PERSPECTIVE

BIOTECHNOLOGY and BIOENGINEERING

Production of Biocommodities and Bioelectricity by Cell-Free Synthetic Enzymatic Pathway Biotransformations: Challenges and Opportunities

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ABSTRACT: Cell-free synthetic (enzymatic) pathway biotransformation (SyPaB) is the assembly of a number of purified enzymes (usually more than 10) and coenzymes for the production of desired products through complicated biochemical reaction networks that a single enzyme cannot do. Cell-free SyPaB, as compared to microbial fermentation, has several distinctive advantages, such as high product yield, great engineering flexibility, high product titer, and fast reaction rate. Biocommodities (e.g., ethanol, hydrogen, and butanol) are low-value products where costs of feedstock carbohydrates often account for ~30–70% of the prices of the products. Therefore, yield of biocommodities is the most important cost factor, and the lowest yields of profitable biofuels are estimated to be ca. 70% of the theoretical yields of sugar-to-biofuels based on sugar prices of ca. US$ 0.18 per kg. The opinion that SyPaB is too costly for producing low-value biocommodities are mainly attributed to the lack of stable standardized building blocks (e.g., enzymes or their complexes), costly labile coenzymes, and replenishment of enzymes and coenzymes. In this perspective, I propose design principles for SyPaB, present several SyPaB examples for generating hydrogen, alcohols, and electricity, and analyze the advantages and limitations of SyPaB. The economical analyses clearly suggest that developments in stable enzymes or their complexes as standardized parts, efficient coenzyme recycling, and use of low-cost and more stable biomimetic coenzyme analogs, would result in much lower production costs than do microbial fermentations because the stabilized enzymes have more than 3 orders of magnitude higher weight-based total turn-over numbers than microbial biocatalysts, although extra costs for enzyme purification and stabilization are spent.

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Introduction

Biocatalysts refer to enzymes or whole cells that accelerate biochemical reactions. Biocatalysts can usually catalyze chemical reactions with high selectivity under mild reaction conditions at temperatures from the freezing point of water to the boiling point and at pressures from ~1 atm to higher.

Biocatalysis or biotransformation mediated by biocatalysts can be classified based on product prices (Fig. 1). High-value products, including protein drugs, antibodies, chiral compounds, taxol, antibiotics, and so on, range from thousands to nearly billions of dollars per kilogram (Lynd et al., 1999). By contrast, biocommodities with low selling prices, such as ethanol, hydrogen, lactic acid, and so on, range from less than one dollar to several dollars per kilogram (Lynd et al., 1999; Zhang and Lynd, 2008). White biotechnology or industrial biotechnology, which is used for the production of biocommodities (fuels and biobased chemicals) (Frazzetto, 2003), is distinct from red biotechnology, which is used for the production of high-value medical products (Bauer, 2005) at multiple levels, including economic driving force, importance of feedstock price, processing cost, capital investment, scale of application, and feedstock availability (Lynd et al., 1999). Biocommodities

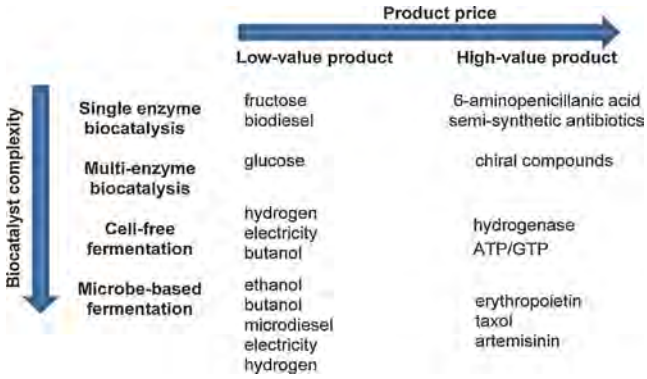

Figure 1. Classification of biotransformations based on biocatalyst complexity and product price.

have low selling prices, so that raw material costs (mainly carbohydrates) are often a dominant factor in determining prices of such items (~30–70%), while raw materials usually account for a very small fraction of the high selling prices of pharmaceuticals (Lynd et al., 1999). Tremendous differences exist with respect to a mass basis. For example, large biocommodity markets often exceed pharmaceutical markets by approximately 11 orders of magnitude (Lynd et al., 1999).

Biotransformation can be divided into four classes, depending on an increasing order in complexity of reactions and biocatalysts: (1) single enzyme, (2) multi-enzyme in one pot, (3) cell-free fermentation, and (4) whole-cell fermentation (Fig. 1). A single enzyme can catalyze a simple chemical reaction and approximately 4,800 enzyme entries have been documented and classified in the Brenda database (http://www.brenda-enzymes.org/) (Chang et al., 2009). Some biotransformations mediated by a single enzyme have been implemented on industrial scales. For example, fructose is produced from glucose as catalyzed by glucose isomerase (Bhosale et al., 1996; Vasic-Racki, 2006), and beta-lactam antibiotics are semi-synthesized by amidases (Demain, 2004; Vasic-Racki, 2006). Relatively complicated chemical reactions can be mediated by multi-enzymes in one pot, for example, cellulose hydrolysis by the synergetic action of endoglucanase, cellobiohydrolase, and beta-glucosidase (Zhang and Lynd, 2004), synthesis of chiral alcohols with NAD(P)H regeneration (Hummel, 1999; Wichmann and Vasic-Racki, 2005), synthesis of carbohydrates (Meyer et al., 2007; Shaeri et al., 2008), and biosynthesis of polymers (Chi et al., 2008). The use of multiple enzymes in one pot has numerous benefits: fewer unit operations, smaller reactor volume, higher volumetric and space–time yields, shorter cycle times, and less waste generation. Also, by coupling steps together, unfavorable equilibria can be driven toward the formation of desired products (Chi et al., 2008; Daines et al., 2004).

For complicated biotransformations that cannot be implemented by a single enzyme or a multi-enzyme mixture, whole cell biocatalysts with or without genetic modifications are a straightforward choice. For example, alcohol fermentation and cheese production mediated by wild-type microorganisms have been used for thousands of years. With the development of modern biotechnology, whole cells have been engineered to produce a large number of products, from low-value products, such as ethanol (Shaw et al., 2008), butanol (Atsumi et al., 2008), fatty acid esters (Kalscheuer et al., 2006), hydrogen (Logan, 2004; Maeda et al., 2008), electricity (Chaudhuri and Lovley, 2003; Logan and Regan, 2006), etc., to high-value products, such as, antibiotics, vitamins, vaccines, enzymes, antibodies, glycoproteins, lycopene, artemisinin, taxol, and so on (Chang and Keasling, 2006; Demain, 2000; Hamilton et al., 2003; Klein-Marcuschamer et al., 2007; Maynard and Georgiou, 2000; Zhong, 2001).

Cell-free fermentation/biotransformation involving a large number of enzymes has been relatively ignored for production purposes but it is not a completely new concept for research purposes. As early as 1897, the Nobel Prize winner Eduard Buchner discovered ethanol fermentation mediated by a yeast extract. Cell-free protein synthesis (CFPS) is becoming a popular alternative for the production of in vivo cytotoxic, regulatory, or unstable proteins that are difficult to be expressed in living cells (Boyer et al., 2008; Wang and Zhang, 2009a) and for incorporation of unnatural amino acids into polypeptides (Hirao et al., 2002; Noren et al., 1989).

