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PERSPECTIVE

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Simpler Is Better: High-Yield and Potential Low-Cost Biofuels Production through Cell-Free Synthetic Pathway Biotransformation (SyPaB)

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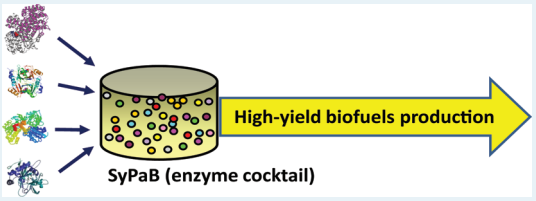
ABSTRACT: The production of biofuels from renewable sugars isolated from plants or produced through artificial photosynthesis would provide a sustainable transportation fuel alternative for decreasing reliance on crude oil, mitigating greenhouse gas emissions, creating new manufacturing jobs, and enhancing national energy security. Since sugar costs usually account for at least 50% of biofuels' selling prices, it is vital to produce desired biofuels with high product yields and at low production costs. Here I suggest high-product yield and potentially low-cost biofuels production through cell-free synthetic enzymatic pathway biotransformation (SyPaB) by in vitro assembly of stable enzymes and (biomimetic) coenzymes. SyPaB can achieve high product yields or high energy efficiencies that living entities cannot achieve. Great potentials of SyPaB, from chiral compounds, biodegradable sugar batteries, sulfur-free jet fuel, hydrogen, sugar hydrogen fuel cell vehicles, high-density electricity storage, to synthetic starch, are motivation to solve the remaining obstacles by using available technologies, such as protein engineering, enzyme immobilization, unit operations, and technology integration. The biotransformation through in vitro assembly of numerous enhanced-performance and stable enzymes in one bioreactor that can last a very long reaction time (e.g., several months or even years) would be an out-of-the-box solution for high-yield and low-cost biofuels production.



KEYWORDS: artificial photosynthesis, biofuels, biological CO_2 fixation, hydrogen, in vitro synthetic biology, biocatalysis and biotransformation, synthetic pathway biotransformation (SyPaB)

1. INTRODUCTION

Biofuels are usually defined as transportation fuels produced from biological resources (e.g., corn kernels, sugar cane, lignocellulosic biomass, and algal biomass) and/or through biological conversions. As compared to the other energy consumption sectors (e.g., industrial, residential, and commercial), transportation fuels that account for approximately 20% of total energy consumption have some special requirements: high energy storage capacity in a small container (e.g., ~50 L), high power output (e.g., ~20−100 kW per vehicle), affordable fuel costs (e.g., $~20−30/GJ), affordable vehicles, low costs for rebuilding the relevant infrastructure, fast charging or refilling of the fuel (e.g., several min per time), safety, and so on.[1−3] Currently, approximately 95% transportation fuels are produced from crude oil. Concerns of depleting crude oil reserves, climate change, national energy security, and wealth transfer are driving the search for sustainable transportation fuel alternatives.[1,3,4]

The production of chemicals mediated by biocatalysts usually has numerous advantages over chemical catalysis, such as higher energy efficiency, higher chemical selectivity (i.e., higher product yield), more modest reaction conditions, and lower costs of bioreactors.[5−7] Different scenarios of biofuels production have been proposed starting from plant biomass, algal biomass, or even CO_2 plus hydrogen or electricity, but nearly all biofuels (secondary energies) originate from the most abundant primary energy—solar energy. Since carbohydrates (e.g., cellulose, hemicellulose, and starch) are the most abundant renewable bioresource (e.g., ~100 billion tons per year), biofuels production through carbohydrates would become a dominant platform in the future. The scope of this perspective is restricted to compare two different biocatalysts, living entities and synthetic cascade enzymes, for the production of the best future biofuel, namely, hydrogen, and the production of synthetic starch from CO_2 but is not involved in bioenergy plants, cellulase engineering, other biofuels production, and algal biofuels. (Note: hydrogen is believed to be the best biofuel in the future because (i) it can be utilized through fuel cells featuring higher energy efficiencies compared to internal combustion engines, (ii) less pollutants are produced, and (iii) it can be produced from diverse energy sources.)

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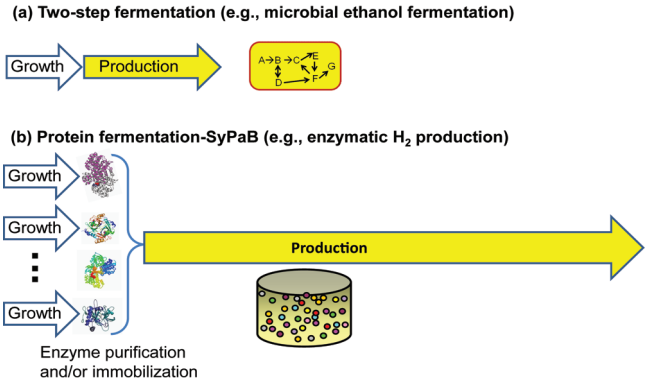

Figure 1. Comparison of microbial two-step biofuels production (a) and a hybrid of microbial fermentation for bulk enzyme production and cell-free synthetic enzymatic biotransformation (SyPaB) (b). Arrows represent microbial fermentation or biotransformation.

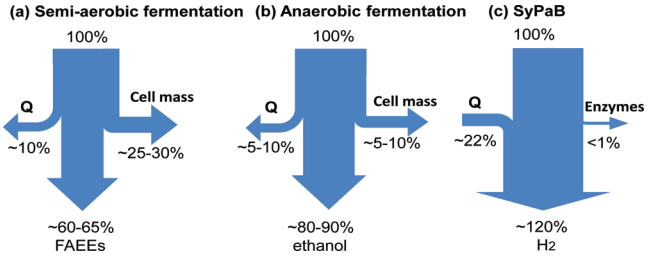

Figure 2. Energy efficiency comparison for fatty acid ethyl esters fermentation by semiaerobic fermentation (a), ethanol fermentation by anaerobic fermentation (b), and hydrogen production by SyPaB (c).

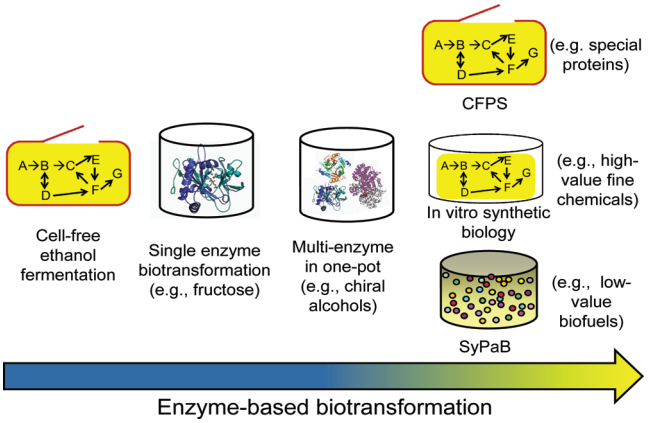

Figure 3. Evolution of enzyme-based biotransformation from cell-free ethanol fermentation (discovery of enzymes), single enzyme biotransformation, multiple-enzyme one pot to cell-free protein synthesis, in vitro synthetic biology, and cell-free synthetic pathway biotransformation in terms of time and increasing system complexity.

Biofuels production R&D is typical of goal-oriented projects with numerous constraints from economical, technological, environmental, social, scalability, competing technologies, and so on. Although so many advanced biofuels, including cellulosic ethanol, long-chain alcohols (e.g., *n*-butanol, iso-butanol), fatty acid ethyl esters, hydrogen, electricity, methane, bioalkanes, and so on, have been produced in laboratories, most of them might not be produced economically in the future. In industrial processes, three key elements—product yield, product titer, and reaction rate—mainly decide process economy. For biofuels production based on available sugars, energy conversion efficiency (or product yield) must be the No.1 cost factor because sugar costs usually account for ~50−70% of prices of mature biofuels.[8−11] The second important factor is product titer, which is closely associated with separation costs, followed by production rate. In it, a default assumption is that reasonable biofuels production rates have been or will be accomplished, for example, ~0.2−1 g sugar consumed per liter per h.

