2
3
4
5 **Production of Biofuels and Biochemicals by in vitro Synthetic**
6 **Biosystems: Opportunities and Challenges**
7

8
9 Yi-Heng Percival Zhang,^{1,2,3 *}

10 1 Biological Systems Engineering Department, Virginia Tech, 304 Seitz Hall, Blacksburg,
11 Virginia 24061, USA

12 2 Cell Free Bioinnovations Inc., 2200 Kraft Drive, Suite 1200B, Blacksburg, VA 24060, USA

13 ³ Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, 32 West 7th
14 Avenue, Tianjin Airport Economic Area, Tianjin 300308, China
15

16
17 * Corresponding author: Y-H Percival Zhang,

18 Email: ypzhang@vt.edu, profCH2O@outlook.com

19 Phone: 001-540-231-7414
20
21
22

Abstract

The largest obstacle to the cost-competitive production of low-value and high-impact biofuels and biochemical (called biocommodities) is high production costs catalyzed by microbes due to their inherent weaknesses, such as low product yield, slow reaction rate, high separation cost, intolerance to toxic products, and so on. This predominant whole-cell platform suffers from a mismatch between the primary goal of living microbes – cell proliferation and the desired biomanufacturing goal – desired products (not cell mass most times). In vitro synthetic biosystems consist of numerous enzymes as building bricks, enzyme complexes as building modules, and/or (biomimetic) coenzymes, which are assembled into synthetic enzymatic pathways for implementing complicated bioreactions. They emerge as an alternative solution for accomplishing desired biotransformation without concerns of cell proliferation, complicated cellular regulation, and side-product formation. In addition to the most important advantage -- high product yield, in vitro synthetic biosystems feature several other biomanufacturing advantages, such as fast reaction rate, easy product separation, open process control, broad reaction condition, tolerance to toxic substrates or products, and so on. In this perspective review, the general design rules of in vitro synthetic pathways are presented with eight supporting examples: hydrogen, n-butanol, isobutanol, electricity, starch, lactate, 1,3-propanediol, and poly-3-hydroxybutyrate. Also, a detailed economic analysis for enzymatic hydrogen production from carbohydrates is presented to illustrate some advantages of this system and remaining challenges. Great market potentials will motivate worldwide efforts from multiple disciplines (i.e., chemistry, biology and engineering) to address remaining obstacles pertaining to cost and stability of enzymes and coenzymes, standardized building parts and modules, biomimetic coenzymes, biosystem optimization and scale-up, soon.

Keywords: biochemicals, biofuels, economic analysis, in vitro synthetic biosystem, in vitro synthetic biology, innovative biomanufacturing, paradigm shift

50	
51	Contents
52	1. Introduction
53	2. Opportunities of in vitro Synthetic Biosystems
54	2.1. General design principles
55	2.2. Examples of biofuels and bioenergy
56	2.2.1. Hydrogen
57	2.2.2. n-Butanol
58	2.2.3. Isobutanol
59	2.2.4. Electricity
60	2.3. Examples of biochemicals
61	2.3.1. Synthetic amylose
62	2.3.2. Lactate
63	2.3.3. 1,3-Propanediol
64	2.3.4. Poly-3-hydroxybutyrate
65	3. Economic Analysis of the Hydrogen Case
66	4. Challenges and Solutions
67	4.1. Stable Enzymes as Standardized Building Blocks
68	4.2. Synthetic Multiple-Enzyme Complexes (Metabolons) as Building Modules
69	4.3. Bulk Enzyme Production and Separation
70	4.4. NAD(P)-Dependent Enzyme Engineering
71	4.5. System optimization and Modeling
72	4.6. Stabilization of Thermolabile Metabolites at Elevating Temperatures
73	4.7. Scale-Up by Integrative Innovation
74	4.8. Other Issues
75	5. Conclusions
76	Acknowledgements
77	References
78	
79	
80	

1. Introduction

The sustainability revolution is taking place in this century mainly due to concerns of depleting fossil fuels and climate changes. At the same time, growing world population and increasing living standards require more natural resource consumption. Biomass produced from terrestrial plants is the most abundant renewable bioresource, approximately five times global energy consumption (Zhang 2013). Biomass and its carbohydrates are the sole cost-competitive energy and carbon sources that will be converted to produce biofuels and biochemicals instead of fossil fuels (Wyman 1999). Compared to low energy concentration (i.e., nonpoint) solar energy, biomass is a concentrated chemical energy, which could be harvested, stored, transported, and converted to other chemical energy forms relatively easily (Zhang 2011c).

Human beings had utilized living whole-cell microorganisms for making numerous fermentative products for thousands of years. Living whole-cell fermentation is the predominant biomanufacturing platform (**Fig. 1a**). However, the primary goal of microorganisms is their proliferation while bioconversions are side effects. Recent advances in synthetic biology and systems biology present numerous breakthroughs, such as the production of non-natural products (e.g., 1,4-butanediol (Yim et al. 2011), isobutanol (Atsumi et al. 2008), etc.). However, some inherent constraints of living microorganisms (e.g., net ATP generation, intact cellular membrane, etc.) prevent them from implementing some important chemical reactions, for example, 12 H_2 production from one glucose and water.

Whole cell lysates (**Fig. 1b**) have been used as an important scientific tool to investigate complicated biological reactions for more than 100 years. For example, Eduard Buchner discovered that the yeast lysate converted glucose to ethanol (Buchner 1897). Due to his paradigm-shifting discovery, he won the Nobel Chemistry Prize in 1907. Later, numerous scientists applied this tool to discover and study natural metabolic pathways. For example, Harden et al. discovered key enzymes in glycolysis (Nobel Chemistry Prize 1929), Calvin elucidated the CO_2 assimilation in plants (Nobel Chemistry Prize 1961), Nirenberg and Matthaei interpreted the genetic code and its function in protein synthesis (Nobel Physiology Prize 1968) (Matthaei et al. 1962). Recently, cell-free protein synthesis has been suggested to be the fastest way to make recombinant proteins, even for membrane or complicated proteins (Carlson et al. 2012; Swartz 2013). Cell-free protein synthesis has been scaled up to 100 liter levels recently (Hodgman and Jewett 2012).

In vitro synthetic biosystems emerge as a manufacturing platform by the assembly of numerous enzymes and enzyme complexes from different sources and/or (biomimetic) coenzymes (**Fig. 1c**). Such systems could surpass the constraints of whole-cells and cell lysates for implementing some biological reactions that microbe cannot do, for example, high yield production of hydrogen (Martín del Campo et al. 2013), or enzymatic transformation of cellulose to starch (You et al. 2013). Although in vitro synthetic biosystems are on their early stage, they have a great potential to becoming a disruptive biomanufacturing platform, especially for low-cost production of biofuels and biochemicals. Table 1 presents the comparison of biomanufacturing advantages between whole cell-based fermentations and in vitro (cell-free) synthetic systems, from product yield, volumetric productivity, reaction condition, product separation, product titer, product purity to process control and optimization. Among them, three key criteria of biomanufacturing are (i) product yield mainly related to feedstock cost, (ii) volumetric productivity (or reaction rate) mainly related to capital investment, and (iii) product separation (or product titer) mainly related to processing costs.