Synthetic biology applies engineering principles (e.g., design, extraction, and standardization) and combines sciences (biology and chemistry) in order to design and build novel biological functions and systems that function unnaturally or function much better than natural counterparts (Benner and Sismour, 2005; Endy, 2005). Synthetic biology is also interpreted as the engineering-driven building of increasingly complicated biological entities (parts, devices, and systems) from simple and basic building blocks.

Synthetic biology projects can be divided into two classes: in vivo and in vitro (Forster and Church, 2007; Meyer et al., 2007). Compared to in vivo living biological entity-based synthetic biology, in vitro cell-free synthetic biology is a largely unexplored approach. However, the benefits of in vitro synthetic biology are clear. Assembling a new system is much easier than modifying a living system. Engineering flexibility in vitro is much greater, that is, it is unshackled from cellular viability, complexity, physiology, and the presence of membranes and/or cell walls (Zhang et al., 2008). Nearly all cell-free synthetic biology projects involve the synthesis of biopolymers from small building blocks, for example, DNAs by PCR amplification, RNAs by in vitro transcription, and polypeptides by in vitro transcription/translation (Forster and Church, 2007; Meyer et al., 2007; Swartz, 2006). The New England Biolabs has commercialized PURExpress, based on a coupled cell-free

transcription/translation system reconstituted from purified *Escherichia coli* components (Shimizu et al., 2001).

Here I discuss the feasibility of biocommodity and bioelectricity production by cell-free synthetic enzymatic pathway biotransformations (SyPaB) through catabolic pathways, which are different from the reconstitution of a cell-free synthetic enzymatic pathway for the production of high-value products (Hirao et al., 2002; Schultheisz et al., 2008; Wang and Zhang, 2009a) or for understanding the basic mechanisms of natural processes (Brown et al., 2008; Buchner, 1897; Nirenberg and Matthaei, 1961). In this perspective, I propose the design principles of SyPaB, present several examples, analyze its advantages and limitations as compared to microbial fermentations, and highlight its R&D challenges and opportunities.

Synthetic Pathway Biotransformation (SyPaB)

SyPaB and microbial fermentation can implement similar-level complicated biochemical reactions. In principle, microbes contain thousands of proteins responsible for metabolism regulation, self-duplication, and formation of a desired product, where a small fraction of cellular enzymes (e.g., ~20 enzymes or less) are usually responsible for converting the substrate to the desired product. By contrast, SyPaB contains only the enzymes responsible for the desired transformation without side pathways or cell duplication.

Figure 2 shows the development cycle of SyPaB, which is composed of five parts: (i) pathway reconstruction, (ii) enzyme selection, (iii) enzyme engineering, (iv) enzyme production, and (v) process engineering. The development of the whole process requires the input of many different specialists. Whole SyPaB processes can be improved in an iterative manner, gradually leading to an efficient industrial process.

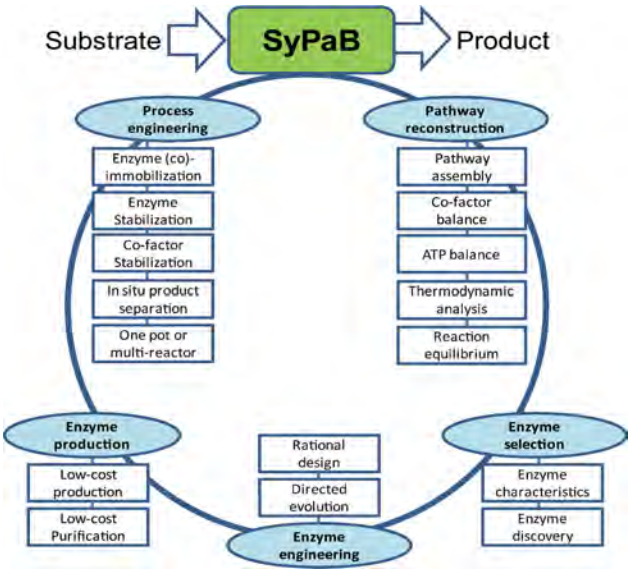

Figure 2. The cycle of cell-free synthetic pathway biotransformation (SyPaB).

The reconstruction of a cell-free synthetic enzymatic pathway is the central point of SyPaB. Synthetic pathway assembly is usually designed based on natural metabolic pathways with necessary modifications. Since the same biochemical reactions can be conducted by several different pathways sometimes, the pathways need to be designed carefully, considering ATP and NAD(P) balance, thermodynamics, reaction equilibrium, product separation, and so on. For example, *Saccharomyces cerevisiae* can produce 2 mol of ethanol and carbon dioxide from each mole of glucose through the glycolysis pathway with 2 mol of ATP formed (Zaldivar et al., 2001), while *Zymomonas mobilis* can implement the same reaction through the Entner–Doudoroff pathway with only 1 mol of ATP formed (Jeffries, 2005). It has been suggested that a larger negative Gibbs energy through the Entner–Doudoroff pathway drives ethanol formation rates faster than through the glycolysis pathway. For sustaining overall reactions, it is vital to keep both ATP and reducing power carriers NAD(P)H balanced. If net ATP is accumulated in the overall process, addition of ATPase or arsenate or phosphatase would dissipate some ATP (Welch and Scopes, 1985) or hydrolyze high-energy phosphate bond-containing metabolites. If a small amount of ATP is required as input for the whole system, several ways can be selected, such as glucose-1-phosphate generation by using phosphorylases and phosphate (Zhang and Lynd, 2005) or ATP generation from the secondary energy sources, such as creatine phosphate, phosphoenolpyruvate, acetate phosphate, or fructose-1,6-biphosphate, through substrate phosphorylation (Wang and Zhang, 2009a). But accumulation of free phosphate and/or costly second energy sources prohibits their application for commodity production. If a large amount of ATP is needed as input, cell-free oxidative phosphorylation may be a choice (Jewett et al., 2008). If net NAD(P)H is accumulated in the system, excess reducing powers can be removed by adding hydrogenase (Wichmann and Vasic-Racki, 2005) or can be used for generation of electricity through biofuel cells (Cooney et al., 2008). If net NAD(P)H input is required, the pentose phosphate pathway or electrical or photochemical NAD(P)H regeneration may be chosen (Palmore et al., 1998; Schroder et al., 2003). In addition, it is important to ensure that there is no accumulation of inhibitors in the pathways. For biocommodity production, any net inputs of costly substrates (such as amino acids, ATP, creatine phosphate, phosphoenolpyruvate, pyruvate, etc.) are economically prohibitive.

Enzyme selection is in principle based on its catalytic reaction and substrate specificity. In addition, enzymes with high substrate selectivity, high catalytic efficiency, low product inhibition, and high stability are highly desired. Although enzymes are well known for their high selectivity, some enzymes with some promiscuous activities may not be good for SyPaB. For example, the *Pyrococcus furiosus*

fructose-1,6-bisphosphatase hydrolyzes numerous other substrates (e.g., inositol-1-phosphate, glycerol-2-phosphate, glucose-1-phosphate) (Verhees et al., 2002). Since different source enzymes may have different optima for pH, temperature, and ionic strength, and may require different metal ions (e.g., Mg^{2+}, Mn^{2+}) as activators, trade-offs have to been made among them. Therefore, it could be preferable that as many enzymes as possible can be obtained from the same microorganism because they may have similar optimum working conditions, and they may form enzyme complexes to allow metabolite channeling (Srivastava and Bernhard, 1986; Srivastava et al., 1989; Zhang, 2009).