Biofuels can be produced from sugars mediated by (i) growing microbes, (ii) resting cells that are not active in the process of cell division, and (iii) cascade enzymes. When some constituent of cells (e.g., fatty acids) is a desired product, the formation rates of such product are directly related to the rates of cell growth, called growth-associated production. At this situation, growing microbes insist on metabolizing sugars for anabolism (i.e., allocation of sugars to other cell components). Therefore, practical product yields are far below from their theoretical yields.[8,12,13] From the point of view of synthetic biology, both cell growth and undesired product formation by living entities are a dissipation of the task that we want them to do, resulting in relative low product yields. To increase biofuels yields, it is vital to insulate basic anabolism from biofuels production (Figure 1a). In practice, industrial ethanol fermentations are usually conducted in two steps. At the first step, yeasts consume sugars to produce a large amount of cell mass with oxygen supplies; at the second step, yeasts produce high-yield ethanol from glucose in the absence of oxygen. When ethanol titer is high, it can stop yeast growth so that yeasts turn to resting cells that produce ethanol only without significant synthesis of cell mass[14] (Figure 1a). Here I extended the concept of high-yield resting cell biotransformation to high-yield cell-free synthetic pathway biotransformation (SyPaB) that can implement complicated biochemical reactions by the in vitro assembly of numerous enzymes and coenzymes.[9,15−17] In SyPaB, the insulation of cell growth from product formation is implemented by process operations (Figure 1b).

An analysis based on thermodynamics and bioenergetics was conducted for assessing upper limits of energy efficiency for the production of fatty acid ethyl esters (FAEEs) by semiaerobic fermentation, ethanol by anaerobic fermentation, and hydrogen by SyPaB (Figure 2).[8] According to their biochemical pathways and thermodynamics, 100% product yields result in ~10% combustion energy loss for FAEEs, ~5% loss for ethanol, and ~22% gain for hydrogen.[8,18,19] Since a fraction of sugar must be consumed for biocatalyst synthesis, potential yields of biofuels should be lower than their theoretical yields. An imbalance of coenzymes in microbial FAEEs production leads to a significant fraction of carbohydrate loss for the synthesis of cell mass in semiaerobic fermentation. As a result, only 60−65% of the combustion energy in sugar would be converted to FAEEs. Ethanol fermentation has much better energy-retaining efficiencies because of (i) anaerobic fermentation and (ii) uncoupling of cell growth and product formation. Therefore, ethanol is a very good liquid biofuel now. The best case would be hydrogen produced by SyPaB because of 22% of extra enthalpy gain by absorbing waste heat (i.e., the combustion energy of hydrogen is more than that of sugar) and a very small amount of sugar consumed for the synthesis of cascade enzymes when enzymes

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have total turnover number (TTN) values of 10^7-10^8 mol product per mol enzyme.[8,15,20]

In this perspective, I present an out-of-the-box solution for a high-yield and potentially low-cost biofuels production platform, SyPaB, featuring very high product yields and fast reaction rates that can insulate protein synthesis from biofuels production, review the brief history of enzyme-based biotransformations, argue SyPaB as a new low-cost biomanufacturing platform, and discuss challenges and opportunities of SyPaB.

2. HISTORY OF ENZYME-BASED BIOTRANSFORMATIONS

Long before people had a clue about the nature of biotransformation, certain properties of microorganisms had been long exploited for commercial processes, such as in the production of beer, wine, vinegar, soy sauce, and cheese, and the preservation of vegetables by pickling. Central to the rational use of biocatalysts has been a stream of theoretical understanding of the nature of living biocatalysts and related enzymes. The developments of enzyme-based biotransformations (Figure 3) can be divided roughly into four phases:

Phase 1 (1897). Recognition of biotransformation occurrence in the absence of living cells (cell-free ethanol fermentation by Eduard Bucher, Nobel Prize in Chemistry, 1907).[9] Later, more studies were focused on studies of enzymes responsible for natural enzymatic pathways in basic metabolisms. For example, Otto Fritz Meyerhof won the Nobel Prize in Physiology or Medicine in 1922 for his elucidation of the glycolytic pathway.[21] Even now, in vitro reconstitution of natural pathways is still an important tool to understand and discover in vivo complicated biochemical reactions or pathways.[22,23]

Phase 2 (1960s). Utilization of one enzyme for simple biotransformation.[5,24] Clearly, the use of isolated enzymes for the production of chemicals has a much shorter history than microbial fermentation. Invertase may be the first immobilized enzyme used commercially for the production of Golden Syrup by Tate & Lyle in World War II. Industrial process for L-amino acid production by soluble aminoacylase was developed in 1954. In 1969, Tanabe Seiyaku Co. (Japan) started the industrial production of L-methionine by using immobilized aminoacylase in a packed bed reactor. In 1967, the Clinton Corn Processing Company (U.S.A.) was the first to produce fructose corn syrup by glucose isomerase. Currently, immobilized glucose isomerase Sweetzyme T (Novo, Denmark) is packed into columns for conversion of glucose into fructose. The longest working lifetime of immobilized glucose isomerase is 687 days at 55 °C and pH 7.5 by Kato Kagaku (Japan). Now, annual enzymatic fructose production from glucose exceeds 9 million tons.[24] Enzymatic acrylamide production was initiated in 1985. Currently, more than 100,000 tons of acrylamide is produced by using immobilized nitrile hydratases per year.[24] Discovery and utilization of thermoenzymes, protein engineering including directed evolution, rational design and their combination, high-cell density fermentation for low-cost recombinant protein production, and enzyme immobilization have enabled the production of very stable recombinant enzyme at very low costs.[25−28]

Phase 3 (1990s). Utilization of multienzyme one pot for relatively complicated biotransformation because most enzymes can function under similar conditions. Multienzyme one pot has numerous benefits: fewer unit operations, smaller reactor volume, higher volumetric and space-time yields, shorter cycle times, and less waste generated. Also, by coupling steps together, unfavorable equilibria can be driven toward the formation of desired products.[9,29,30] For cofactor-dependent enzyme reactions, it is not economically feasible to continuously provide costly cofactors in biomanufacturing. Therefore, in situ NAD(P)H-regenerated by another enzyme is becoming more and more accepted, especially for the synthesis of high-value chiral compounds in the pharmaceutical industry.[17,31,32] NAD(P)H is usually generated by using a pair of a hydrogen-donor substrate and a single enzyme, including formate/formate dehydrogenase,[33] glucose/glucose dehydrogenase,[34] glucose-6-phosphate/glucose-6-phosphate dehydrogenase,[26] dihydrogen/hydrogenase,[35] and phosphite/phosphite dehydrogenase.[36] In another case, enzymatic hydrolysis of crystalline cellulose require a synergetic action of endoglucanases, cellobiohydrolases, and beta-glucosidases.[37−39] In the organic chemistry field, the synthesis of monosaccharides, activated monosaccharides, oligosaccharides, and glycopeptides by using multienzyme one pot has been intensively investigated.[40−46]

Phase 4 (2000s). Utilization of numerous cascade enzymes for very complicated biotransformation. It includes three representative directions: (1) cell-free protein synthesis (CFPS), which utilizes natural protein synthesis systems in cell lysates for fast synthesis of proteins for research purpose and the production of high-value antibodies or other proteins,[47,48] (2) in vitro synthetic biology for the production of high-value products,[25,49−52] and (3) synthetic pathway biotransformation (SyPaB) for low-value biofuels production.[9,15,17] Different from CFPS and high-value product formation, SyPaB must have balanced cofactors and ATP in vitro.[17] In addition, thermodynamics must be analyzed to ensure designed non-natural processes to take place as expected. The development cycle of SyPaB is composed of five parts: (i) pathway reconstruction, (ii) enzyme selection, (iii) enzyme engineering, (iv) enzyme production, and (v) bioprocess engineering.[9,15,17] Whole SyPaB processes can be improved in an iterative manner, gradually leading to a low-cost industrial bioprocess.[9,15,17] The SyPaB technology has successfully achieved some breakthroughs that neither microbes nor chemical catalysts could implement before, such as production of nearly 12 mol of hydrogen from per mol of anhydroglucose and water,[19,53] ultrahigh-yield regeneration of NAD(P)H in microbe-toxic biomass hydrolysate,[20] enzymatic conversion of ethanol and CO_2 to lactate,[54] and so on.

3. BIOCATALYSTS: LIVING ENTITIES VERSUS SYPAB

Although SyPaB and living entities are responsible for transforming similar-level complicated biochemical reactions, SyPaB featuring high product yields and fast reaction rates enable it to play more important roles in biofuels production because (energy) conversion efficiencies will be important to decide their production economics in a long-term[8] and their reaction rates will be vital to their potential applications.[1,55] Here we present two SyPaB examples, which do much better than do natural living entities.