The history of biomanufacturing platforms accompanied with key milestones is presented in **Figure 2**. Before Louis Pasteur clearly proposed the theory of biogenesis (1864), human beings had used microorganisms for making wine, beer, bread, cheese and so on by solid state fermentation for thousands of years. Weizmann developed anaerobic liquid fermentation by using a mono-culture to produce acetone in World War I. In World War II, the submerged aerobic fermentation technology was invented to produce penicillin at low costs. Next major breakthroughs were recombinant DNA technology and mammalian cell cultures for the production of erythropoietin (EPO), insulin, vaccines, antibodies, and so on in 1980s. In this century, new biomanufacturing breakthroughs will revolutionize industrial bioprocess by producing a myriad of new or old products through more cost-effective approaches.

The development of in vitro (cell-free) technologies lagged far behind the whole cell-based technologies (Bornscheuer et al. 2012; Vasic-Racki 2006). Although Eduard Buchner discovered the phenomenon of cell-free ethanol fermentation in 1890s, the use of one purified enzyme along with enzyme immobilization for industrial production began in 1960s-1970s, for example, high fructose corn syrup (i.e., more than 10 billion US dollars yearly) and semi-synthetic antibiotics (e.g., cephalosporin) (Demain 2004). Such cell-free systems evolved to more complicated ones – multi-enzyme one pot for enhancing volumetric productivity, decreasing product inhibition, and shifting reaction equilibrium (Santacoloma et al. 2010;

Schoffelen and van Hest 2012). For example, the pharmaceutical and fine chemistry industries adopt this platform to produce high-value chiral alcohols, α -hydroxy acids, and α -amino acids, such as, (S)-2-butanol, L-*tert*-leucine, (S)-ethyl-4-chloro-3-hydroxybutyrate, atorvastatin, and so on (Bornscheuer et al. 2012; Huisman et al. 2010; Wildeman et al. 2007). In the organic chemistry field, the synthesis of monosaccharides, activated monosaccharides, oligosaccharides, and glycopeptides by using multi-enzyme one pot has been intensively investigated, such as, L-fructose, 5-deoxy-5-ethyl-D-xylulose, amylose, and so on (Endo and Koizumi 2000; Fessner 1998; Fessner and Helaine 2001; Franke et al. 2003; Huang et al. 2006; Qi et al. 2014; Schoevaart et al. 2000; Zhang et al. 2005). In this century, a few researchers propose to put more than four biocatalytic components in one vessel to implement very complicated reactions comparable to microbial cell factories. Because in vitro synthetic biosystems for future biomanufacturing are on their early stage, different names have been suggested in the literature, such as, synthetic pathway biotransformation (SyPaB) (Zhang 2010a; Zhang 2011a), synthetic cascade biomanufacturing (Steffler and Sieber 2013), cell-free bioprocessing (Swartz 2013), synthetic biochemistry (Korman et al. 2014; Opgenorth et al. 2014), cell-free biosystems for biomanufacturing or cell-free biomanufacturing (You and Zhang 2013), in vitro metabolic engineering (Honda et al. 2004), cascade enzyme factories (Zhang and Huang 2012), and so on.

Here I present pathway design principles of in vitro synthetic biosystems for biomanufacturing of biofuels and biochemicals (called biocommodities) with eight examples, provide a detailed economic analysis for enzymatic hydrogen production for identifying remaining challenges, and suggest respective solutions. The other applications of in vitro synthetic systems or cell extracts are not covered here, for example, the synthesis of fine chemicals (Bruggink et al. 2003; Bujara et al. 2011), of radiolabelled products (Rodriguez and Leyh 2014; Schultheisz et al. 2008; Schultheisz et al. 2009), of special compounds (Schultheisz et al. 2008; Scism and Bachmann 2010), of proteins (Carlson et al. 2012; Hodgman and Jewett 2012; Swartz 2013), and of drugs (Boltje et al. 2009; Xu et al. 2014; Xu et al. 2011), in vitro genetic or protein circuits and computation (Hockenberry and Jewett 2012; Katz and Privman 2010; Kim and Winfree 2011; Noireaux 2003; Yordanov et al. 2014), in-depth understanding of in vivo biosystems (Bogorad et al. 2013; Keller et al. 2013), and so on.

2. Opportunities of in vitro Synthetic Biosystems

For the production of low-value and high-impact biocommodities, the major cost factor is carbohydrate costs, accounting for at least a half or even more than 80% product price (Lynd et al. 1999; Zhang 2010a). In vitro synthetic biosystems could lower feedstock costs than whole-cell systems because of high product yields (that is, neither the synthesis of cell mass nor the formation of side-products) (Table 1). Furthermore, because balanced coenzymes are designed in in vitro synthetic pathways (Section 2.1), in vitro synthetic biosystems could have very low processing costs (that is, no aeration, low cooling energy, etc.) and low capital investment, especially important for large-scale production of biocommodities. As shown in Table 1, in vitro synthetic biosystems could have numerous biomanufacturing advantages over traditional whole-cell fermentation, leading to great opportunities to the production of biocommodities. Here we present the design principles of in vitro synthetic enzymatic pathways with eight supporting examples – four for biofuels and bioelectricity and four for biochemicals.

2.1. General Design Principles

The design principles of in vitro synthetic biosystems for manufacturing include (i) in vitro pathway reconstruction, (ii) enzyme selection, (iii) enzyme engineering, (iv) enzyme production, and (v) process engineering (Zhang 2010a). Whole processes can be improved in an iterative manner by inputs of different specialists (e.g., enzyme engineers, bioprocess engineers, biochemists, bulk enzyme producers, bioinformaticians, organic chemists, and so on), gradually leading to an efficient industrial process (Zhang 2010a). The design of an in vitro synthetic enzymatic pathway is central, while the others (i.e., enzyme selection, enzyme engineering, enzyme production, and process engineering) are pretty mature. Synthetic enzymatic pathways are usually designed based on natural metabolic pathways with some modifications. Because the same biochemical reactions can be designed by different pathways, synthetic pathways need to be designed carefully by considering ATP balance, NAD(P) balance, NAD and NADP matching, coenzyme involvement, enzyme chosen, thermodynamics, reaction equilibrium, product separation, and so on (Rollin et al. 2013; Zhang 2010a).