Enzymes can be further engineered to enhance their catalytic efficiency for industrial applications, such as thermostability (Given et al., 1998; Liu et al., 2009), cofactor preference (Banta et al., 2002a; Woodyer et al., 2003), and so on, by rational design, directed evolution, or a combination (Luetz et al., 2008; Zhang et al., 2006).

Decreasing enzyme production costs are of importance for industrial processes. In general, low-cost recombinant proteins can be produced in high cell density fermentations (Barnard et al., 2004; Hartley, 2006; Zhang et al., 2006). Low-cost enzyme purification can be conducted by scalable purification means, such as precipitation (Banki et al., 2005; Scopes, 1993), heat precipitation (Blumer-Schuette et al., 2008; Wang and Zhang, 2009b), or adsorption/desorption (Hong et al., 2008a,b). It is estimated that production costs of recombinant protein may be as low as \$20–40 per kg of proteins (dry weight) (Zhang et al., 2006, 2008). Recently, we have over-expressed more than 250 mg of *Thermotoga maritima* 6-phosphogluconate dehydrogenase per liter of the *E. coli* culture, accounting for more than 30% of total cellular protein (Wang and Zhang, 2009b).

Process engineering includes enzyme immobilization, cofactor stabilization, in situ product separation, reactor engineering, and so on. In addition to the reuse of enzymes and recycling cofactors as well as stabilizing enzymes, cofactors, and metabolites, in situ product removal is desired sometimes because it decreases product inhibition, shifts the overall reaction toward product formation, and increases productivity (Freeman et al., 1993).

Systems engineering techniques can be applied for setting priorities for improvements of SyPaB, a detailed understanding of the costs and potential for improvement of each step in the whole process.

Examples of SyPaB

Most biocommodities (e.g., biofuels and biobased chemicals) are or will be produced from fermentable carbohydrates isolated from biomass. Low-cost renewable carbohydrates (~\$0.18 per kg) will likely become a key future industrial feedstock, similar to the role of crude oil in modern oil refineries (Lynd et al., 2008; Zhang, 2008b, 2009). Utilization of renewable carbohydrates for the production of biocommodities would decrease greenhouse gas emissions, promote the rural economy, and solve the challenges associated with scattered solar energy utilization and storage (Zhang, 2008a). Several examples using SyPaB are presented here.

High-Yield Production of Hydrogen

Hydrogen is a promising future clean energy carrier with high-energy utilization efficiency. The production of hydrogen from less costly abundant biomass is a shortcut for producing low-cost hydrogen without net carbon emissions (Adams and Stiefel, 1998; Cortright et al., 2002; Salge et al., 2006; Zhang, 2009).

A synthetic enzymatic pathway has been designed for generating high-yield hydrogen (Fig. 3). This synthetic pathway contains (i) a chain-shortening phosphorylation reaction for producing glucose-1-phosphate (G-1-P) catalyzed by glucan phosphorylase (Eq. 1); (ii) conversion of G-1-P to glucose-6-phosphate (G-6-P) catalyzed by phosphoglucomutase (Eq. 2); (iii) a pentose phosphate pathway containing 10 enzymes for producing 12 NADPH per G-6-P (Eq. 3); and (iv) hydrogen generation from NADPH catalyzed by hydrogenase (Eq. 4)

$$(C_6H_{10}O_5)_n + H_2O + P_i \leftrightarrow (C_6H_{10}O_5)_{n-1} + G{-}1{-}P \quad (1)$$

$$G{-}1{-}P \leftrightarrow G{-}6{-}P \quad (2)$$

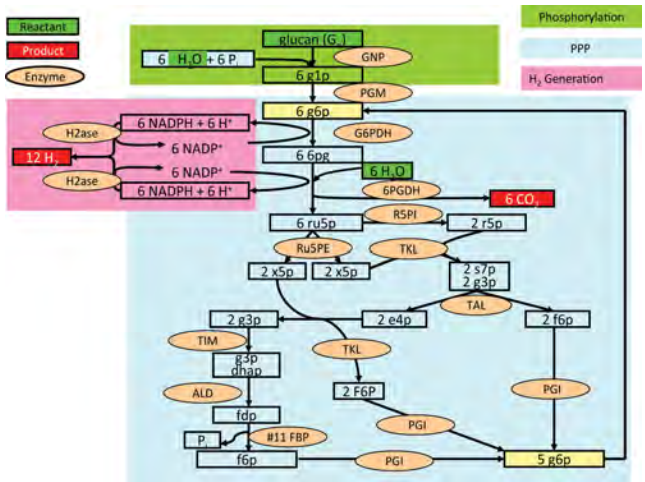

Figure 3. The synthetic pathway for complete conversion of glucan and water to hydrogen and carbon dioxide. PPP, pentose phosphate pathway. The enzymes are: GNP, glucan phosphorylase; PGM, phosphoglucomutase; G6PDH, G-6-P dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; R5PI, phosphoribose isomerase; Ru5PE, ribulose 5-phosphate epimerase; TKL, transketolase; TAL, transaldolase; TIM, triose phosphate isomerase; ALD, aldolase; FBP, fructose-1,6-bisphosphatase; PGI, phosphoglucose isomerase; and H2ase, hydrogenase. The metabolites and chemicals are: G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; 6pg, 6-phosphogluconate; ru5p, ribulose-5-phosphate; x5p, xylulose-5-phosphate; r5p, ribose-5-phosphate; s7p, sedoheptulose-7-phosphate; g3p, glyceraldehyde-3-phosphate; e4p, erythrose-4-phosphate; dhap, dihydroxyacetone phosphate; fdp, fructose-1,6-diphosphate; f6p, fructose-6-phosphate; and P_i, inorganic phosphate.

$$\mathrm{G{-}6{-}P} + 12\mathrm{NADP^+} + 6\mathrm{H_2O} \leftrightarrow 12\mathrm{NADPH} + 12\mathrm{H^+} + 6\mathrm{CO_2} + \mathrm{P_i} \quad (3)$$

$$12\mathrm{NADPH} + 12\mathrm{H^+} \leftrightarrow 12\mathrm{H_2} + 2\mathrm{NADP^+} \quad (4)$$

The combination of Equations (1)–(4):

$$\mathrm{C_6H_{10}O_5(aqueous)} + 7\mathrm{H_2O(liquid)} \rightarrow 12\mathrm{H_2(gas)} + 6\mathrm{CO_2(gas)} \quad (5)$$

Thermodynamic analysis suggests that the overall reaction (Eq. 5) is a spontaneous endothermic process ($\Delta G^\circ = -48.9$ kJ/mol and $\Delta H^\circ = 596$ kJ/mol) (Ye et al., 2009; Zhang et al., 2007). This enzymatic reaction is among rare entropy-driven chemical reactions because the two final products are gaseous under the experimental conditions ($\sim$1 atm and $<100^\circ$C). Great increases in the entropy due to the phase change enable the positive-enthalpy reactions to occur. For example, two entropy-driven reactions of N_2O_5 (s) $\rightarrow 2NO_2$ (g) $+ {}^1/_2O_2$ (g) and $Ba(OH)_2{\cdot}8H_2O$ (s) $+ 2NH_4SCN$ (s) $\rightarrow Ba(SCN)_2$ (aq) $+ 2NH_3$ (aq) $+ 10H_2O$ (l) also involve phase changes.

