engineering**. *Biotechnol Prog* 1999, **15**:777-793.

32. Shuler M, Kargi F: *Bioprocess Engineering: Basic Concepts*Prentice-Hall; 1991.

33. Walther T, Hensirisak P, Agblevor FA: **The influence of aeration and hemicellulosic sugars on xylitol production by *Candida tropicalis***. *Bioresour Technol* 2001, **76**:213-220.

34. Zhang Y-HP, Lynd LR: **Cellulose utilization by *Clostridium thermocellum*: bioenergetics and hydrolysis product assimilation**. *Proc Natl Acad Sci USA* 2005, **102**:7321-7325.

35. Papoutsakis ET: **Equations and calculations for fermentations of butyric-acid bacteria**. *Biotechnol Bioeng* 1984, **26**:174-187.

36. Zhang Y-HP: **Reviving the carbohydrate economy via multi-product biorefineries**. *J Ind Microbiol Biotechnol* 2008, **35**:367-375.

37. Rude MA, Schirmer A: **New microbial fuels: a biotech perspective**. *Curr Opin Microbiol* 2009, **12**:274-281.

38. Banta S, Swanson BA, Wu S, Jarnagin A, Anderson S: **Optimizing an artificial metabolic pathway: engineering the cofactor specificity of *Corynebacterium* 2,5-Diketo-D-gluconic acid reductase for use in vitamin C biosynthesis**. *Biochemistry* 2002, **41**:6226-6236.

39. Sanli G, Banta S, Anderson S, Blaber M: **Structural alteration of cofactor specificity in Corynebacterium 2,5-diketo-D-gluconic acid reductase**. *Protein Eng* 2004, **13**:504-512.

40. • Lo HC, Fish RH: **Biomimetic NAD^+ models for tandem cofactor regeneration, horse liver alcohol dehydrogenase recognition of 1,4-NADH derivatives, and chiral synthesis**. *Angew Chem Int Ed* 2002, **41**:478-481.
The wild-type horse liver alcohol dehydrogenase can utilize biomimetic cofactors.

41. Lutz J, Hollmann F, Ho TV, Schnyder A, Fish RH, Schmid A: **Bioorganometallic chemistry: biocatalytic oxidation reactions with biomimetic NAD^+/NADH co-factors and $[Cp^*Rh(bpy)H]^+$ for selective organic synthesis**. *J Organomet Chem* 2004, **689**:4783-4790.

42. Fish RH, Kerr JB, Lo HC: Agents for replacement of NAD^+/NADH system in enzymatic reaction. US Patent 2004.

43. •• Ryan JD, Fish RH, Clark DS: **Engineering cytochrome P450 enzymes for improved activity towards biomimetic 1,4-NADH cofactors**. *ChemBioChem* 2008, **9**:2579-2582.
This paper presents that P450 can be engineered to work on biomemitic NAD cofactors more efficiently.

44. Grunwald J, Wirz B, Scollar MP, Klibanov AM: **Asymmetric oxidoreductions catalyzed by alcohol dehydrogenase in organic solvents**. *J Am Chem Soc* 1986, **108**:6732-6734.

45. Panke S, Held M, Wubbolts M: **Trends and innovations in industrial biocatalysis for the production of fine chemicals**. *Curr Opin Biotechnol* 2004, **15**:272-279.

46. Wang Y, Zhang Y-HP: **Cell-free protein synthesis energized by slowly-metabolized maltodextrin**. *BMC Biotechnol* 2009, **9**:58.

47. Boyer ME, Stapleton JA, Kuchenreuther JM, Wang C-W, Swartz JR: **Cell-free synthesis and maturation of [FeFe] hydrogenases**. *Biotechnol Bioeng* 2008, **99**:59-67.

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