In vitro synthetic enzymatic pathways must have a balance in ATP production and consumption while in vivo synthetic pathways in whole cells can obtain or depose extra ATP from or to cellular metabolisms. If net ATP is generated for the case of in vitro ethanol fermentation via the glycolysis pathway, the accumulation of ATPs stops systems from running for a long time (Welch and Scopes 1985). If ATP input is needed, it is important to have low-cost ATP

regeneration systems. The best solution is careful design of pathways without ATP involvement or with ATP balance. Different from the typical glycolytic pathway from glucose to two pyruvate that generates two moles of ATP, in vitro pathways can produce pyruvate without ATP involvement (Guterl et al. 2012) or produce zero net ATP (Krutsakorn et al. 2013a; Ye et al. 2012a). If net ATP is accumulated in the overall process, the addition of ATPase or arsenate or phosphatase could dissipate some ATP (Welch and Scopes 1985) or hydrolyze high-energy phosphate bond-containing metabolites (Moradian and Benner 1992). If a small amount of ATP is required as an input for the whole system, several ways can be selected, such as the generation of glucose 1-phosphate by using glucan phosphorylases (Wang and Zhang 2009a; Ye et al. 2009; Zhang et al. 2007), the generation of sugar phosphates from polyphosphate (Liao et al. 2012; Martin del Campo et al. 2013b), or the regeneration of ATP from the secondary energy sources, such as, polyphosphate, creatine phosphate, phosphoenolpyruvate, and so on, through substrate phosphorylation (Resnick and Zehnder 2000; Wang and Zhang 2009a). It is noted that the use of costly second energy sources prohibits their application for biocommodity production. If a large amount of ATP is needed as an input, cell-free oxidative phosphorylation (Jewett et al. 2008) or novel ATP synthase-based regeneration systems may be a choice (Rupp 2013). But the long-term stability of synthetic membranes should be addressed.

Similar to ATP balance, it is essentially important to design NAD(P)H-balanced pathways. First, the type of reduced NAD(P)H generated from substrates should match that of NAD(P)H consumption for the production of desired products. If not, it can be addressed to find out new enzymes using the same type of coenzymes from nature (Krutsakorn et al. 2013a), engineer coenzyme preferences by rational protein design (Opgenorth et al. 2014; Rollin et al. 2013) or add another enzyme transhydrogenase. Second, when in vitro pathways generate extra reduced coenzymes, such reduced coenzymes should be removed by using hydrogenase under anaerobic conditions (Wichmann and Vasic-Racki 2005), by using NAD(H) oxidase under aerobic conditions (Opgenorth et al. 2014), or via electron mediators in enzymatic fuel cells (Zhu et al. 2012). When pathways need extra reduced coenzymes inputs, NAD(P)H can be usually generated by using a hydrogen-donor substrate and one of the following: a single enzyme, cascade enzymes, and electro-enzymes. Single-enzyme systems include alcohol/alcohol dehydrogenase (Wichmann and Vasic-Racki 2005), formate/formate dehydrogenase (Bozic et al. 2010), glucose/glucose dehydrogenase (Xu et al. 2007), glucose 6-phosphate/glucose 6-phosphate dehydrogenase (Wong and Whitesides 1981), dihydrogen/hydrogenase (Mertens

and Liese 2004; Wong et al. 1981), and phosphite/phosphite dehydrogenase (Johannes et al. 2007). Single-enzyme NAD(P)H regeneration systems are widely used in the synthesis of high-value of chiral compounds in the pharmaceutical industry. A 12-enzyme system is used to produce nearly 12 NADPH from one glucose unit of cellobiose (Wang et al. 2011). Among all hydrogen-donor compounds, renewable sugars have the lowest substrate costs, but this 12-enzyme system requires more enzymes and increases system complexity (Wang et al. 2011). The utilization of electrochemistry to generate reduced coenzymes is low-cost and clean, but the instability of NAD(P)H under high over-potential must be solved before this technique becomes industrially feasible (Kohlmann et al. 2008; Paul et al. 2014; Zheng et al. 2009).

Thermodynamics should be calculated between substrates and desired products, especially for the cases of non-natural biochemical reactions designed. An online calculator for biochemical thermodynamics – eEquilibrator is available (<http://equilibrator.weizmann.ac.il/>) (Jankowski et al. 2008). Also, it is important to identify reduced power sources as substrates, which may be carbohydrates, glycerol, H₂, CO, methane, methanol, electricity and so on. For example, butanol production based on glucose is spontaneous (i.e., $\Delta G < 0$) mainly due to a negative enthalpy (Li et al. 2010). In contrast, methane generation from acetate occurs spontaneously due to a gain of entropy but a positive enthalpy (Thauer et al. 1977; Zhang 2011a). Synthetic pathways or special enzymatic reactions cannot be accomplished against thermodynamics laws (Bornscheuer et al. 2012). To ensure to drive the reaction towards the desired direction, Gibbs free energy under standard or custom conditions need be checked. To drive the reactions towards desired products, we could apply a push-pull strategy sometimes. In situ product removal (for example, hydrogen removal by vacuum or gas flush (Martin del Campo et al. 2013a; Myung et al. 2014), butanol stripping (Smith et al. 2010)) provides more pull forces, while increasing the pressure or concentration of gaseous substrates could generate more push forces for desired reactions (Keller et al. 2013; Rieckenberg et al. 2014; Zhang and Huang 2012).

Generally speaking, enzymes used in vitro are highly exchangeable regardless of their resources (Zhang et al. 2010). However, a choice of right enzymes is important sometimes. In the case of enzymatic transformation of cellulose to synthetic starch, only alpha-glucan phosphorylase from potato can synthesize amylose from cellobiose while the other two bacterial alpha-glucan enzymes having the same enzyme catalog number cannot do (You et al. 2013). Because potato alpha-glucan phosphorylase equipped with a special cap on its catalytic domain

has a lower activation energy for the amylose synthesis reaction than bacterial enzymes (You et al. 2013).

2.2. Examples of Biofuels and Bioelectricity

The combined markets of hydrogen for hydrogen fuel cell vehicles, liquid biofuels for jet planes, and bioelectricity for portable electronics are expected to be trillions of US dollars annually. Production of biofuels and bioelectricity from renewable carbohydrates could be economically viable because carbohydrates are less costly in term of energy content compared to most fossil fuels (Zhang 2009) and are expected to decline annually.

2.2.1. Hydrogen

Natural and genetically-modified microorganisms cannot produce hydrogen with a yield of more than 4 H₂ per glucose, that is, the Thauer limit (Thauer et al. 2008; Zhang 2011a), although a theoretical yield is 12 H₂ per glucose. Nature cannot evolve such high-yield hydrogen generation pathways due to two reasons. First, theoretical yield of hydrogen production is an endothermic reaction so that it cannot co-generate ATP. Second, if a small fraction of reduced NAD(P)H was used to generate ATP via oxidative phosphorylation (Swartz 2013), the presence of oxygen would inhibit oxygen-sensitive hydrogenase activity greatly.