Figure 4 presents the profile of hydrogen and carbon dioxide evolution from cellopentaose (beta-1,4-oligosaccharide, a soluble fragment of cellulose) and water in a batch reaction at 32°C. Clearly, CO_2 evolves before H_2, in good agreement with the mechanism by which CO_2 is released by 6-phosphogluconate dehydrogenase before NADPH accumulates beyond the critical value for hydrogen generation by hydrogenase (Fig. 3). A maximum hydrogen production rate is 3.92 mmol of hydrogen per hour per liter of reactor. When cellobiose is used as the substrate with a reaction time of 150 h for a complete reaction, the overall yields of H_2 and CO_2 are 11.2 mol of H_2 and 5.64 mol of CO_2 per mole of anhydroglucose unit of cellobiose, corresponding to 93.1% and 94% of the theoretical yields, respectively (Ye et al., 2009). The slightly less than theoretical value is readily explained by accumulated equilibrium intermediates (e.g., G-1-P, G-6-P, NADPH) in batch operation. The theoretical yield (12 H_2 per glucose unit) is expected to be obtained when a continuous reactor is run.

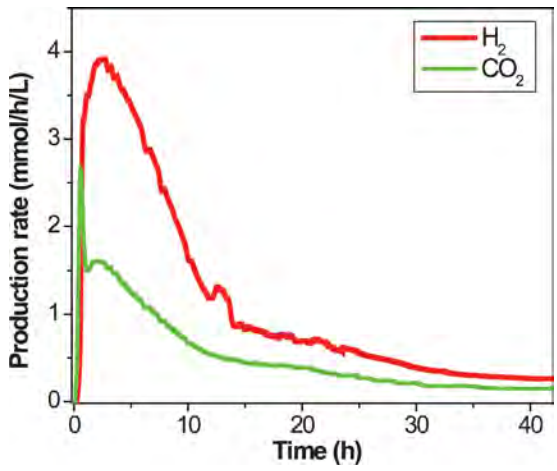

Figure 4. Experimental profile for evolution of hydrogen and carbon dioxide from cellopentaose and water.

The reaction conditions are chosen based on several criteria: (i) all enzymes are active at 30–32°C and pH 7.5; (ii) the HEPES buffer is used because some buffers (e.g., Tris) inhibit activities of some enzymes; (iii) 4 mM phosphate and 2 mM $NADP^+$ are chosen because both are substrates and inhibitors (i.e., neither a high nor low concentration is good); (iv) 10 mM Mg^{2+} and 0.5 mM Mn^{2+} are needed as activators for several enzymes (Mg^{2+} levels that are too high can precipitate free inorganic phosphate); and (v) thiamine pyrophosphate is added as an coenzyme for transketolase.

Woodward et al. (2000) have demonstrated high-yield hydrogen generation from glucose-6-phosphate, that is, 11.6 mol of H_2 per mole of G-6-P in a batch reaction. This pathway design has several drawbacks: (1) use of costly glucose-6-phosphate, which is proposed to be produced with an ATP regeneration system (Woodward et al., 2000); (2) free phosphate as the final product, which precipitates free Mg^{2+} ions that are of importance for several enzyme activities (Jewett and Swartz, 2004; Wang and Zhang, 2009a); and (3) the pH shift due to phosphate accumulation (Wang and Zhang, 2009a). According to the design principles of SyPaB (Synthetic Pathway Biotransformation (SyPaB) Section), this process cannot be used for sustainable production of low-cost hydrogen. Also, their calculation that 98% of the potential energy of glucose-6-phosphate is recoverable through hydrogen combustion (Woodward et al., 2000) is wrong. In fact, the entropy-driven reactions of hydrogen generation from polysaccharides or G-6-P or glucose and water through complete oxidation have enthalpy-based energy efficiencies of more than 100% (Chheda et al., 2007; Ye et al., 2009; Zhang, 2009; Zhang et al., 2007).

Production of Alcohols

Ethanol, an attractive liquid biofuel, can be produced through microbial anaerobic fermentation by the yeast *S. cerevisiae* or by other microorganisms such as *E. coli* and *Z. mobilis*. Ethanol yields from hexose are as high as 85–95% of the theoretical yield, and final concentrations are up to 10–12% (w/v) (Bai et al., 2008). Although the cell-free ethanol fermentation experiment catalyzed by yeast extract demonstrates the feasibility of ethanol production without live cells (Buchner, 1897), ATP imbalance (i.e., two ATP produced per glucose) prevents complete conversion of glucose to ethanol (Welch and Scopes, 1985; Xu and Taylor, 1993). Welch and Scopes (1985) demonstrated the feasibility of high-yield ethanol production by a reconstituted

yeast glycolytic enzyme system with supplementation of ATPase, which is responsible for keeping ATP balanced. Consequently, the cell-free system is capable of totally converting 180 g/L glucose to 90 g/L ethanol within 8 h with a yield of 99% (Welch and Scopes, 1985). Alternatively, arsenate can replace costly ATPase (Welch and Scopes, 1985).

Butanol, a four-carbon liquid alcohol, has several advantages over ethanol, such as a higher energy content, lower water absorption, better blending ability, and easier usage in internal combustion engines (Lee et al., 2008). Acetone, butanol, and ethanol (ABE) fermentation by *Clostridium* sp. is a two-step process, which is complicated and difficult to control (Jones and Woods, 1986; Lee et al., 2008). In addition, butanol yields in most ABE fermentations are fairly low and rarely exceed 80% (Jones and Woods, 1986). Also, butanol is a stronger inhibitor of microbial metabolism and membrane integrity than is ethanol (Ingram and Buttke, 1984). Hydrophobicity of butanol can destabilize the phospholipid component of the cell membrane (Bowles and Ellefson, 1985; Vollherbst-Schneck et al., 1984). One percent of butanol significantly inhibits cell growth and the fermentation process (Huang et al., 2004; Jones and Woods, 1986); 1.6% of butanol completely inhibits cell growth and terminates the fermentation (Jones and Woods, 1986). A lack of clear understanding of alcohol toleration mechanisms results in great challenges in engineering microbes that can grow and produce alcohol in the presence of high-titer alcohols (Borden and Papoutsakis, 2007; Ezeji et al., 2007; Paredes et al., 2005; Tomas et al., 2003).

Butanol production by cell-free SyPaB has been proposed based on the natural butanol fermentation pathway with some modifications (e.g., ATP balanced) (Zhang et al., 2008). Similar to cell-free ethanol fermentation, addition of ATPase or arsenate or phosphatase would enable sustainable butanol conversion by keeping ATP balanced. SyPaB is believed to solve several problems associated with microbial fermentations because of (i) a high product yield due to neither byproduct formation nor cell mass synthesis, (ii) possibly a high product titer because most enzymes can tolerate higher levels of organic solvents than can cellular membranes (Zhang et al., 2008), (iii) much easier process control (i.e., one-step conversion), (iv) no risk of bacteriophage contamination (Jones et al., 2000), and (v) no costly medium nutrients for microbial growth. Similar to microbial butanol fermentation (Ezeji et al., 2004), in situ product removals for SyPaB by pervaporation, liquid–liquid extraction, or gas stripping are desired because these downstream operations can drive the overall reaction toward butanol formation and can increase productivity.