3.1. Hydrogen Production from Sugars. The global biosphere produces more than 250 million tons of biohydrogen per year.[56] Most hydrogen arises from anaerobic fermentation of carbohydrate previously fixed by photosynthesis, followed by its consumption along with CO_2 or organic acid reduction by methanogenic archaebacteria. On oceanic continental shelves

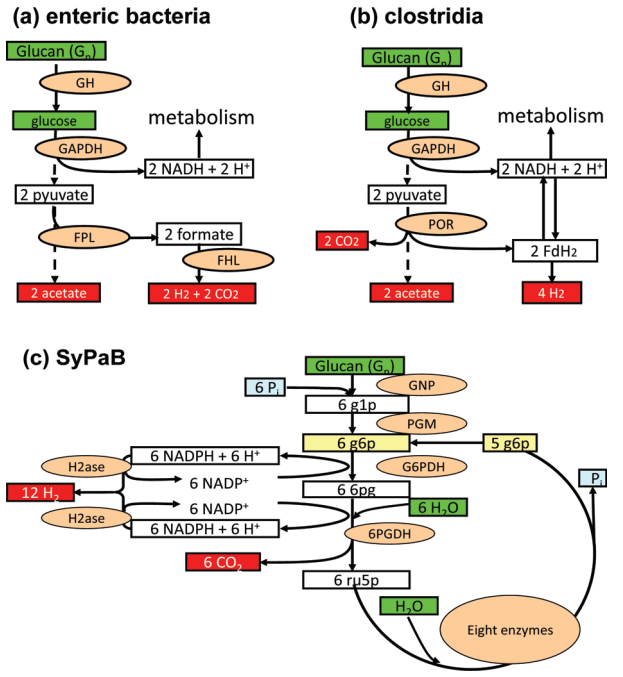

Figure 4. Scheme of microbial hydrogen production by enteric bacteria (a), clostridia (b), and by SyPaB (c).

and in permafrost regions, methane has been accumulated extensively as methane hydrate deposits, which exceed petroleum, coal, and natural gas deposits combined. In other locales, methane is released to the atmosphere, a much stronger greenhouse gas than carbon dioxide.

Natural microorganisms can produce hydrogen from sugars through different pathways.[38,57−59] The upper limit of hydrogen yield for living entities is 4 mol of H_2 produced per mol of glucose equivalent consumed plus 2 acetic acids, called the Thauer limit (eq 1).[58,59]

$$C_6H_{12}O_6(aq) + 2H_2O(l) \rightarrow 4H_2(g) + 2CO_2(g) + 2C_2H_4O_2(l) \quad (1)$$

Enteric microorganisms include facultative anaerobic bacteria, such as *Enterobacter aerogenes, Enterobacter cloacae,* and *Escherichia coli*. Since they can grow under aerobic conditions for high-cell mass concentrations, such high-cell masses result in extraordinarily high volumetric H_2 productivities. However, specific H_2 yields of enteric bacteria are relatively low, usually less than two mol of H_2 per mol of glucose because of their central metabolism, where hydrogen is generated from pyruvate−formate lyase and formate hydrogen lyase[60] (Figure 4a). NADH is generated from the glycolytic pathway, but this coenzyme is not a favorable electron carrier for hydrogen generation at moderate temperatures. The clostridia are obligate anaerobes capable of producing organic solvents as well as H_2 through a mixed acid pathway (Figure 4b). These microorganisms are the predominant organisms in mixed microflora capable of producing H_2 from biomass waste treatment.[61] Different from enteric bacteria, H_2 production by many clostridia species is catalyzed by the combination of pyruvate:ferredoxin oxidoreductases (POR) and Fe-only hydrogenase.[62] NADH can donate electrons to ferredoxin by NADH:ferredoxin oxidoreductase.[38] Several clostridial species have been evaluated for their potential as biohydrogen producers.[61] Hyperthermophiles can produce nearly 4 mol of H_2 produced per mol of glucose equivalent, albeit at lower volumetric productivities than observed for mesophilic bacteria.[57] In spite of intensive efforts in metabolic engineering and synthetic biology, none of natural or engineered microorganisms can produce hydrogen more than the Thauer limit.[57,63−66]

To break the Thauer limit, a non-natural synthetic pathway has been designed to split water by using the chemical energy in starch.[19] As a result, far more than 4 mol of hydrogen per mol of glucose unit from starch and water is produced.[19]

$$C_6H_{10}O_5(aq) + 7H_2O(l) \rightarrow 12H_2(g) + 6CO_2(g) \quad (2)$$

This non-natural synthetic catabolic pathway is composed of 13 enzymes together (Figure 4c). The pathway contains four biocatalytic modules: (i) a chain-shortening phosphorylation reaction for producing glucose-1-phosphate (g1p) catalyzed by glucan phosphorylase (eq 3); (ii) generation of glucose-6-phosphate (g6p) from g1p catalyzed by phosphoglucomutase (eq 4); (iii) generation of 12 NADPH from g6p through a pentose phosphate pathway plus four enzymes in the glycolysis and gluconeogenesis pathways (eq 5); and (iv) generation of hydrogen from NADPH catalyzed by hydrogenase (eq 6).

$$(C_6H_{10}O_5)_n + P_i \rightleftharpoons (C_6H_{10}O_5)_{n-1} + g1p \quad (3)$$

$$g1p \rightleftharpoons g6p \quad (4)$$

$$g6p + 12NADP^+ + 7H_2O \rightleftharpoons 12NADPH + 12H^+ + 6CO_2 + P_i \quad (5)$$

$$12NADPH + 12H^+ \rightleftharpoons 12H_2 + 12NADP^+ \quad (6)$$

Thermodynamic analysis suggests that the overall reactions from starch or cellulosic materials and water are spontaneous and endothermic (i.e., $\Delta G^\circ = -49.8$ kJ/mol and $\Delta H^\circ = +598$ kJ/mol).[19,53] Such reactions are driven by entropy gains rather than enthalpy losses. These entropy-driven chemical reactions can generate the chemical energy in the form of hydrogen more than the chemical energy in polysaccharides by absorbing ambient-temperature thermal energy.[19,53] The removal of gaseous products, H_2 and CO_2, from the aqueous phase under mild reaction conditions (<100 °C and ~1 atm) favors the unidirectional reactions for the formation of hydrogen.[19,53] Similarly, another entropy-driven bioreaction is $C_2H_4O_2(aq) \rightarrow CH_4(g) + CO_2(g)$ mediated by methanogenesis microorganisms, resulting in 1.7% combustion energy gain. Two spontaneous endothermic chemical reactions are $N_2O_5(s) \rightarrow 2\,NO_2(g) + {}^1/_2O_2(g)$ and $Ba(OH)_2 \cdot 8H_2O(s) + 2NH_4SCN(s) \rightarrow Ba(SCN)_2(aq) + 2NH_3(aq) + 10H_2O(l)$.[9] All of the above entropy-driven reactions involve phase changes from more orderly to less orderly.

3.2. Biological CO_2 Fixation. Carbon dioxide can be biologically fixed by plants, microorganisms, and animals. Most plants fix carbon dioxide by using chloroplasts through the reductive pentose-phosphate cycle, that is, the Calvin−Benson cycle. Microorganisms can fix CO_2 through six pathways: the reductive citric acid cycle,[67] the reductive acetyl-CoA pathway (Wood−Ljungdahl pathway),[68] the 3-hydroxypropionate pathway,[69] the 3-hydroxypropionate-4-hydroxybutyrate cycle, and dicarboxylate-4-hydroxylbutyrate cycle.[70] Sometimes, animal tissues, such

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as liver cells, have been found to fix CO_2 to produce cell constituents (e.g., glycogen).[71] All of natural biological CO_2 fixation pathways require 12 mol of the reduced cofactor (NADPH) or its equivalents plus several mol of ATP for the generation of one mol of glucose from 6 mol of CO_2. The ATP number consumed depends on the pathways in microorganisms and plants, ranging from 2 to 30.[70,72] Here ATP is an extra energy driving force for implementing thermodynamically-unfavorable reactions because most times CO_2 concentrations in the environments are very low. When high concentration of CO_2 is available, the number of ATP consumed per glucose unit may be decreased greatly.

Plant photosynthesis utilizes intermittent low-energy concentration solar energy (e.g., ~170 W/m^2) and fixes CO_2 in the form of carbohydrate. But natural plant photosynthesis has pretty low energy efficiencies from solar energy to chemical energy of 4.6−6.0% (theoretical), ~3−4% (peak), ~1−2% (dedicated crops), and ~0.2−0.3% (global average).[73−75] Such low efficiencies are mainly attributed to four factors: (i) narrow light absorption spectrum by chlorophylls, (ii) unmatched reaction rates between light reactions and dark reactions, (iii) relatively low efficiencies of carbohydrate synthesis, and (iv) carbohydrate losses because of respiration of living entities.[73−76]

To surpass low-efficiency plant photosynthesis for CO_2 fixation, another potential application of SyPaB is to fix CO_2 through a non-natural ATP-neutral high-efficiency pathway[77] (Figure 4, eq 7)

$$8CO_2(g) + 18H_2(g) \rightarrow C_6H_{10}O_5(s) + C_2H_6O(l) + 10H_2O(l) \quad (7)$$

where the inputs are CO_2 and hydrogen; the outputs are water-insoluble amylose (linear starch), volatile ethanol (C_2H_6O), and water.