Woodward and his coworkers (Woodward et al. 2000) produced nearly 12 H₂ from costly glucose 6-phosphate (G-6-P). This pathway is comprised of three modules: (i) two NADPH generation from G-6-P mediated by two dehydrogenases (equation 1), (ii) hydrogen generation from NADPH mediated by hydrogenase (equation 2), and (iii) re-generation of G-6-P from ribulose 5-phosphate (equation 3). However, costly substrate G-6-P prevents its potential application so that Woodward did not file a patent for this in vitro synthetic pathway.

Furthermore, I proposed to generate glucose 1-phosphate (G-1-P) from starch and phosphate catalyzed by alpha-glucan phosphorylase (equation 4) and then converted to G-6-P by phosphoglucomutase (equation 5).

G-1-P = G-6-P [5]

As a result, the whole stoichiometric reaction can be written as equation 6, that is, neither costly G-6-P nor ATP is needed for high-yield hydrogen production, suggesting a great potential of low-cost green hydrogen production from polysaccharides.

The proof-of-concept experiment was conducted by using 13 enzymes from five different sources (e.g., bacterium, yeast, plant, animal, and archaea) (Zhang et al. 2007). Furthermore, oligosaccharides (e.g., cellobiose, cellopentaose and sucrose) along with their respective phosphorylases (e.g., cellobiose phosphorylase, cellodextrin phosphorylase, and sucrose phosphorylase) can be used to produce high-yield hydrogen (Myung et al. 2014; Ye et al. 2009). However, one glucose unit per mole of polysaccharide or oligosaccharide cannot be converted to G-1-P. To completely utilize all hexose units, G-6-P can be produced from low-cost polyphosphate instead of ATP mediated by polyphosphate glucokinase (Liao et al. 2012). Therefore, high-yield hydrogen can be produced from all hexose units of sucrose (Myung et al. 2014) and glucose (Rollin et al. 2014). To utilize xylose, the most abundant pentose for the production of low-cost hydrogen, a novel polyphosphate xylulokinase was discovered (Martín del Campo et al. 2013b). By using this enzyme plus polyphosphate, nearly 10 mol of hydrogen was produced from one xylose and water (Martín del Campo et al. 2013).

The overall reaction (Eq. 6) is spontaneous and endothermic (i.e., $\Delta G^\circ = \sim -50$ kJ/mol and $\Delta H^\circ = +598$ kJ/mol) (Zhang et al. 2007). This entropy-driven reaction can generate the chemical energy output in the form of hydrogen more than the chemical energy of polysaccharides by absorbing low temperature waste heat, which is widely available from refrigerators, air conditioners, cooling towers, and fuel cells. These overall reactions are spontaneous due to the phase changes from more orderly liquid to less orderly gas, resulting in a large positive ΔS (Ye et al. 2009; Zhang et al. 2007).

High-yield enzymatic hydrogen production from a variety of carbohydrates from starch, cellodextrins, sucrose, glucose, fructose, and xylose (**Figure 3**) brings multiple implications: (i) low-cost green hydrogen production for hydrogen fuel cell vehicles, (ii) a new hydrogen carrier –

carbohydrate for on-demand hydrogen production, and (iii) an outside solution to the costly infrastructure of the hydrogen economy (Zhang 2009; Zhang 2010b; Zhang et al. 2013).

2.2.2. n-Butanol

n-Butanol, a primary 4-carbon alcohol, is a better liquid biofuel than ethanol due to higher energy density and less water adsorption. It used to be produced by acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum*. However, its fermentation involves a complicated transition from acidogenesis to solvogenesis and suffers from low product yields and severe product inhibition, resulting in low product titers (Ezeji et al. 2007).

Honda and his coworkers designed an in vitro non-natural, ATP-balanced pathway for n-butanol production from glucose (Krutsakorn et al. 2013a). This pathway is comprised of 16 thermostable enzymes (**Figure 4**). The pathway has three modules: (i) generation of two pyruvate and two NADH from one glucose without ATP accumulation (equation 7, different from glycolysis), (ii) generation of acetyl-CoA from pyruvate (equation 8); and (iii) n-butanol production from 2 acetyl-CoA (equation 9). As a result, one glucose can produce one n-butanol, two CO₂ and one water (equation 10).

This synthetic pathway has three features: (i) ATP balance, where the ATP consumption during the conversion of glucose to fructose-1,6-bisphosphate matches the ATP regeneration from phosphoenolpyruvate to pyruvate mediated by pyruvate kinase; (ii) NADH balance, where NADH regeneration by non-phosphorylating GAP dehydrogenase and CoA-acylating aldehyde dehydrogenase (ADDH) matches its consumption by 3-hydroxyacyl-CoA dehydrogenase, ADDH, NADH-dependent flavinoxidoreductase, and hydroxybutyryl-CoA dehydrogenase; and (iii) CoA balance, where CoA is needed by ADDH and is released by acetyl-CoA acetyltransferase and ADDH. The overall reaction is an enthalpy-driven reaction, meaning a slight chemical energy loss (Huang and Zhang 2011). By compromising reaction temperatures and optimizing enzyme loading, the maximum n-butanol yield was 82% (Krutsakorn et al. 2013a), comparable with best product yields in ABE fermentation (Ezeji et al. 2007).

2.2.3. Isobutanol

Isobutanol, a branched C₄ alcohol, has a similar energy density to n-butanol, limited miscibility with water, and is completely miscible with gasoline (Peralta-Yahya et al. 2012). Isobutanol's branching gives it a better octane number than n-butanol. Isobutanol can be produced through the Ehrlich pathway, also called the 2-keto-acid pathway (Hazelwood et al. 2008; Peralta-Yahya et al. 2012). Heterologous pathway for isobutanol production from carbohydrates have been introduced to a number of microorganisms, such as *E. coli* (Atsumi et al. 2008), *Bacillus subtilis* (Li et al. 2011), *Corynebacterium glutamicum* (Smith et al. 2010), *S. cerevisiae* (Chen et al. 2011), and *Clostridium acetobutylicum* (Higashide et al. 2011). However, its hydrophobic effect is toxic to cellular membranes, resulting on low isobutanol titers (e.g., 1-2%) (Atsumi et al. 2010).

Sieber and his coworkers designed an in vitro non-natural, ATP- and CoA-free pathway for isobutanol production from glucose (**Figure 5**) (Guterl et al. 2012). This synthetic pathway contains two modules: (i) generation of two pyruvate from glucose without involvement of ATP and CoA mediated by four enzymes (equation 11), and (ii) production of one isobutanol from two pyruvate (equation 12). As a result, one glucose can produce one isobutanol, two CO₂ and one water (equation 13).