Electricity Generation

Enzymatic fuel cells are a specific type of fuel cell, which uses enzymes rather than chemical catalysts to convert energy in the chemicals to electrical energy (Calabrese Barton et al., 2004; Cooney et al., 2008). Enzymes are used to catalytically oxidize the fuels (sugars or alcohols or organic acids) at the anode and reduce the oxidant at the cathode. Like fuel cells, enzymatic fuels cells can be refilled rapidly and have high-energy storage densities (Calabrese Barton et al., 2004; Moehlenbrock and Minteer, 2008). To date, no commercial enzymatic biofuel cell exists, mainly due to poor enzyme stability and low power output. But a number of companies (e.g., Sony) are working on enzymatic fuel cells (Sakai et al., 2009). Complete conversion of the chemical energy stored in sugars or complicated chemical compounds to electricity has fourfold benefits: high energy utilization efficiency, high energy storage density, little product inhibition, and high power density (Cooney et al., 2008; Minteer et al., 2007). Most enzymatic fuel cell designs extract only a small fraction of chemical energy in the compounds and convert it to electricity, that is, incomplete oxidation of chemicals (Bullen et al., 2006; Glykys and Banta, 2009; Ramanavicius et al., 2008; Sakai et al., 2009). Recently, Sokic-Lazic and Minteer (2008) have demonstrated immobilization of up to 11 enzymes, including 6 dehydrogenases responsible for generating NAD(P)H and $FADH_2$ and 5 non-energy producing enzymes necessary for the whole cycle, on the surface of an electrode (Fig. 5). Current densities increase when more dehydrogenases are immobilized on an anode with polymethylene green as a mediator, which is responsible for electron transfer. By mimicking the complete citric acid cycle on a carbon electrode, power density is increased by 8.71-fold as compared to a single enzyme (alcohol dehydrogenase)-based ethanol/air fuel cell (Sokic-Lazic and Minteer, 2008). However, this pathway needs

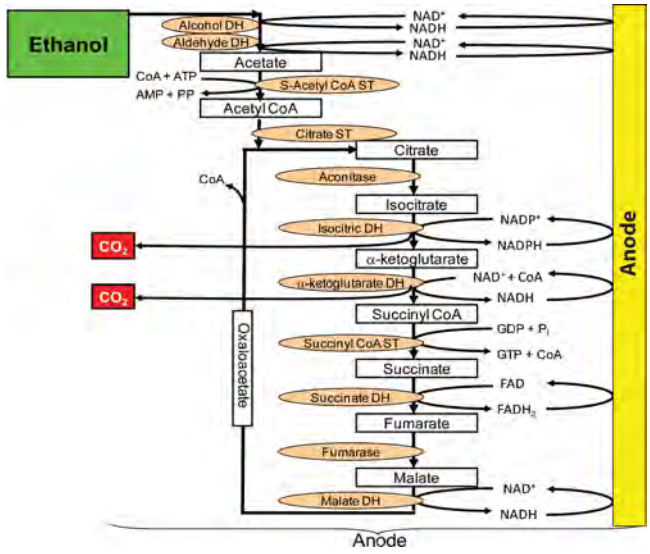

Figure 5. The synthetic pathway for complete conversion of ethanol and water to electricity and carbon dioxide (modified by Sokic-Lazic and Minteer, 2008). DH, dehydrogenase; ST, synthetase.

some improvements, according to the design principles of SyPaB.

Key Features for Industrial Processes

Industrial processes must trade off three key factors: product yield (gram product per gram substrate), product titer (gram product per liter), and productivity (gram product per liter per hour). For the production of biocommodities, product yield is the most important because a large fraction of product costs come from feedstock carbohydrate (e.g., ~30–70%) (Lynd et al., 1999; Zhang, 2009; Zhang et al., 2007). Product titer is second in importance because it is closely associated with product separation. For the production of high-value products, product titer is the most important because downstream separation usually accounts for more than 80% of processing costs for the final products (Belter et al., 1988; Lightfoot and Moscariello, 2004). Product yield is not important at all because raw feedstock or substrate accounts for only a small fraction of the prices of final products (e.g., <1%, sometimes).

For the production of biocommodities, feedstock costs usually account for ~30–70% of final product price and the remaining costs comprised processing costs for biocatalysts, product separation, waste treatment, utilities, capital depreciation, labor, taxes, and so on. Considering the cost of carbohydrates (~$0.18 per kg or $10.6 per GJ) and selling prices of ethanol ($2 per gallon or $22.1 per GJ) (Zhang, 2009), the bottom yield for profitable ethanol production is estimated to be 70–80%. Similar analyses can be applied to other advanced biofuels such as butanol, fatty acid esters, hydrocarbon, and so on; minimum yields for profitable production of advanced biofuels may be ~70% of theoretical yields of sugar to biofuels. Furthermore, considering the fact that anaerobic fermentations usually consume ~10% of carbohydrates for cell mass synthesis (Zhang and Lynd, 2003, 2005) while aerobic fermentations consume 50–90% of carbohydrates, it is clear that the production of advanced biofuels from fermentable carbohydrates must be conducted by microbes under anaerobic or micro-aerobic conditions or by resting microbes.

Table I presents a comparison between microbial fermentation and SyPaB based on several factors related to industrial production. SyPaB gives a yield of the desired product that is often very close to a theoretical value (100%) when the reactions are irreversible or the products can be removed in situ. Most times, microbial fermentations have low or modest yields since a significant fraction of carbohydrate is used for duplication of cells and formation of side-products. Ethanol fermentation may be among the most efficient microbial fermentations, with yields close to 90–95% of a theoretical yield, depending on the microbes and cultivation conditions, especially where cells can be recycled for decreasing the formation of cell mass. On the other hand, it is difficult for microbial butanol fermentation to reach a yield of more than 90% (Atsumi et al., 2008; Connor and Liao, 2009) possibly due to strong product inhibition and the large amount of cell mass synthesized to lessen product inhibition.

Table I. Comparison of cell-free fermentation (synthetic pathway biotransformation, SyPaB) and microbial fermentation.

Features	SyPaB	Microbial fermentation
Product yield	Theoretic or high[a]	Low or modest
Product titer	High	Low or modest
Productivity	High	Low or modest
Process control	Easy	Modest or hard
Reaction conditions	Broad	Narrow
Product purity	High	Low

[a]If the reactions are irreversible or products are removed in situ.

Much higher productivity is believed to be obtained for SyPaB than for microbial fermentation because (i) no cell membrane or wall is present to slow down the substrate/product transport, (ii) no energy is needed for transport of substrate/product across the membrane, and (iii) much higher concentrations of biocatalysts can be present in the reactors because of the absence of most cellular proteins and other biomacromolecules. Recently, production rates of hydrogen by SyPaB have been increased by nearly 20-fold through enzyme ratio optimization and higher substrate concentration (Ye et al., 2009). It is expected that another 1,000-fold increase could be implemented using higher temperatures, higher enzyme loading, higher substrate concentration, optimization of the key enzyme ratio, higher specific activity enzymes, metabolite channeling, etc. (Zhang, 2009). A kinetic model has predicted that ethanol productivity by SyPaB could be higher than microbial fermentation by using the same protein concentration (Allain, 2007). Comparison of power densities generated by enzymatic biofuel cells and by microbial biofuel cells clearly suggests that cell-free enzymatic systems can have 1 or 2 orders of magnitude higher productivities (power densities) than microbial systems (Cooney et al., 2008).