The hypothetical hydrogen/CO_2-to-carbohydrate process is composed of six biocatalytic modules, including

(1) NADH is generated from hydrogen by using hydrogenase (eq 8)[78,79]

$$18NAD^+ + 18H_2 \rightleftharpoons 18NADH + 18H^+ \quad (8)$$

(2) CO_2 fixation to formaldehyde (CH_2O) mediated by formate dehydrogenase and formaldehyde dehydrogenase (eq 9),[80−82]

$$9CO_2 + 18NADH + 18H^+ \rightleftharpoons 9CH_2O + 9H_2O + 18NAD^+ \quad (9)$$

(3) conversion of formaldehyde to fructose-6-phosphate (f6p) by 3-hexulose-6-phosphate synthase and hexulose phosphate isomerase from the ribulose monophosphate pathway (eq 10),[83,84]

$$9CH_2O + 9ru5p \rightleftharpoons 9f6p \quad (10)$$

(4) ribulose-5-phosphate (ru5p) regeneration by the eight enzymes from the nonoxidative pentose phosphate pathway (eq 11),[19,72]

$$8f6p + 2ATP \rightarrow 9ru5p + g3p + 2ADP \quad (11)$$

(5) ethanol production from glyceraldehydes-3-phosphate (g3p) by the seven enzymes from the glycolysis and ethanogenesis pathway (eq 12),[72]

$$g3p + P_i + 2ADP \rightarrow C_2H_6O + CO_2 + H_2O + 2ATP \quad (12)$$

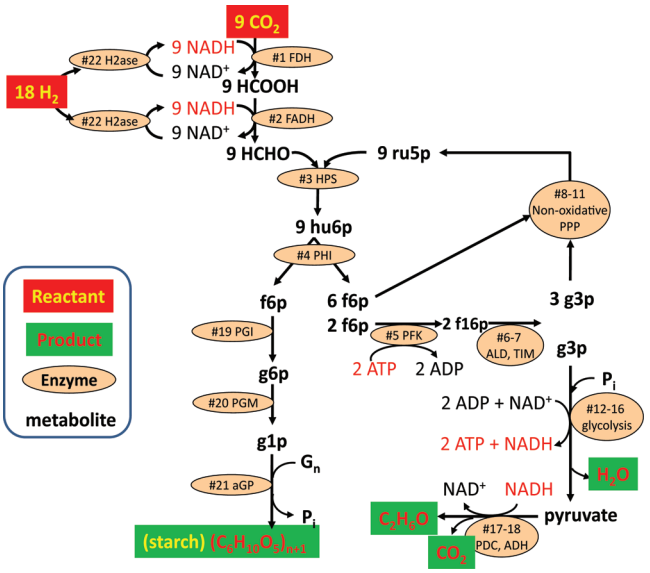

Figure 5. In vitro ATP-balanced synthetic pathway of CO_2 fixation by using hydrogen for the production of synthetic starch and ethanol.

(6) starch (amylose, a linear α-1,4-glucosidic bond starch) lengthening reaction mediated by starch phosphorylase along with phosphoglucose isomerase and phosphoglucomutase (eq 13),[19,72]

$$f6p + (C_6H_{10}O_5)_n \rightleftharpoons (C_6H_{10}O_5)_{n+1} + P_i \quad (13)$$

The combination of eqs 8−13 results in eq 7 with an energy conversion efficiency of 81%.[77] The standard Gibbs free energy of eq 7 is −54.5 kJ/mol, implying that the above reaction may occur spontaneously under standard conditions. The overall reaction could be operative since (i) nearly each reaction is reversible, except 6-phosphofructokinase and pyruvate kinase, both of which control the overall reaction direction, (ii) each module (eqs 8−13) involving several enzymatic steps has been implemented successfully in the literature, and (iii) the Gibbs free energy is negative. This process can drive forward the desired products through several process operations: (i) high-pressure and high concentration CO_2 from a power station or a CO_2 storage site is used for a high driving force for this artificial photosynthesis, (ii) the amylose-lengthening reaction occurs on the nonreducing ends of amylose and amylose is more insoluble in the presence of ethanol, and (iii) ethanol can be stripped from the aqueous phase. Instead of putting all of enzymes in SyPaB in one reactor, it is possible to separate several cascade reactions into several bioreactors in series, as demonstrated in the synthesis of D-ribulose-1,5-bisphosphate from 3-phospho-glycerate.[85] In the starch synthesis step (eq 13), this reaction may be run like solid-phase synthesis, where anhydroglucose units are added on the nonreducing ends of amylose one by one.

The major potential applications of such artificial photosynthesis could be the storage of low-cost renewable hydrogen or electricity in the form of starch and ethanol on a large scale

Table 1. Challenges on SyPaB Technology and Their Respective Solutions and Supportive Examples

challenge	solution	example	ref.
enzyme instability	utilization of thermoenzymes	Taq polymerase, amylase, glucose isomerase	121,122
	protein engineering (directed evolution and rational design)	subtilisin, cellulase	123−125
	enzyme immobilization	immobilized glucose isomerase, immobilized phosphoglucose isomerase	5,126
	TTN value $>10^7$	CthPGI, CthPGM, Tm6PGDH, TmFBP [a]	26,28,94,95
costly enzymes	high-cell density microbial fermentation production	cellulase ($5/kg), amylase ($∼10/kg), Hyperthermophilic 6PGDH	14,26,37
	recombinant overexpression by *E. coli*	*P. furiosus* hydrogenase, CthPGI, CthPGM, Tm6PGDH, TmFBP,	26,28,94,95,127
	simple scalable purification techniques (e.g., heat precipitation, $(NH_4)_2SO_4$ precipitation, adsorption/desorption)	heat precipitation (Tm6PGDH), one-step CthPGI purification and immobilization	14,26,28,96,97
costly and labile coenzymes	coenzyme immobilization and recycling	chiral alcohol synthesis in biopharmaceutical industry	128,129
	stable and low-cost biomimetic coenzyme replacement	P450, horse peroxidase, alcohol dehydrogenase	100,101,103,104
lack of thermoenzyme library	meta-genomics, bioinformatics tools, robotic automation, and high throughput cloning	screening ∼500 recombinant enzymes in one biocatalysis reaction >1400 *T. thermophilus* HB8 thermoenzyme library	14
different optimal conditions for different enzymes	reaction conditions compromised	sugar-to-hydrogen, biohydrogenation	19,20,53
	numerous enzymes obtained from one source or modify them	*T. thermophilus* HB8 thermoenzyme library	14
scalability potential	production of 75 million tons of H_2 replacing 450 million tons of gasoline (i.e., 150 billion gallons)	∼250,000 tons of enzyme mixtures (i.e., ∼300 kg of H_2 per kg of enzyme mixture) [b]	120

[a] CthPGI, *C. thermocellum* phosphoglucose isomerase; CthPGM, *C. thermocellum* phosphoglucomutase; Tm6PGDH, *T. maritima* 6-phosphogluconate dehydrogenase; and TmFBP, *T. maritima* fructose-fructose-1,6-bisphosphatase. [b] One kg of enzyme mixture can produce 300 kg of hydrogen based on two assumptions: (i) all enzymes have TTN values of 30,000,000 mol product per mol enzyme and (ii) the average molecular weight of the enzyme mixture is 50,000.[20]

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Table 2. Analysis of Potential Hydrogen Rate Increases for Sugar-to-Hydrogen Mediated by SyPaB

technology	potential fold	ref.	predicted fold[a]
increasing reaction temperatures from 30 to 80 °C or even higher	32	Q10 effect for hyperthermophilic hydrogenase[b130]	4−20
increasing the use of enzymes responsible for rate-limiting reactions	10	53	2−5
increasing overall enzyme concentration	10	106	5
increasing substrate concentration by 50-fold	10	53	5
creating metabolite channeling among cascade enzymes	∼2−50	95,131,132	2
increasing catalytic efficiency of enzymes	∼10		
overall accelerating rates	640,000−32,000,000		500−5,000

[a] Predicted folds based on each technology may change greatly. It is feasible to increase reaction rates by 3000-fold to be the same level as compared to the highest microbial hydrogen generation rates.[106] [b] *P. furiosus* hydrogenase responsible for the rate-limited step in the sugar-to-hydrogen production, exhibited approximately 1% of its maximum activity at ∼30 °C. Increasing reaction temperature along with the use of other thermoenzymes would accelerate hydrogen generation rates greatly.