As compared to the in vitro n-butanol pathway, this pathway requires eight enzymes without ATP and CoA. The proof-of-concept one-pot experiment achieved a 53% isobutanol yield (Guterl et al. 2012). What is more important, this in vitro system can produce isobutanol even in the presence of 4% isobutanol (Guterl et al. 2012), suggesting that enzyme cocktails can tolerate organic solvents far better than cellular membranes even at elevated temperatures (Zhang 2010a).

2.2.4. Bioelectricity

Enzymatic fuel cells (EFCs) are an emerging electrobiochemical device that directly converts chemical energy of a variety of fuels to electricity through enzymes (Calabrese Barton et al.

2004; Moehlenbrock and Minteer 2008; Zhu et al. 2011). Inspired by living cells that can utilize complex organic compounds (e.g., starch, glycogen) as energy sources, sugar-powered EFCs could be next generation of biodegradable, highly safe, metal-free biobatteries. Compared to microbial fuel cells, EFCs usually generate much higher power density in terms of mW cm^{-2} of membrane or electrode, suggesting their great potentials in powering portable electronics (Cooney et al. 2008; Sakai et al. 2009; Zebda et al. 2011).

To release all electron potentials (i.e., 24) from each glucose unit, a non-natural enzymatic pathway containing 13 enzymes is designed (**Figure 6**). This synthetic pathway contains four modules: (i) G-6-P generation from maltodextrin mediated by alpha-glucan phosphorylase and phosphoglucomutase (equation 14); (ii) 2 NADH generated from G-6-P mediated by two NAD-dependent G-6-P dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) (equation 15); (iii) NADH electro-oxidation through diaphorase (DI) to VK_3 that can generate 2 electrons per NADH (equation 16); and (iv) 5/6 moles of G-6-P regeneration from one mole of ribulose-5-phosphate through a hybrid pathway comprised of enzymes in the pentose phosphate, glycolysis, and gluconeogenesis pathways (equation 17). As a result, each glucose unit of maltodextrin can generate 24 electrons on the anode through this de novo pathway (equation 18). This pathway utilizes two NAD-dependent G6PDH and 6PGDH that can generate NADH in a different way from that of natural NADP-dependent enzymes in the typical pentose phosphate pathway used in anabolism. The above pathway does require neither ATP nor CoA, which are too costly and not stable for EFCs (Sokic-Lazic and Minteer 2008).

Nearly 24 electrons are produced per glucose unit of maltodextrin via this pathway in an air-breathing EFC (Zhu et al. 2014b) and its Coulombic efficiency is higher than that of the best microbial fuel cell (Chaudhuri and Lovley 2003). This EFC also exhibited a maximum power output of 0.8 mW cm^{-2} and a maximum current density of 6 mA cm^{-2} (Zhu et al. 2014b), faster than those of the best microbial fuel cells (Logan 2009). EFCs containing a 15% (wt./v.) maltodextrin solution have an energy storage density of 596 Ah kg^{-1} , which is one order of

magnitude higher than that of lithium-ion batteries. Sugar-powered biobatteries could serve as next-generation green power sources, particularly for portable electronics (Zhu et al. 2014b).

2.3. Examples of Biochemicals

The production of biochemicals from renewable carbohydrates is of importance to not only decrease reliance on fossil fuels but also increase economics of next-generation cellulosic biorefineries, although the market size of biochemicals is at least one order of magnitude lower than that of biofuels.

2.3.1. Synthetic Amylose

Synthetic amylose, a linear glucan linked by alpha-1,4-glycosidic bonds, could be a very important compound as a precursor of low-oxygen diffusion biodegradable plastic films, a promising health food additive, and a potential high-density hydrogen carrier (Qi et al. 2014; You et al. 2013). Cellulose, a linear glucan linked by beta-1,4-glycosidic bonds, is the supporting material of plant cell walls. Annual cellulose resource is approximately 40 times the starch produced by cultivated crops as food and feed (You et al. 2013). In addition, (perennial) cellulosic plants and dedicated bioenergy crops can grow on low-quality or marginal land, and require less input such as fertilizers, herbicides, pesticides, and water (Zhang 2013).

An enzymatic pathway is designed to convert cellulose to amylose without involvement of any coenzymes (You et al. 2013) (**Figure 7**). This pathway includes three modules: (i) partial hydrolysis of cellulose to cellobiose by endoglucanase and cellobiohydrolase (equation 19), (ii) generation of amylose and glucose from cellobiose catalyzed by cellobiose phosphorylase (CBP) and potato alpha-glucan phosphorylase (PGP) (equation 20), and (iii) selective glucose removal by a yeast (equation 21). As a result, a half of cellulose could be used to produce synthetic amylose and the other could be used to produce ethanol (equation 22). Thanks to relatively low yields of cellobiose from cellulose, it leads to a lower amylose yield less than 0.5 g amylose per g of cellulose (You et al. 2013).

Simultaneous enzymatic biotransformation and microbial fermentation can be regarded as modified simultaneous saccharification and fermentation in second generation cellulosic biorefineries, where beta-glucosidase is replaced with CBP/PGP. In this process, all glucan units in cellulose can be utilized to produce amylose, ethanol, and yeast without sugar losses. Therefore, this process has a great potential for future commercialization after solving enzyme costs and enzyme stability. The cost-effective transformation of non-food cellulose to synthetic starch could revolutionize agriculture and reshape the bioeconomy, while maintaining biodiversity, minimizing agriculture's environmental footprint, and conserving fresh water (Somerville et al. 2010; Zhang 2013). This transformation would not only promote the cultivation of plants chosen for rapid growth rather than those optimized for starch-rich seed production, but it would also efficiently utilize marginal land for the production of the biomass required to meet the increasing needs of biofuels and biochemicals (Casillas and Kammen 2010; Sheppard et al. 2011; Zhang 2013).

2.3.2. Lactate

Lactate is the most naturally-occurring hydroxycarboxylic acid (Datta and Henry 2006). It has an exploding application as the precursor of biodegradable plastic polylactic acid (PLA) (Datta and Henry 2006; Joglekar et al. 2006; Zhang et al. 2011a). High yield and high-titer lactate is usually fermented from carbohydrates by anaerobic fermentation, where one glucose can generate two lactate along with two ATP, which supports cell duplication and basic metabolism of whole cells. However, its separation is relatively costly because of impurities, such as residual sugars, nutrients, and other organic acids in broths, the consumption of sulfuric acid and alkali, and the generation of a side-product calcium sulfate (Joglekar et al. 2006; Patnaik et al. 2002).

Honda and his coworkers demonstrated an alternative in vitro ATP-balanced enzymatic pathway that can convert glucose to lactate (Ye et al. 2012a) (**Figure 8**). This pathway is comprised of ten enzymes, containing two modules: (i) generation of two pyruvate and two NADH from one glucose (equation 7), and (ii) production of two lactate from two pyruvate catalyzed by lactate dehydrogenase (equation 23). As a result, one glucose can produce two lactate only (equation 24).