High concentrations of fermentation products (e.g., alcohols and organic acids) inhibit microbial fermentations, resulting in low product titers, which are accompanied by high separation costs. Such inhibitions are usually attributed to cell membrane dissociation effects from alcohols or undissociated acids (Demain et al., 2005; Ingram and Buttke, 1984). In principle, enzymes can tolerate higher levels of organic solvents than can the microbial hydrophobic membranes. For example, fungal cellulase loses only 20% of its activity at 200 g/L ethanol or 100 g/L butanol (Holtzapple et al., 1990). Enzyme-mediated catalysis is often conducted in the presence of organic solvents or in a non-aqueous phase (Hudson et al., 2005; Koeller and Wong, 2001; Lee and Dordick, 2002; Schmid et al., 2000; Serdakowski and Dordick, 2008). In addition, protein engineering has been used to improve enzyme performance in organic solvents (Arnold, 1990). For example, the activity of subtilisin E in 20% (v/v) dimethylformamide solution has been improved 10-fold through mutagenesis of two amino

acids (Chen and Arnold, 1991). The specific activity of cytochrome P450 BM-3 monooxygenase in 25% (v/v) dimethyl sulfoxide has been improved sixfold (Wong et al., 2004). Thus, SyPaB is believed to work better than microbes in the presence of high-titer (inhibitive) products.

SyPaB can be controlled more easily than can fermentation because the latter has complicated feedback control loops for gene regulation, protein transcription, translation, and regulation of enzyme activities. For example, a typical acetone–butanol–ethanol microbial fermentation involves acidogenic and solventogenic stages (Jones and Woods, 1986).

SyPaB is conducted under much broader reaction conditions than microbial fermentation because microbial physiology and metabolism are very sensitive to environmental changes. Enzymes, especially those discovered from extremophiles, can tolerate more severe conditions, such as very low or high temperatures, low or high pH, high concentration organic solvents, or high salts (Antranikian et al., 2005; Auernik et al., 2008; Blumer-Schuette et al., 2008; van den Burg, 2003). Furthermore, the recombinant DNA technologies that can modify microbial hosts are limited to a small number of microorganisms, such as *E. coli*, *Bacillus subtilis*, and *S. cerevisiae*, and are not available for most microorganisms (Demain, 2009; Henstra et al., 2007; Tyurin et al., 2004).

Cost Analysis and Perspectives

The large difference between microbial fermentation and SyPaB is that microbes can duplicate themselves but enzymes cannot. Since microbes can self-duplicate, there are no extra costs associated with biocatalyst separation and stabilization of enzymes and cofactors. By contrast, SyPaB requires enzyme production, purification, and stabilization of enzymes as well as the addition of costly coenzymes.

Here we analyze whether SyPaB would compete with microbial fermentations based on costs of biocatalysts. The cost for producing microbes (C_M) can be estimated based on

$$C_M = \frac{F_M C_S}{Y^M_{X/S}} \tag{6}$$

where F_M is a cost correction coefficient for fermentation relative to sugar, ranging from nearly 1 to very large, C_S is the cost of sugar ($ per kg of sugar); and $Y^M_{X/S}$ is the cell mass yield based on sugar (kg cell mass per kg sugar), being ~0.1 for anaerobic fermentations for the production of biocommodities.

The production cost of stabilized purified enzymes (C_E) can be calculated based on

$$C_E = \frac{F_M C_S}{Y^E_{X/S} Y_{E/X}} F_P F_S \tag{7}$$

where $Y_{E/X}$ is the yield of desired enzyme based on microbe mass (kg enzyme per kg cell mass); $Y^E_{X/S}$ is the cell mass yield based on sugar (kg cell mass per kg sugar), being ~0.5 for aerobic fermentations for the production of desired enzymes; F_P is a coefficient for purified relative to crude enzyme, ranging from 1 to higher; and F_S is a coefficient for stabilized to free enzyme, ranging from 1 to 10 or higher.

For over-production of intracellular proteins, values of $Y_{E/X}$ vary from less than 0.1 to 0.4. For example, we have over-produced a recombinant hyperthermostable 6-phosphogluconate dehydrogenase in *E. coli* with a $Y_{E/X}$ of 0.15, that is, accounting for 30% of the intracellular *E. coli* protein (Wang and Zhang, 2009b). For the production of extracellular proteins (cellulase and amylase), $Y_{E/X}$ could be much higher than 0.3 or even more than 1.0, suggesting relatively low production costs for secretory proteins, such as cellulases, amylases, or proteases (Zhang et al., 2006, 2008).

Weight-based total turnover number (TTN_W) can be calculated in terms of kilogram of product per kilogram of biocatalyst

$$TTN_W = \frac{\text{product}}{\text{biocatalyst}} \tag{8}$$

Figure 6 shows typical values of TTN_W. In general, microbial fermentations have low TTN_W values from less than 0.001 to up to 20. For example, ethanol fermentation (~90% of sugars to ~45% of ethanol and 8% of cell mass) results in a $TTN_W = 6$. When yeast cells are recycled, less cell mass is generated and more ethanol is produced (Wheals et al., 1999), leading to higher TTN_W values (e.g., 10–20). The high-yield ethanol-producing microorganism *Z. mobilis* ZM4 has a TTN_W value of up to 27 (Gunasekaran and Raj, 1999). For the production of single cell protein, TTN_W is essentially one because cells are both the product and the

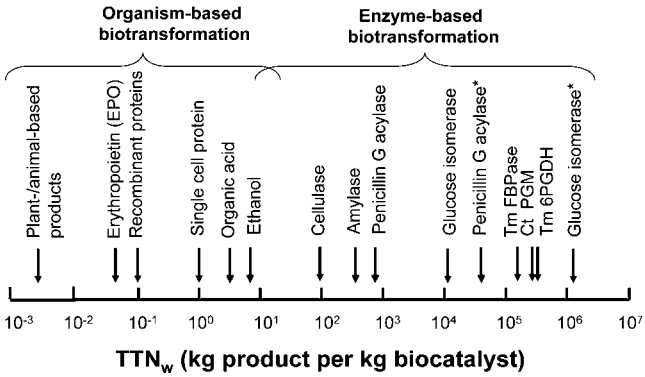

Figure 6. Comparison of weight-based total turnover number (TTN_W). Asterisk (*) denotes immobilized enzymes. Ct PGM, *C. thermocellum* phosphoglucomutase (Wang and Zhang, 2009c); Tm 6PGDH, *T. maritima* 6-phosphogluconate dehydrogenase (Wang and Zhang, 2009b); Tm FBPase. *T. maritima* fructose-1,6-bisphosphatase.

biocatalyst. When plants or animals are used to produce the desired products, the products account for a small fraction of the total weight of biocatalysts, resulting in very low TTN_W values (e.g., ~0.01 or lower).