and the production of feed and food in emergency cases, such as volcanic winter. A combination of high efficiency solar cells with solar-to-electricity efficiencies of 18−42%,[86] water electrolysis with electricity-to-hydrogen efficiencies of ∼85%,[87] and carbohydrate generation from H_2 and CO_2 with an efficiency of ∼80% here would have overall solar energy-to-carbohydrate efficiencies from 12 to 29%, much higher than those of natural plant photosynthesis.[73,75] The much higher efficiencies of this artificial photosynthesis are mainly attributed to (i) higher efficiency solar cells that can utilize a broader wavelength range of solar insolation, (ii) no respiration (energy) losses in cell-free biocatalysis systems, and (iii) higher-energy efficiency synthetic pathway of starch (Figure 5). Since solar/wind electricity can be easily collected by wires and be distributed by grids, it would be feasible to produce synthetic starch 24/7 at well-controlled bioreactors. More appealing, this artificial photosynthesis does not require a large amount of water for plant transpiration, resulting in potential conservation of fresh water by about 500-fold or higher.[88,89] The pollutants generated from bioreactors can be treated more easily than those from agricultural land because they are point pollution sources.[90] Modern farming requires significantly high inputs from nutrients (e.g., nitrogen and phosphorus), herbicides, and pesticides for high crop productivities.[91] Only a fraction of fertilizers (e.g., ∼30−50%) are utilized by plants, resulting in severe nonpoint water pollution from agricultural land.[92] Waste water pretreatment for bioreactors would be much easier than those from agricultural land.

Approximately 10−60 fold increases in area-specific starch productivity and ∼500−1000 fold water reduction per weight of starch synthesis through this artificial photosynthesis would drastically decrease land uses for biofuels production and reduce or eliminate land/water competition with food and feed production. Also, the conversion of starch to biofuels and value-added chemicals is much more easy than that of nonfood biomass.[38,73,93]

4. CHALLENGES AND OPPORTUNITIES

Construction of in vitro synthetic enzymatic pathways is much easier than modification of living biological entities so that in vitro reconstitution of enzymatic pathways has long been used for understanding natural pathways.[22,23] In the future, in vitro synthetic cascade enzymes would become a low-cost biomanufacturing platform, where product yield is the most critical factor for economically viable production of biofuels. Different from living biological entities operated far from thermodynamic equilibrium and their complicated regulation mechanisms, which are being elucidated by intensive efforts of systems biology and synthetic biology, cell-free systems can be accessed, regulated, operated, and scaled up easily. For example, it is relatively easy to get very high product yields, although all of the enzymes are obtained from different sources and their optimal conditions are not matched well.[20,50,53]

The challenges or doubts of low-cost biomanufacturing SyPaB are attributed to a fixed paradigm of most bioengineers and scientists. The possible causes include (i) enzyme instability, (ii) costly enzymes, (iii) costly and labile coenzymes, (iv) a lack of stable enzymes, (v) different optimal conditions for different enzymes, and (vi) scalability potential.[9,14] To address the above challenges, the respective solutions and supportive examples are listed in Table 1. For example, enzyme instability can be addressed by thermoenzymes, protein engineering through directed evolution and rational design, enzyme immobilization, and their combinations. The previous economic analyses suggest that enzyme costs would be minimal when total turnover numbers (TTN) of all enzymes are larger than 10^7−10^8 mol of product per mol of enzyme.[14,15,20] In practice, it is very feasible to obtain enzymes with such high TTN values from natural thermoenzymes, for example, *Clostridium thermocellum* phosphoglucomutase,[94] *Thermotoga maritima* 6-phosphogluconate dehydrogenase,[26] *T. maritima* fructose-1,6-bisphosphatase,[95] and *C. thermocellum* phosphoglucose isomerase.[28] With respect to costly enzyme, bulk industrial enzymes can be produced and obtained at very low costs, for example, $∼5 per kg of crude protease produced by *Bacillus subtilis*, $5−10 per kg of cellulase produced by *Trichoderma* spp., and tens of U.S. dollars per kg of recombinant proteins produced in *E. coli*.[14] Several low-cost scalable protein purification approaches are available, for example, simple centrifugation for secretory enzymes, adsorption/desorption on low-cost cellulosic materials,[96,97] heat precipitation for thermostable enzymes,[26,98] ammonia precipitation,[14,99] and one-step enzyme purification and immobilization.[28] Therefore, purification costs for bulk recombinant thermoenzymes would become minor.

Currently, the largest obstacle to SyPaB may be costly coenzymes, NADH and NADPH. The labile coenzyme issue can be addressed by the use of low-cost and stable NAD biomimetic coenzymes. But this research area is in its infancy[100,101] because there were no large markets before. Several redox enzymes (e.g., P450 and alcohol dehydrogenase) have been engineered for better performance on biomimetic coenzymes.[102−104] With developments in (i) engineered oxidoreductases that can use biomimetic NAD coenzymes and (ii) stable enzymes as building blocks of SyPaB, we

Table 3. Selected SyPaB-Based Applications, As Compared to Competing Technologies, Their Technology Readiness Levels (TRL) for the Y-12 National Security Complex,[108] Their Remaining Obstacles, and Respective Solutions

application	competing technology	market size* (US $/year)	TRL	remaining obstacle	solution	ref.
biosynthesis of chiral drugs via biohydrogenation	one-enzyme NAD(P)H regeneration	~ billions	TRL 6	separation of metabolites/products with enzymes	enzyme immobilization, membrane reactor	20,36
environmentally friendly sugar batteries (enzymatic biofuel cells)	primary batteries, rechargeable batteries, DMFC	~ 2 billion	TRL 4	low power output, incomplete oxidation, short lifetime	system optimization, nanobiotechnology, cascade pathways, thermoenzymes, enzyme engineering and immobilization	9,110,111
sugary H_2 for local hydrogen users	made from natural gas and coal, or biomass, solar, or wind energy	~20 billion (e.g., ~8 million tons of H_2)	TRL 4	enzyme stability, enzyme costs, labile coenzymes, slow reaction rates	Tables 1 and 2	9,14,19,53
sulfur-free jet biofuel	microbial fermentations, FT process, pyrolysis	~ 50 billion (e.g., 75 million tons of jet fuel)	TRL 3	*ditto* as biohydrogenation, metabolite, and enzyme removal	Table 1, membrane reactor	9,14,20
electricity generators	Diesel electricity generators	~ billions	TRL 2	*ditto* as sugary H_2	Tables 1 and 2	1
sugar fuel cell vehicles (SFCV)	BEV [a] FCV ICE	~500 billion (e.g., 450 million tons of gasoline)	TRL 2	*ditto* as sugary H_2 slow reaction rate	Tables 1 and 2	1,3,16
CO_2 fixation for starch production	dedicated bioenergy plants, mass electricity storage	NA	TRL 2	*ditto* as enzymatic fuel cells	Tables 1 and 2	77

* U.S. market only. [a] BEV, battery electric vehicle; FCV, (hydrogen) fuel cell vehicle; ICE, internal combustion engineer-based vehicle.[120]

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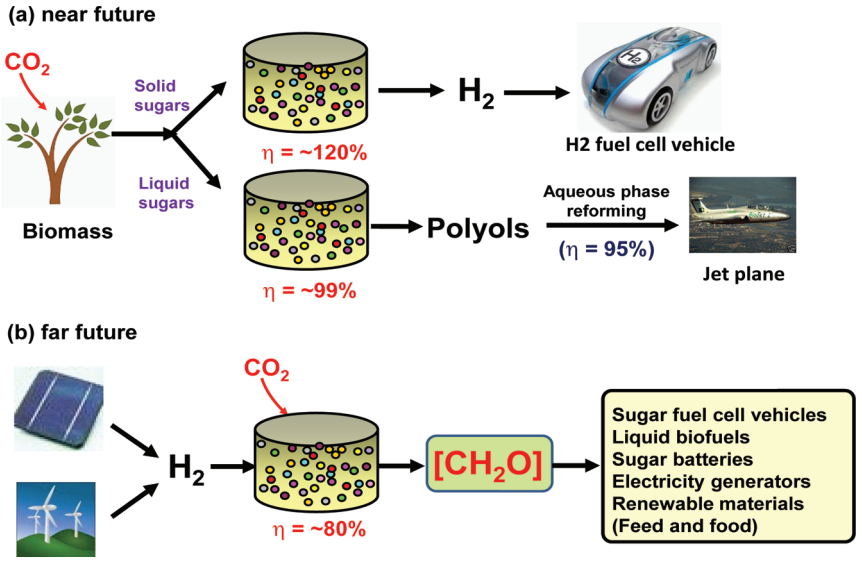