By compromising reaction temperature and optimizing enzyme loading, the maximum lactate yield reached 100% (Ye et al. 2012a). This pathway could be further improved. For example, it is possible to decrease the number of enzymes used from glucose to pyruvate in the isobutanol pathway (**Figure 5**) instead of nine enzymes here (**Figure 8**). Also, this change can avoid using ATP/ADP. Furthermore, in vitro enzymatic lactate production could be conducted at low pHs where enzymes could tolerate low pHs better than whole cells so to decrease separation cost of lactate (Patnaik et al. 2002). Low pH fermentation or biotransformation will bring several benefits: facilitating lactic acid precipitation at its isoelectric point, decreasing the consumption of an alkali for neutralization of lactate and of sulfuric acid for regeneration of lactic acid, and lessen the generation of gypsum waste.

2.3.3. 1,3-Propanediol

1,3-propanediol (1,3-PDO) is a colorless viscous liquid, which can be used as monomer in the production of polymers such as polytrimethylene terephthalate. 1,3-PDO can be produced from glucose (Nakamura and Whited 2003) or glycerol (Dietz and Zeng 2014) by whole-cell fermentation. However, its yields based on glycerol are approximately 0.50-0.76 mol 1,3-PDO/mol glycerol (Celińska 2010; Dietz and Zeng 2014; Zeng and Sabra 2011) due to the synthesis of cell mass, the generation of extra reducing power by branching glycerol utilization pathways under micro-aerobic conditions, and the production of undesired side-products.

Zeng and his coworkers developed a straightforward in vitro pathway for converting glycerol to 1,3-PDO under strictly anaerobic condition (**Figure 9**) (Rieckenberg et al. 2014). This pathway includes two modules: (i) generation of 1,3-PDO from glycerol by consuming one NADPH catalyzed by glycerol dehydratase and NADPH-dependent propanediol oxidoreductase-isoenzyme (equation 25); and (ii) NADPH regeneration from H₂ by hydrogenase (equation 26). As a result, one mole of 1,3-PDO was produced from one mole of glycerol and one mole of H₂ (equation 27). Via this pathway, a very high 1,3-PDO yield of about 0.95 mol/mol has been accomplished (Rieckenberg et al. 2014).

2.3.4. Poly-3-hydroxybutyrate

Poly-3-hydroxybutyrate (P3HB) consisting of 1,000 to 30,000 hydroxy fatty acid monomers is the simplest and most commonly occurring form of polyhydroxyalkanoates, which can be either thermoplastic or elastomeric biodegradable plastics. P3HB is usually produced by microbial fermentation. Its synthesis is usually caused by certain deficiency conditions (e.g., a lack of phosphorous, nitrogen, trace elements, or oxygen) and the excess supply of carbon sources. The intracellular accumulated polyhydroxyalkanoates can be as high as 80% of the dry cell weight. However, its purification is costly and its intracellular polymerization synthesis is difficult to control (Gerngross and Slater 2003).

Munekata and his coworkers (Sato et al. 2003) demonstrate an in vitro pathway to produce P3HB from acetate, where one extra NADPH input is generated from glucose mediated by glucose dehydrogenase. The weight-average molecular weight and polydispersity of in vitro synthesized P3HB are 6.64×10^6 and 1.36, respectively (Sato et al. 2003). Recently, Bowie and his coworkers (Opgenorth et al. 2014) design an in vitro pathway for the production of P3HB from pyruvate without an external NAD(P)H input (**Figure 10**). This in vitro pathway contains three modules: (i) acetyl-CoA generation from pyruvate, co-producing NADH and NADPH by using two NADP-dependent and NAD-dependent pyruvate dehydrogenase, respectively (equation 28); (ii) addition of one unit of P3HB₃ from 2 acetyl-CoA, accompanies with the consumption of NADPH (equation 29); and (iii) oxidation of NADH by using NADH oxidase (NOX). As a result, the whole system has a reaction of equation 31 with balanced coenzymes. This delicate purge valve system based on PDH-1/PDH-2/NOX relieves extra NADPH accumulation and allows the carbon flux to drive forward the desired product. In the optimized system, the maximum yield of P3HB was approximately 94% (Opgenorth et al. 2014).

3. Economic Analysis of the Hydrogen Case

The most appealing biomanufacturing feature of in vitro synthetic biosystems may be nearly theoretical yield of a desired product. Here we conduct a detailed economic analysis of

hydrogen production from carbohydrates. Hydrogen, one of the most important industrial compounds (i.e., annual market size of more than 100 billion US dollars), is mainly produced from natural gas and coal. If hydrogen is cost-effectively produced from carbohydrates by in vitro synthetic biosystems, it is likely that others, more valuable biofuels and biochemicals, will also be amenable to cost-competitive production via in vitro synthetic biosystems.

In vitro synthetic biosystems for biomanufacturing can be regarded as two-step biotransformation: (i) the production of numerous enzymes by whole-cell fermentation followed by their purification and (ii) in vitro biotransformation mediated by enzyme cocktails (Zhang 2011a). Therefore, it is very important to estimate a carbohydrate fraction of hydrogen production to overall sugar consumption for both production of enzymes and hydrogen. All enzymes except hydrogenase (**Figure 3**) can be produced in *E. coli* under aerobic conditions (Myung et al. 2014), where recombinant enzyme yields (Y_{rE}) could range from 0.05 to 0.1 kg protein/kg glucose (Huang and Zhang 2011; Wang et al. 2011; Zhang et al. 2010). In contrast, recombinant active hydrogenase must be produced under anaerobic conditions, and its yields (Y_{H_2ase}) could range from 0.001 to 0.01 kg hydrogenase/kg glucose (Chandrayan et al. 2012; Sun et al. 2010).

Figure 11a shows the effects of enzyme stability (i.e., total turn-over number, TTN) on the carbohydrate fraction of hydrogen production to overall sugar consumption. When enzyme TTN values are low (e.g., 10^{3-5}), most carbohydrate is used to produce enzymes instead of the desired product – hydrogen. When enzyme TTN values are more than 10^7 regardless of recombinant protein yields, more than 99% carbohydrate is used to produce hydrogen. This analysis suggests that enhancing enzyme TTN values is the first priority for high-yield production of hydrogen and high-product yields are possible even considering carbohydrate consumption for enzyme production.

The overall production cost of hydrogen from carbohydrates before tax include (i) feedstock (mainly, carbohydrates) used for enzyme synthesis and hydrogen production, (ii) biocatalysts -- enzymes, (iii) coenzymes, and (iv) capital investment and processing costs. Three key inputs/assumptions are (i) carbohydrate price = \$0.30/kg glucose equivalent (or $6 \times CH_2O$), (ii) coenzyme costs are \$4,500/kg NADP, \$1,500/kg NAD, and \$50/kg benzyl nicotinamide (BNA); and (iii) enzyme costs are \$20/kg recombinant protein and \$100/kg hydrogenase (Rollin et al. 2013).