By contrast, biotransformations mediated by enzymes usually have higher values for TTN_W, from 50 to more than 1,000,000. The values of free enzyme are usually several orders of magnitude lower than those of immobilized or stabilized enzymes because the latter can be stabilized for a long reaction time and can be recycled efficiently (Cao et al., 2003; Sheldon, 2007; Zhang, 2009). Enzymatic hydrolysis of pretreated lignocellulosic biomass without enzyme recycling has low TTN_W values ranging ~50–100 (i.e., 1 kg of glucose produced from cellulose needs 0.01–0.02 kg of cellulase or ~50–100 g of cellulase per gallon of cellulosic ethanol produced) (Taylor et al., 2008; Zhang et al., 2006; Zhu et al., 2009). Starch hydrolysis requires much lesser amounts of amylase and glucoamylase, resulting in TTN_W of ~500. Recently, we have succeeded in increasing the TTN_W value of enzymatic cellulose hydrolysis to ~1,500 by increasing substrate reactivity and reducing enzyme loadings by 15-fold (Sathitsuksanoh et al., 2009, 2010). One kg of immobilized glucose isomerase and immobilized penicillin-G amidase can produce 11,000 kg of fructose and 600 kg of 6-aminopenicillanic acid, corresponding to their TTN_W values of 11,000 and 600, respectively (Tischer and Kasche, 1999; Vasic-Racki, 2006). Considering the fact that the immobilized enzymes account for about 1% of the mass weight of biocatalysts (Cao et al., 2003), the TTN_W values are approximately 1,100,000 and 60,000 for pure glucose isomerase and penicillin-G amidase, respectively. When TTN_W values reach the levels of 1,000,000, there is no cost motivation to increase TTN_W of a single enzyme biocatalysis. Recently, we have obtained a highly active phosphoglucomutase ($V_{max} = 880$ μmol of product per milligram of the enzyme per second) with a half lifetime of 72 h at 60°C (i.e., $TTN_W = 415{,}000$) (Wang and Zhang, 2010). Another recombinant hyperthermostable 6-phosphogluconate dehydrogenase ($V_{max} = 320$ μmol of product per milligram of the enzyme per second, $T_{1/2} = 140$ h at 80°C) has a TTN_W value of 420,000 (Wang and Zhang, 2009b). Much higher TTN_W values of these thermos table enzymes are expected after immobilization, like those of immobilized thermophilic glucose isomerase (Vasic-Racki, 2006).

A cost ratio (R) between SyPaB and microbial fermentation based on the product relative to the biocatalyst can be calculated as follows:

$$R = \frac{\text{SyPaB}}{\text{MF}} = \frac{TTN_W^E/(C_E n)}{TTN_W^M/C_M} = \frac{TTN_W^E Y_{X/S}^E Y_{E/X}}{TTN_W^E Y_{X/S}^M (F_P F_S n)} \quad (9)$$

where n is the number of enzymes involving the SyPaB. When R values are greater than 1, it suggests that SyPaB has comparative advantages over microbial fermentation based on biocatalyst cost. The larger the R values, the smaller the costs of SyPaB.

Taking enzymatic hydrogen production by SyPaB as an example, the R value would be 1.8 with the assumptions that (i) the TTN_W values are 20 and 20,000 for microbe and enzymes, respectively; (ii) $Y_{E/X}^E = 0.1$, a typical value for recombinant protein production; and (iii) a combination of enzyme purification and stability, $F_P F_S = 20$. $R = 1.8$ means that the costs of stabilized enzyme cocktails would account for only 56% of the cost of microbes as biocatalysts. Clearly, SyPaB requires more costs for enzyme purification and stabilization but has advantages for at least 3 orders of magnitude higher TTN_W values plus neither synthesis of unnecessary biomacromolecules nor formation of side-products. The above comparison is based on an assumption that the future microbes can produce nearly 12 mol of hydrogen per mole of glucose, threefold higher of the theoretical yield of dark anaerobic fermentations. In fact, the production costs of cell-free SyPaB would be far less than that of developing hydrogen-producing microbes (Maeda et al., 2008; Veit et al., 2008).

It is vital to estimate carbohydrate allocation between the products and biocatalysts, all of which are made from fermentable carbohydrates through fermentations. $Y_{X/S}^M = \sim 0.1$ for anaerobic fermentations, that is, approximately 10% of the carbohydrate is allocated to synthesis of microbial cell mass and ~90% to formation of products. For aerobic fermentations for enzyme production, $Y_{X/S}^E = \sim 0.5$. By using the assumptions of $TTN_W^E = 20{,}000$, $Y_{E/X}^E = 0.1$, and $n = 14$ as above, approximately 1.4% of carbohydrate is allocated to synthesis of the enzymes and ~98% of carbohydrate to the final products.

The above analyses were based on relatively conservative assumptions for the values of TTN_W, $Y_{E/X}^E$, and $F_P F_S$. First, TTN_W values can be enhanced greatly to 1,000,000 or higher, the same level as industrial glucose isomerase by (i) discovery and utilization of stable wild-type enzymes (Blumer-Schuette et al., 2008; Danson et al., 1996; Razvi and Scholtz, 2006; Unsworth et al., 2007; Vieille and Zeikus, 2001; Wang and Zhang, 2009b, 2010), (ii) engineering wild-type enzymes to greater stability and activity (Bornscheuer and Pohl, 2001; Chautard et al., 2007; Given et al., 1998; Liu et al., 2009; Roodveldt et al., 2005; Rubingh, 1997), (iii) immobilization on traditional materials or nanomaterials (Bornscheuer, 2003; Govardhan, 1999; Kim et al., 2005; Lee et al., 2007; Polizzi et al., 2007), and (iv) formulation of enzyme buffers (Fedunová and Antalík, 2006; Wang and Zhang, 2010). Stabilization of one enzyme or multiple enzymes on solid supporters is a well-known technology. With the rapid development of nanomaterials with much larger surface areas (i.e., more enzymes can be immobilized), ultra-stable immobilized enzymes have been reported to be active for several months or years (Bornscheuer, 2003; Lee et al., 2005, 2007; Polizzi et al., 2007; St. Clair et al., 2000; Vasic-Racki, 2006).

Second, the $Y_{E/X}^E$ value can be increased from 0.1 to 0.3 or higher through (i) the production of over-expressed recombinant enzymes (Barnard et al., 2004; Hartley, 2006; Sørensen and Mortensen, 2005; Wang and Zhang, 2009b),

(ii) the use of high-cell density fermentation plus low-cost fermentation media (Barnard et al., 2004; Shiloach and Fass, 2005), and (iii) the production of high-level secretory enzymes (Choi and Lee, 2004; Hong et al., 2008b; Zhang et al., 2006).

Third, the combined cost coefficient of enzyme purification and immobilization ($F_P F_S$) can be decreased from 20 to 5 or lower by (i) simple adsorption/desorption (Barnard et al., 2005; Hong et al., 2008a,b) or simple heat precipitation (Wang and Zhang, 2009b), (ii) direct immobilization of dead microbes that contain active enzyme (Bhosale et al., 1996), and (iii) low-cost immobilization technologies (Mateo et al., 2001; Pessela et al., 2003).

Based on the potentials for improved $TTN_W = 1{,}000{,}000$, $Y^E_{E/X} = 0.3$, and $F_P F_S = 5$, the R value is expected to increase to 1,000, that is, the enzyme costs for SyPaB would be 0.1% of the cost of microbial biocatalysts and the carbohydrate allocation to the enzymes would be lower than 0.1%. Therefore, the development of low-cost stable enzyme building blocks as off-the-shelf, ready-to-use enzymes will be one of the top research topics for in vitro synthetic biology projects. Future enzymes or enzyme complexes could be ready for different designs, like assembling of different standardized computer parts to build a customized computer.