Figure 6. Different biofuels scenarios based on plant biomass through natural photosynthesis (near future) and starch produced by artificial photosynthesis (far future), where high-yield and low-cost SyPaB would have a central role for different biofuels production. The data in red represent energy efficiencies mediated by SyPaB featuring ~99% mass conversion.

estimate that ultimate hydrogen production costs may decrease to ~$1.50 per kg of hydrogen, where carbohydrate ($0.22/kg) accounts for ~95% of its production costs, in part because biohydrogen has very low separation and purification costs and the other chemicals in reactors can be recycled.[14,15]

Enzymatic reactions are usually faster than microbial fermentations[9,105] mainly because neither the dilution of biomacromolecules (e.g., DNA, RNA, other proteins, etc.) nor the mass transfer barriers resulted from the cellular membrane.[1,14] Current enzymatic hydrogen generation rates are comparable with those of anaerobic hydrogen fermentation and are much faster than photobiological hydrogen fermentation.[53] As compared to the highest microbial hydrogen production rates (i.e., 23.6 g H_2/L/h) in the literature,[106] the current enzymatic hydrogen rate[53] would have a potential of ~3000-fold reaction rate increases. Table 2 shows potential methods for increasing reaction rates for sugary hydrogen mediated by SyPaB. They are: (i) increasing reaction temperatures, (ii) increasing the use of enzymes responsible for rate-limiting reactions, (iii) increasing substrate concentrations, (iv) increasing overall enzyme concentrations, (v) accelerating the reaction rates by metabolite (product) channeling, and (vi) increasing the catalytic efficiency of enzymes to catalytically perfect enzymes. With more collaboration among biologists, chemists, and engineers all round the world and system optimization, the reaction rates of SyPaB would be accelerated by several orders of magnitude.[1] In partial support to this prediction, power densities of microbial fuel cells have been enhanced by nearly 10,000,000 fold through intensive efforts during the past one and a half decade.[107]

SyPaB-based applications are increasing greatly. Table 3 presents several potential applications, as compared to their competing technologies, technology readiness levels (TRL),[108] remaining obstacles, and respective solutions. Since each application has its unique market, it has different technology challenges (Table 3). For example, a promising application is enzymatic fuel cells (EFC) powering (low-power) portable electronics, such as cellular phones and MP3 players.[105,109,110] Several big companies (e.g., Sony and Nokia) and small companies (e.g., Gate Fuels and Akermin) are developing enzymatic fuel cells. To our knowledge, the highest power densities of enzymatic fuel cells based on sugar are about 5−10 mW/cm^2 of anode, sufficient to power a Sony Walkman.[111,112] To increase fuel utilization efficiency, cascade enzymes are usually employed.[110,113−115] Complete conversion of sugar energy to electricity would have 4-fold benefits: high energy utilization efficiency, high energy storage density, low product inhibition, and high power density.[9,105,116] It is estimated that complete oxidization of a 20% sugar/water solution (17 MJ/kg sugar ×20%) would lead to energy storage densities of up to 1.7 MJ (i.e., 470 Wh) electricity per kg of the fuel solution based on ~100% Coulombic efficiency and ~50% voltage efficiency. Clearly, such high-energy density biodegradable EFCs might replace some primary batteries and secondary batteries in the future.[55,117]

5. BIOFUELS PERSPECTIVE

Enzyme-based biotransformations are evolving from a single enzyme to multienzyme one pot to synthetic cascade enzymes. SyPaB features unique advantages: great engineering flexibility, high product yields, fast reaction rates, broad reaction conditions (e.g., high temperature and/or low pH), easy operation and control, and tolerance of microorganism-toxic compounds.[9,15,16,20] Therefore, SyPaB would play more important roles in some yield-sensitive applications, such as biofuels production, because thermodynamics (energy efficiency) determines economics (cost) in the long term.[118]

What biofuels would be short-term (e.g., 5 years), middle-term (e.g., 10−20 years), and long-term (e.g., > 20 years) winners is under debate. But it is worth pointing out that high-yield conversion would defeat low-yield conversion eventually because of a megatrend of increasing energy utilization efficiency. In the future, transportation fuels could mainly consist of hydrogen from carbohydrates for light-duty vehicles, electricity from renewable energy sources for short-distance vehicles, and high-energy density liquid biofuels (e.g., hydrocarbons and

butanol) made from biomass for jet planes.[1,119] On the basis of available biomass resources and pretreatment (Figure 6a), liquid hemicellulose sugars and solid cellulosic materials may be converted to jet fuel and hydrogen through high-yield SyPaB, respectively. Liquid jet fuel can be produced through a hybrid of high-yield SyPaB and aqueous phase reforming with an overall energy retaining efficiency (~95%), much higher than fatty acid ester fermentation (~60−65%) and butanol fermentation (~85%).[8,20] Cellulosic materials can be converted to hydrogen in local stations for providing hydrogen for proton exchange membrane fuel cell vehicles.[3,14] In the far future, synthetic starch used for electricity/hydrogen storage (e.g., > 8 mass H_2% or 11−14 MJ electricity/kg starch) may be generated through artificial photosynthesis with an hydrogen-to-starch efficiency of ~80% mediated by SyPaB. Also, starch can be converted back to hydrogen or electricity for different applications. For example, fuel cell-based sugar vehicles that would store starch as a high-density hydrogen carrier might become ultrahigh energy efficiency prime movers.[1,3,120]

In a word, great potentials of high-yield SyPaB (Table 3) would motivate the transformation of basic research to real applications by integrating well-known technologies (Table 1). The maturation of genomics, molecular biology, techniques for enzyme engineering, low-cost enzyme production, purification, and immobilization has led to highly efficient, tunable enzymes tailored for specific large-scale industrial production. The biotransformation through in vitro assembly of numerous enhanced performance and stable enzymes in one bioreactor that can last a very long reaction time (e.g., several months or even years) would become a disruptive technology for low-cost biomanufacturing, especially for the production of biofuels where product yield is the most important cost factor.