Figure 11b shows the effects of enzyme stability on hydrogen production costs, when TTN of coenzyme is 10^6 . Clearly, hydrogen production costs decrease exponentially from more than \$100,000 to several US dollars/kg H_2 where TTN of all enzymes increases from 10^3 to 10^8 , suggesting that enhancing enzyme stability is the most important factor for low-cost biomanufacturing. When TTN of enzymes are more than 10^9 , further improvement of enzyme stability has nearly no impact on lowering hydrogen production costs. Similar to this case, no R&D efforts are being made to improve stability of industrial glucose isomerase. When enzymes are stable enough (e.g., TTN = 10^9), the cost of coenzyme greatly determines final hydrogen production cost (e.g., \$8.43/kg H_2 using NADP, \$4.64/kg H_2 using NAD, and \$2.61/kg H_2 using BNA), suggesting the importance of the replacement of high-cost and labile natural coenzymes with low-cost and stable biomimetic coenzymes (Paul et al. 2014; Rollin et al. 2013).

Figure 11c shows the effects of coenzymes' TTN and costs on hydrogen production costs, when TTN of all enzymes is 10^9 . Clearly, increasing TTN of coenzymes exponentially decrease hydrogen production costs, when TTNs are less than 10^5 . When TTN of coenzymes are larger than 10^8 , hydrogen production cost could be as low as \$2.38/kg H_2 . However, the highest TTN for NAD reported is 10^6 (Kazandjian and Klibanov 1985), it is essentially important to replace natural coenzymes with biomimics (Rollin et al. 2013) by considering an increasing stability order of coenzymes from NADP to NAD to BNA (Paul et al. 2014).

Figure 11d shows the effects of carbohydrate costs from \$0.05/kg to \$0.5/kg on the overall hydrogen production cost, where the sum of capital expenses and operation expense is \$0.35/kg H_2 , overall enzyme cost is \$0.13/kg H_2 (i.e., TTN of enzymes = 10^9), and coenzyme cost is \$0.01/kg H_2 (i.e., TTN of BNA = 10^8). It is noted that hydrogen separation costs from the aqueous enzymatic solution are very low; hydrogen and CO_2 can be separated by membrane technology, pressure swing adsorption, or a hybrid of both (Zhang 2009); high-purity hydrogen without CO can be used by proton exchange membrane fuel cells easily (Huang and Zhang 2011). **Figure 11d** shows that the overall hydrogen production costs are proportional to carbohydrate cost. When carbohydrate cost is \$0.2/kg and \$0.3/kg, the hydrogen production costs could be \$1.99/kg and \$2.74/kg of H_2 , respectively. These data are very cost-competitively with hydrogen produced from natural gas without considering two extra benefits: carbon-neutral hydrogen production and distributed hydrogen production systems based on scattered biomass resources. Also, the fraction of carbohydrate cost to overall hydrogen production cost ranges

from 0.435 to 0.885 when carbohydrate costs increase from \$0.05/kg to \$0.5/kg (**Figure 11d**). These data are similar to those ratios of feedstock to final product in mature industrial processes, such as vegetable oil to biodiesel, crude oil to gasoline and diesel, corn kernels to ethanol (Lynd et al. 1999; Zhang 2008).

Distributed hydrogen production from local biomass carbohydrates has multiple advantages over centered hydrogen production from fossil fuels. High capital costs of fossil fuel-to-hydrogen result from its technologically complex, multistep process involving high-pressure and high-temperature gasification, and steam shifting and separation of hydrogen from other products. Critical challenges associated with a lack of high density hydrogen storage approaches, a lack of infrastructure for compressed hydrogen distribution, and the high cost of hydrogen compression lead to serious doubt about the feasibility of the hydrogen economy. Beyond the low-cost and green hydrogen production, the production of high-yield and high-purity hydrogen from local carbohydrate resources via in vitro synthetic biosystems is an out-of-the-box solution to address numerous challenges of the hydrogen economy (Zhang 2009; Zhang 2010b; Zhang 2013; Zhang et al. 2013).

The above analysis suggests that it could be economically feasible to produce cost-competitively hydrogen from carbohydrates and water via in vitro synthetic biosystems. Similar economic analyses may be conducted for the production of other biocommodities. In short, in vitro assembly of purified, stabilized enzymes plus biomimetic coenzymes could have much lower production costs than microbial fermentations, because the former can work much longer, achieve higher product yields, implement faster volumetric productivity, and facilitate easier product separation, and so on (Zhang 2011a).

4. Challenges and Solutions

Large-scale implementation of in vitro synthetic biosystems for biomanufacturing requires synergistic efforts in enzyme engineering and discovery, bulk enzyme production and purification, bioinformatics, synthetic biology, bioprocess engineering, organic chemistry, etc.

4.1. Stable Enzymes as Standardized Building Blocks

As discussed in Section 3, extending the stability of enzymes as building blocks of in vitro synthetic biosystems is essentially important to increase the carbohydrate allocation to the desired product and decrease production costs (**Figure 11a&b**). In general, three strategies can

be conducted to meet the challenge of enzyme stability: immobilization, use of enzymes from thermophilic hosts (thermoenzymes), and enzyme engineering. Whenever possible, a good starting point is to use thermoenzymes. Many examples exist of enzyme discovery from hosts like *Thermotoga maritima*, *Thermus thermophilus*, *Clostridium thermocellum*, *Geobacillus* spp. and so on. Now several websites have provided good collections for putative enzyme sources, for example, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>). When a special enzyme is only available from a mesophilic source, or activity modification is desired, the next tool often used is enzyme engineering, involving rational design and directed evolution or their combination. For example, a large size enzyme cellobiose phosphorylase has been enhanced greatly through rational design and directed evolution combined (Ye et al. 2012b). All recombinant thermoenzymes used in the enzymatic hydrogen production have been produced using *E. coli* (Martín del Campo et al. 2013; Myung et al. 2014).

Enzyme immobilization technologies have been developed for more than a half century. A variety of techniques include physical adsorption, covalent binding to support structures, cross-linked enzyme aggregates, and so on (Zhang et al. 2011b). For example, a combination of thermostable enzyme and enzyme immobilization leads to TTN of phosphoglucose isomerase more than 10^9 at 60 °C (Myung et al. 2011). Another immobilized thermostable glucose isomerase has a working lifetime from a half year to two years at 55 °C (Vasic-Racki 2006). Recent advances in enzyme immobilization on nanomaterials (e.g., polymeric nanogels, nanocrystals, nanomagnetic particles) may preserve enzyme activity to a large degree, while simultaneously increasing stability and facilitating great mass transfer (Ge et al. 2012; Myung et al. 2013). Also, immobilized enzymes can be easily recycled and separated from soluble products/substrates if necessary.