In addition to building blocks (enzymes or their complexes), labile cofactors may be another bottleneck for the production of biocommodities. Living biological systems regenerate or resynthesize NAD(P)H, relying on their complete enzyme apparatus of the cells (Noctor et al., 2006; Pollak et al., 2007), while costly NAD(P) must be kept balanced and recycled for cell-free SyPaB. NAD(P)H are known to be unstable in acid solutions due to a general acid-catalyzed hydration reaction (Johnson and Tuazon, 1977; Wu et al., 1986). NAD(P)H is more stable in basic than in acidic solution, and NAD(P) is more stable in acidic than in basic solutions (Wong and Whitesides, 1981). Since NADPH is generally less stable than NADH (Wong and Whitesides, 1981; Wu et al., 1986) and the former is more costly (Woodyer et al., 2003), some efforts have been made to change the enzymes' preferences from NADPH to NADH (Banta and Anderson, 2002; Banta et al., 2002a,b; Sanli et al., 2004).

The previous prejudice that the costs of labile cofactors are so high that cofactor-dependent cell-free biochemical reactions cannot be carried out economically has been discarded in the pharmaceutical industry for the production of high-value chiral compounds (Chartrain et al., 2001; Moore et al., 2007; Wandrey, 2004; Wichmann et al., 2000; Wildeman et al., 2007). Two typical NAD(P)H-recycling techniques are entrapped cofactors by membrane technologies or immobilized cofactors (Betancor et al., 2006; Johannes et al., 2007; Liu and Wang, 2007). For example, NAD linked with polyethelene glycol has been regenerated more than 80,000 and 600,000 times for production of L-leucine and L-phenylalanine, respectively (Hummel et al., 1987; Kragl et al., 1996; Wichmann and Vasic-Racki, 2005). The total turnover number for NAD has been reported to be more than 1 million for chemical synthesis in organic solvents (Kazandjian and Klibanov, 1985). In the pharmaceutical industry, costs of NAD(P) for the synthesis of chiral compounds have been decreased to negligible levels relative to high-value products (Chartrain et al., 2001; Moore et al., 2007; Wildeman et al., 2007).

In addition to recycling costly natural NAD(P) cofactors, another promising direction is replacing NAD(P) with more stable and low-cost artificial biomimetic NAD(P) analogs (Ansell and Lowe, 1999; Lo and Fish, 2002; Ryan et al., 2008). Lowe and his coworkers have developed a range of NAD(P)H analogs based on the structure of a triazine dry template (Ansell and Lowe, 1999; Ansell et al., 1997a,b, 1999; Burton et al., 1996). These biomimetic redox coenzymes with apparently minimal structural similarity to the natural NAD are inexpensive to synthesize (Ansell and Lowe, 1999). The most active biomimetic cofactor is Nap 1 with a k_{cat} value of an order of magnitude lower and a K_m value of 2 orders of magnitude higher relative to native NAD. *N*-benzyl-1,4-dihydrononicotinamide and its derivatives, which are somewhat structurally similar to natural NAD, represent another type of biomimetic NADH analogs, which have been utilized with wild-type enzymes (Fish et al., 2004; Lo and Fish, 2002; Lutz et al., 2004). Cytochrome P450, a heme-containing enzyme, has been engineered for improving its performance on these NADH biomimics (Ryan et al., 2008). It is expected that further engineering wild-type oxidoreductases would increase their catalytic efficiencies based on economically advantageous and more stable biomimetic cofactors.

Considering an NAD price of $28/mmol (Sigma, 2009 catalog), a 20-fold reduction of NAD price due to mass production and demand, engineering two dehydrogenases and hydrogenase with the substrate preferences from NADP to NAD, and a TTN of 1,000,000 for NAD, cofactor costs would account for $0.70 per kg of hydrogen. Including the costs of stabilized enzymes and carbohydrate ($1.5 per kg of hydrogen), hydrogen generation by SyPaB would be competitive with that made from natural gas ($2.70 per kg of hydrogen). Considering the benefits associated with the use of biomass carbohydrates, decreasing greenhouse gas emissions, and higher fuel prices for mobile applications, enzymatic generation of hydrogen/electricity will be more competitive after full development. Further cost reductions in the use of biomimetic cofactors, engineered dehydrogenases, and recombinant hydrogenase would pave the way to ultra-low cost production of hydrogen from renewable carbohydrates. It is estimated that ultimate hydrogen production costs could be as low as $1.5 per kg based on sugar costs of $0.18 per kg.

Closing Remarks

The concept of cell-free SyPaB is not brand new. But the production of biocommodities and bioelectricity by SyPaB

is an emerging frontier because low-value products require careful pathway designs with a balance of ATP and NAD(P), low-cost stabilized enzymes, recycled coenzymes, biomimetic coenzymes, and low-cost substrates. SyPaB featuring very high product yields and great potentials for drastically decreasing production costs presents a future platform for ultra-low-cost production of biocommodities and bioelectricity.

Two major obstacles for SyPaB are the lack of stable building blocks (enzymes or enzyme complexes) as standardized parts and instability of costly coenzymes. Two of the most important lessons in the modern world are standardization and mass production, as has occurred with cellular phones, digital cameras, personal computers, railroad gauges, screw threads, and so on. Strong motivations have driven intensive efforts in discovering, engineering, and producing thermostable enzymes, such as DNA polymerase, amylase, glucose isomerase, cellulase, and so on (Adams and Kelly, 1998; Auernik et al., 2008; Blumer-Schuette et al., 2008; van de Werken et al., 2008; Zhang et al., 2006). These enzymes are affordable for most users. Before the potential of SyPaB for biocommodity production was recognized, limited efforts had been made to clone, characterize, and produce thermostable building blocks associated with catabolic pathways (Wang and Zhang, 2009b, 2010) and develop biomimetic cofactor analogs and their associated enzymes (Ansell and Lowe, 1999; Lo and Fish, 2002; Ryan et al., 2008). After the concept of SyPaB is accepted and more stable enzyme building blocks and biomimetic cofactor analogs are available, cell-free SyPaB will eventually compete with microbial fermentation for the production of low-value biocommodities.

Another important research direction for SyPaB is to increase reaction rate (productivity). This can be addressed via optimization of enzyme components by kinetic modeling, metabolic flux analysis, metabolic control analysis, high substrate concentration, high enzyme loading, and high reaction temperatures by using thermophilic or even hyperthermophilic enzymes (Zhang, 2009; Zhang et al., 2007). For enzymatic hydrogen production projects, we have increased the reaction rate by nearly 20-fold during the past 2 years (Ye et al., 2009; Zhang et al., 2007), and we expect at least a 1,000-fold increase over the next several years (Zhang, 2009). A similar example is more than 1,000,000-fold increase in power densities of microbial fuel cells during the past decade, which has been achieved through the intensive efforts of numerous laboratories (Logan and Regan, 2006).

Nomenclature

C_S	cost of sugar ($ per kilogram of sugar, e.g., $0.18/kg)
F_M	cost correction coefficient for fermentation relative to sugar
F_P	coefficient for purified relative to crude enzyme
F_S	coefficient from stabilized to free enzyme
R	cost ratio of SyPaB to microbe, based on the product relative to biocatalyst cost
TTN	total turnover number (mole catalysis per mole biocatalyst)
TTN_W	total turnover number based on weight (kilogram product per kilogram of biocatalyst)
$Y_{E/X}$	enzyme yield based on microbe mass (kilogram enzyme per kilogram cell mass)
$Y^M_{X/S}$	cell mass yield based on sugar (kilogram cell mass per kilogram sugar) in anaerobic fermentation for producing biocommodities
$Y^E_{X/S}$	cell mass yield based on sugar (kilogram cell mass per kilogram sugar) in aerobic fermentation for producing enzymes

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