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REFERENCES

(1) Zhang, Y.-H. P. *Int. J. Hydrogen Energy* **2010**, *35*, 10334–10342.
(2) Smil, V. *Oil: A beginner's guide*; Oneworld Publications: Oxford, England, 2008.
(3) Zhang, Y.-H. P. *Energy Environ. Sci.* **2009**, *2*, 272–282.
(4) Lynd, L. R. *Energy Environ. Sci.* **2010**, *3*, 1150–1152.
(5) Vasic-Racki, D. In *Industrial Biotransformations*; Liese, A., Seebald, S., Wandrey, C., Eds.; Wiley-VCH, KGaA: Weinheim, Germany, 2006; pp 1−37.
(6) Yuryev, R.; Liese, A. *ChemCatChem* **2009**, *2*, 103–107.
(7) Lynd, L. R.; Laser, M. S.; Bransby, D.; Dale, B. E.; Davison, B.; Hamilton, R.; Himmel, M.; Keller, M.; McMillan, J. D.; Sheehan, J.; Wyman, C. E. *Nat. Biotechnol.* **2008**, *26*, 169–172.
(8) Huang, W. D.; Zhang, Y.-H. P. *Energy Environ. Sci.* **2011**, *4*, 784–792.
(9) Zhang, Y.-H. P. *Biotechnol. Bioeng.* **2010**, *105*, 663–677.
(10) Lynd, L. R.; Wyman, C. E.; Gerngross, T. U. *Biotechnol. Prog.* **1999**, *15*, 777–793.
(11) Wooley, R.; Ruth, M.; Glassner, D.; Sheehan, J. *Biotechnol. Prog.* **1999**, *15*, 794–803.
(12) Lu, X.; Vora, H.; Khosla, C. *Metab. Eng.* **2008**, *10*, 333–339.
(13) Steen, E. J.; Kang, Y.; Bokinsky, G.; Hu, Z.; Schirmer, A.; McClure, A.; del Cardayre, S. B.; Keasling, J. D. *Nature* **2010**, *463*, 559–562.
(14) Zhang, Y.-H. P.; Mielenz, J. R. *Energies* **2011**, *4*, 254–275.
(15) Zhang, Y.-H. P.; Sun, J.-B.; Zhong, J.-J. *Curr. Opin. Biotechnol.* **2010**, *21*, 663–669.
(16) Zhang, Y.-H. P. *Microbe* **2009**, *4*, 560–565.
(17) Zhang, Y.-H. P.; Myung, S.; You, C.; Zhu, Z. G.; Rollin, J. *J. Mater. Chem.* **2011**, DOI: 10.1039/C1031JM12078F.
(18) Li, H.; Cann, A. F.; Liao, J. C. *Annu. Rev. Chem. Biomol. Eng.* **2010**, *1*, 19–36.
(19) Zhang, Y.-H. P.; Evans, B. R.; Mielenz, J. R.; Hopkins, R. C.; Adams, M. W. W. *PLoS One* **2007**, *2*, e456.
(20) Wang, Y.; Huang, W.; Sathitsuksanoh, N.; Zhu, Z.; Zhang, Y.-H. P. *Chem. Biol.* **2011**, *18*, 372–380.
(21) Kresge, N.; Simoni, R. D.; Hill, R. L. *J. Biol. Chem.* **2005**, *280*, e3.
(22) Brown, S.; Zhang, Y.-H.; Walker, S. *Chem. Biol.* **2008**, *15*, 12–21.
(23) Grunden, A. M.; Jenney, F. E., Jr.; Ma, K.; Ji, M.; Weinberg, M. V.; Adams, M. W. W. *Appl. Environ. Microbiol.* **2005**, *71*, 1522–1530.
(24) Michels, P.; Rosazza, J. *SIM News* **2009**, *2009*, 36–52.
(25) Panke, S.; Held, M.; Wubbolts, M. *Curr. Opin. Biotechnol.* **2004**, *15*, 272–279.
(26) Wang, Y.; Zhang, Y.-H. P. *Microb. Cell Fact.* **2009**, *8*, 30.
(27) Luetz, S.; Giver, L.; Lalonde, J. *Biotechnol. Bioeng.* **2008**, *101*, 647–653.
(28) Myung, S.; Zhang, X.-Z.; Zhang, Y.-H. P. *Biotechnol. Prog.* **2011**, DOI: 10.1002/btpr.606.
(29) Daines, A. M.; Maltman, B. A.; Flitsch, S. L. *Curr. Opin. Chem. Biol.* **2004**, *8*, 106–113.
(30) Chi, Y.; Scroggins, S. T.; Frechet, J. M. J. *J. Am. Chem. Soc.* **2008**, *130*, 6322–6323.
(31) Wildeman, S. M. A. D.; Sonke, T.; Schoemaker, H. E.; May, O. *Acc. Chem. Res.* **2007**, *40*, 1260–1266.
(32) Wichmann, R.; Vasic-Racki, D. *Adv. Biochem. Eng. Biotechnol.* **2005**, *92*, 225–260.
(33) Bozic, M.; Pricelius, S.; Guebitz, G. M.; Kokol, V. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 563–571.
(34) Xu, Z.; Jing, K.; Liu, Y.; Cen, P. *J Ind. Microbiol. Biotechnol.* **2007**, *34*, 83–90.
(35) Mertens, R.; Liese, A. *Curr. Opin. Biotechnol.* **2004**, *15*, 343–348.
(36) Johannes, T. W.; Woodyer, R. D.; Zhao, H. *Biotechnol. Bioeng.* **2007**, *96*, 18–26.
(37) Zhang, Y.-H. P.; Himmel, M.; Mielenz, J. R. *Biotechnol. Adv.* **2006**, *24*, 452–481.
(38) Lynd, L. R.; Weimer, P. J.; van Zyl, W. H.; Pretorius, I. S. *Microbiol. Mol. Biol. Rev.* **2002**, *66*, 506–577.
(39) Liao, H. H.; Zhang, X. Z.; Rollin, J. A.; Zhang, Y.-H. P. *Biotechnol. J.* **2011**, DOI: 10.1002/biot.201100157.
(40) Franke, D.; Machajewski, T.; Hsu, C.-C.; Wong, C.-H. *J. Org. Chem.* **2003**, *68*, 6828–6831.
(41) Schoevaart, R.; van Rantwijk, F.; Sheldon, R. A. *J. Org. Chem.* **2000**, *65*, 6940–6943.
(42) Huang, K.-T.; Wu, B.-C.; Lin, C.-C.; Luo, S.-C.; Chen, C.; Wong, C.-H.; Lin, C.-C. *Carbohydr. Res.* **2006**, *341*, 2151–2155.
(43) Zhang, J.; Shao, J.; Kowal, P.; Wang, P. G. *Enzymatic Synthesis of Oligosaccharides*; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2005.
(44) Fessner, W.-D.; Helaine, V. *Curr. Opin. Biotechnol.* **2001**, *12*, 574–586.
(45) Fessner, W.-D. *Curr. Opin. Chem. Biol.* **1998**, *2*, 85–97.
(46) Endo, T.; Koizumi, S. *Curr. Opin. Struct. Biol.* **2000**, *10*, 536–541.
(47) Wang, Y.; Zhang, Y.-H. P. *BMC Biotechnol.* **2009**, *9*, 58.
(48) Calhoun, K. A.; Swartz, J. R. *Biotechnol. Prog.* **2005**, *21*, 1146–1153.

(49) Hold, C.; Panke, S. *J. R. Soc. Interface* **2009**, *6*, S507–S521.
(50) Schultheisz, H. L.; Szymczyna, B. R.; Scott, L. G.; Williamson, J. R. *ACS Chem. Biol.* **2008**, *3*, 499–511.
(51) Bujara, M.; Schümperli, M.; Pellaux, R.; Heinemann, M.; Panke, S. *Nat. Chem. Biol.* **2011**, *7*, 271–277.
(52) Schultheisz, H. L.; Szymczyna, B. R.; Williamson, J. R. *J. Am. Chem. Soc.* **2009**, *131*, 14571–14578.
(53) Ye, X.; Wang, Y.; Hopkins, R. C.; Adams, M. W. W.; Evans, B. R.; Mielenz, J. R.; Zhang, Y.-H. P. *ChemSusChem* **2009**, *2*, 149–152.
(54) Tong, X.; El-Zahab, B.; Zhao, X.; Liu, Y.; Wang, P. *Biotechnol. Bioeng.* **2011**, *108*, 465–469.
(55) Calabrese Barton, S.; Gallaway, J.; Atanassov, P. *Chem. Rev.* **2004**, *104*, 4867–4886.
(56) Hoehler, T. M.; Bebout, B. M.; Des Marais, D. J. *Nature* **2001**, *412*, 324–327.
(57) Chou, C.-J.; Jenney, F. E., Jr.; Adams, M. W. W.; Kelly, R. M. *Metab. Eng.* **2008**, *10*, 394–404.
(58) Thauer, K.; Jungermann, K.; Decker, K. *Bacteriol. Rev.* **1977**, *41*, 100–180.
(59) Thauer, R. K.; Kaster, A. K.; Seedorf, H.; Buckel, W.; Hedderich, R. *Nat. Rev. Microbiol.* **2008**, *6*, 579–591.
(60) Hallenbeck, P. C. *Water Sci. Technol.* **2005**, *52*, 21–29.
(61) Manish, S.; Banerjee, R. *Int. J. Hydrogen Energy* **2008**, *33*, 279–286.
(62) Morimoto, K.; Kimura, T.; Sakka, K.; Ohmiya, K. *FEMS Microbiol. Lett.* **2005**, *246*, 229–234.
(63) Maeda, T.; Sanchez-Torres, V.; Wood, T. K. *Microb. Biotechnol.* **2008**, *1*, 30–39.
(64) Agapakis, C.; Ducat, D.; Boyle, P.; Wintermute, E.; Way, J.; Silver, P. *J. Biol. Eng.* **2010**, *4*, 3.
(65) Maeda, T.; Sanchez-Torres, V.; Wood, T. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 77–86.
(66) Veit, A.; Akhtar, M. K.; Mizutani, T.; Jones, P. R. *Microb. Biotechnol.* **2008**, *1*, 382–394.
(67) Pereto, J.; Velasco, A.; Becarra, A.; Lazcano, A. *Int. Microbiol.* **1999**, *2*, 3–10.
(68) Wood, H. *FASEB J.* **1991**, *5*, 156–163.
(69) Berg, I. A.; Kockelkorn, D.; Buckel, W.; Fuchs, G. *Science* **2007**, *318*, 1782–1786.
(70) Berg, I. A.; Kockelkorn, D.; Ramos-Vera, W. H.; Say, R. F.; Zarzycki, J.; Hügler, M.; Alber, B. E.; Fuchs, G. *Nat. Rev. Microbiol.* **2010**, *8*, 447–460.
(71) Krebs, H. A. *Mol. Cell. Biochem.* **1974**, *5*, 79–94.
(72) Berg, J. M.; Tymoczko, J. L.; Stryer, L., *Biochemistry (fifth ed.)*, W. H. Freeman & Co., New York, 2002.
(73) Zhang, Y.-H. P. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 367–375.
(74) Smil, V. *Energy in Nature and Society*; MIT Press: Cambridge, MA, 2008.
(75) Zhu, X.-G.; Long, S. P.; Ort, D. R. *Curr. Opin. Biotechnol.* **2008**, *19*, 153–159.
(76) Williams, P. J. L.; Laurens, L. M. L. *Energy Environ. Sci.* **2010**, *3*, 554–590.
(77) Zhang, Y.-H. P. *Nature Precedings* **2010**, *2010.4167.1*.
(78) Greiner, L.; Schröder, I.; Müller, D. H.; Liese, A. *Green Chem.* **2003**, *5*, 697–700.
(79) De Lacey, A. L.; Detcheverry, M.; Moiroux, J.; Bourdillon, C. *Biotechnol. Bioeng.* **2000**, *68*, 1–10.
(80) Kuwabata, S.; Tsuda, R.; Yoneyama, H. *J. Am. Chem. Soc.* **1994**, *116*, 5437–5443.
(81) Obert, R.; Dave, B. C. *J. Am. Chem. Soc.* **1999**, *121*, 12192–12193.
(82) El-Zahab, B.; Donnelly, D.; Wang, P. *Biotechnol. Bioeng.* **2008**, *99*, 508–514.
(83) Kato, N.; Yurimoto, H.; Thauer, R. K. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 10–21.
(84) Orita, I.; Sato, T.; Yurimoto, H.; Kato, N.; Atomi, H.; Imanaka, T.; Sakai, Y. *J. Bacteriol.* **2006**, *188*, 4698–4704.
(85) Bhattacharya, S.; Schiavone, M.; Gomes, J.; Bhattacharya, S. K. *J. Biotechnol.* **2004**, *111*, 203–217.
(86) Luque, A.; Martí, A. *Phys. Rev. Lett.* **1997**, *78*, 5014–5017.
(87) Kanan, M. W.; Nocera, D. G. *Science* **2008**, *321*, 1072–1075.
(88) *Water security: the water-food-energy-climate nexus: The World Economic Forum Water Initiative*; Waughray, D., Ed.; Island Press: Washington, DC, 2011.
(89) Hightower, M.; Pierce, S. A. *Nature* **2008**, *452*, 285–286.
(90) Grady, G. P. L.; Daigger, G. T.; G, L. N.; Filipe, C. D. M. *Biological Waste Treatment* 3rd ed.; CRC Press: Boca Raton, FL, 2011.
(91) Smil, V. *Feeding the World: A Challenge for the Twenty-First Century*; MIT Press: Boston, MA, 2000.
(92) Braskerud, B. C. *Eco. Eng.* **2002**, *19*, 41–61.
(93) Zhang, Y.-H. P.; Lynd, L. R. *Biotechnol. Bioeng.* **2004**, *88*, 797–824.
(94) Wang, Y.; Zhang, Y.-H. P. *J. Appl. Microbiol.* **2010**, *108*, 39–46.
(95) Myung, S.; Wang, Y. R.; Zhang, Y.-H. P. *Process Biochem.* **2010**, *45*, 1882–1887.
(96) Hong, J.; Wang, Y.; Ye, X.; Zhang, Y.-H. P. *J. Chromatogr., A* **2008**, *1194*, 150–154.
(97) Hong, J.; Ye, X.; Wang, Y.; Zhang, Y.-H. P. *Anal. Chim. Acta* **2008**, *621*, 193–199.
(98) Iyer, R.; Wang, J.; Bachas, L. *Extremophiles* **2002**, *6*, 283–289.
(99) Scopes, R. K. *Protein purification: principles and practice*, 3rd ed.; Springer-Verlag: New York, 1993.
(100) Lutz, J.; Hollmann, F.; Ho, T. V.; Schnyder, A.; Fish, R. H.; Schmid, A. *J. Organomet. Chem.* **2004**, *689*, 4783–4790.
(101) Ansell, R. J.; Lowe, C. R. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 703–710.
(102) Lo, H. C.; Fish, R. H. *Angew. Chem., Int. Ed.* **2002**, *41*, 478–481.
(103) Ryan, J. D.; Fish, R. H.; Clark, D. S. *ChemBioChem* **2008**, *9*, 2579–2582.
(104) Campbell, E.; Wheeldon, I. R.; Banta, S. *Biotechnol. Bioeng.* **2010**, *107*, 763–774.
(105) Cooney, M. J.; Svoboda, V.; Lau, C.; Martin, G.; Minteer, S. D. *Energy Environ. Sci.* **2008**, *1*, 320–337.
(106) Yoshida, A.; Nishimura, T.; Kawaguchi, H.; Inui, M.; Yukawa, H. *Appl. Environ. Microbiol.* **2005**, *71*, 6762–6768.
(107) Logan, B. E. *Nat. Rev. Microbiol.* **2009**, *7*, 375–381.
(108) http://www.y12.doe.gov/business/technologies/.
(109) Moehlenbrock, M.; Minteer, S. *Chem. Soc. Rev.* **2008**, *37*, 1188–1196.
(110) Zhu, Z.; Wang, Y.; Minteer, S.; Zhang, Y.-H. P. *J. Power Sources* **2011**, *196*, 7505–7509.
(111) Sakai, H.; Nakagawa, T.; Tokita, Y.; Hatazawa, T.; Ikeda, T.; Tsujimura, S.; Kano, K. *Energy Environ. Sci.* **2009**, *2*, 133–138.
(112) Sakai, H.; Nakagawa, T.; Mita, H.; Matsumoto, R.; Sugiyama, T.; Kumita, H.; Tokita, Y.; Hatazawa, T. *ECS Trans.* **2009**, *16*, 9–15.
(113) Palmore, G. T. R.; Bertschy, H.; Bergens, S. H.; Whitesides, G. M. *J. Electroanal. Chem.* **1998**, *443*, 155–161.
(114) Sokic-Lazic, D.; Minteer, S. D. *Electrochem. Solid-State Lett.* **2009**, *12*, F26–F28.
(115) Moehlenbrock, M. J.; Toby, T. K.; Waheed, A.; Minteer, S. D. *J. Am. Chem. Soc.* **2010**, *132*, 6288–6289.
(116) Minteer, S. D.; Liaw, B. Y.; Cooney, M. J. *Curr. Opin. Biotechnol.* **2007**, *18*, 228–234.
(117) Armand, M.; Tarascon, J. M. *Nature* **2008**, *451*, 652–657.
(118) Smil, V. *Energy Transitions: History, Requirements, Prospects*; ABC-CLIO, LLC: Santa Barbara, CA, 2010.
(119) Thomas, C. E. *Int. J. Hydrogen Energy* **2009**, *34*, 6005–6020.
(120) Huang, W. D.; Zhang, Y.-H. P. *PLoS One* **2011**, *6*, e22113.
(121) Demain, A. L.; Vaishnav, P. *Biotechnol. Adv.* **2009**, *27*, 297–306.
(122) Kirk, O.; Borchert, T. V.; Fuglsang, C. C. *Curr. Opin. Biotechnol.* **2002**, *13*, 345–351.
(123) Zhao, H.; Arnold, F. H. *Protein Eng.* **1999**, *12*, 47–53.

(124) Liu, W.; Zhang, X.-Z.; Zhang, Z.-M.; Zhang, Y.-H. P. *Appl. Environ. Microbiol.* **2010**, *76*, 4914–4917.
(125) Liu, W.; Hong, J.; Bevan, D. R.; Zhang, Y.-H. P. *Biotechnol. Bioeng.* **2009**, *103*, 1087–1094.
(126) Cao, L.; Langen, L. v.; Sheldon, R. A. *Curr. Opin. Biotechnol.* **2003**, *14*, 387–394.
(127) Sun, J.; Hopkins, R. C.; Jenney, F. E.; McTernan, P. M.; Adams, M. W. W. *PLoS One* **2010**, *5*, e10526.
(128) Liu, W.; Wang, P. *Biotechnol. Adv.* **2007**, *25*, 369–384.
(129) Kazandjian, R.; Klibanov, A. *J. Am. Chem. Soc.* **1985**, *107*, 5448–5450.
(130) Ma, K.; Zhou, Z. H.; Adams, M. W. W. *FEMS Microbiol. Lett.* **1994**, *122*, 245–250.
(131) Srivastava, D. K.; Bernhard, S. A. *Science* **1986**, *234*, 1081–1086.
(132) Zhang, Y.-H. P. *Biotechnol. Adv.* **2011**, DOI: 10.1016/j.biotechadv.2011.05.020.