4.2. Synthetic Multiple-Enzyme Complexes (Metabolons) as Building Modules

Natural biological systems have evolved numerous enzyme complexes or create microcompartments containing several cascade enzymes together (Jandt et al. 2013). Such structures promote specific metabolic processes by encapsulating and colocalizing cascade enzymes with their substrates and cofactors, by facilitating substrate channeling, protecting vulnerable enzymes in defined microenvironments, and by sequestering toxic, labile, or volatile intermediates (Kerfeld et al. 2010; You et al. 2012; Zhang 2011b).

The simplest way to obtain multi-function enzymes is the creation of chimeric proteins, in which two or more cascade enzymes are combined by a linker to form a multi-functional single polypeptide (Bulow et al. 1985; Conrado et al. 2008). For example, Bulow et al. constructed a bifunctional enzyme containing cascade reactions mediated by *E. coli* β -galactosidase and galactokinase (Bulow et al. 1985). This synthetic fusion enzyme displayed kinetic advantages (1.5-2.4 fold) over free enzyme mixtures. Similar effects have been reported in other fusion enzymatic systems (Agapakis et al. 2010; Meynial-Salles et al. 2007; Orita et al. 2007; Riedel and Bronnenmeier 1998). However, the misfolding of large multi-domain proteins often happens and decreases their apparent activities (Chang et al. 2005), resulting in a large uncertainty of this strategy.

Scaffolding molecules, includes proteins and nucleic acids, can recruit enzymes to form multienzyme complexes (Aldaye et al. 2008; You et al. 2012) (Wei et al. 2012). Inspired by cellulosomes (Bayer et al. 1994), a synthetic scaffold containing different cohesins from different microorganisms is used for the assembly of three metabolic enzymes (You et al. 2012). Triosephosphate isomerase (TIM), aldolase (ALD), and fructose 1,6-bisphosphatase (FBP) are engineered to have a dockerin at their C-terminals. These three dockerin-containing enzymes can be self-assembled into a static trifunctional enzyme complex through the interaction with a mini-scaffold protein consisting of three different matching cohesins. The synthetic metabolon showed more than one order of magnitude enhancements in reaction rates compared to the non-complexed TIM, ALD, and FBP mixture (You et al. 2012). Furthermore, it is found that the annexation of more high-activity ALD in the synthetic enzyme complexes drastically decreases the degree of substrate channeling by five times (You and Zhang 2014). Either DNA or RNA can be designed to fold into various structures in vitro, forming simple structures such as sheets to very more complicated structures such as tubes and capsules (Aldaye et al. 2008; Wei et al. 2012). It is more convenient to in vitro synthesize oligonucleotide as DNA or RNA scaffolds by using automate oligonucleotide synthesis machines than protein scaffolds. However, the costs of DNA and RNA is far more expensive than that of protein scaffolds produced by whole cells, which may impair their applications on a large scale, especially for the production of biocommodities.

Co-immobilization of multiple enzymes is another very practical choice. Enzyme components can be randomly distributed (Betancor et al. 2006; El-Zahab et al. 2004), positionally assembled (El-Zahab et al. 2004), and even the active site of an enzyme face to the one of

another enzyme (Mansson et al. 1983) on solid supports. The enhanced reaction rates among co-immobilization of cascade enzymes have been observed for several systems (Mateo et al. 2006; Myung et al. 2013; Zhang 2011b), but direct cross linking could lead to loss of enzyme activity (Sheldon and van Pelt 2013).

Regardless of fusion proteins, the use of scaffolds, and co-immobilization, the general rules of constructing such synthetic enzyme complexes need further investigation for their optimal ratios and their orientation.

4.3. Bulk Enzyme Production and Separation

A wide range of bulk enzyme production methods have been explored in recent years (Demain and Vaishnav 2009). Desired enzymes are produced in recombinant forms, using bacteria, fungal, yeast and plant platforms, preferred due to in-depth knowledge of the organisms, ease of genetic manipulation, and low production cost (Demain and Vaishnav 2009). For example, protease is produced at a cost of \$~10 per kg by *B. subtilis*, cellulase is produced at a cost of \$~10 per kg by *Trichoderma* spp. and *A. niger* spp., and recombinant proteins produced at costs of tens of dollars per kg in *E. coli* (Taylor et al. 2008; Zhang and Zhang 2010; Zhang et al. 2006). The DOE Biomass Program even anticipated industrial enzyme production costs as low as \$0.7 per kg of dry enzyme in the future, equaling that of soybean protein (Rollin et al. 2013). To increase recombinant protein expression levels, systematic efforts have been conducted by optimization of codon usage, expression plasmid and host, inducer type, concentration, addition time, and so on. It resulted in ~500-fold enhancement of a hyperthermophilic *T. maritima* 6-phosphogluconate dehydrogenase in *E. coli* (Wang and Zhang 2009b). The other option is overexpression of the target protein in its native organism, such as hydrogenase by *Pyrococcus furiosus* (Chandrayan et al. 2012).

Instead of costly chromatographic separation techniques, several low-cost, scalable enzyme purification approaches have been developed, for example, simple centrifugation for secretory enzymes (e.g., cellulase, amylase, protease), one-step cellulose binding module-tagged enzyme purification and immobilization on low-cost cellulosic materials (Liao et al. 2012; Myung et al. 2011), heat precipitation for thermostable enzymes (Sun et al. 2012; Ye et al. 2012a), and ammonia precipitation (Myung and Zhang 2013). It is noted that the protein of thermostable enzymes in mesophilic hosts can decrease the enzyme purification cost because enzymes cloned from thermophilic microbes can be stable at high temperature (60-80 °C). Therefore,

heat treatment can deactivate *E. coli* endogenous proteins; after centrifugation, only soluble thermostable proteins remain in the supernatant. This heat precipitation provides a simple and less-costly purification method for numerous thermostable enzymes (Krutsakorn et al. 2013a; Krutsakorn et al. 2013b; Sun et al. 2012; Wang and Zhang 2009b; Ye et al. 2012a). Honda and his coworkers attempted to co-expressing nine thermoenzymes in one *E. coli* cell (Ninh et al. 2014). After heat precipitation, they harvested the thermoenzyme cocktail suitable for one-pot biotransformation (Ninh et al. 2014).

Therefore, there are no clear technical obstacles to producing low-cost bulk enzymes suitable for in vitro synthetic biosystems for biomanufacturing.

4.4. NAD(P)-Dependent Enzyme Engineering

NAD and NADP are the predominant energy carriers for cellular metabolisms but they cannot be self-regenerated or self-repaired in vitro. These coenzymes are too costly and easily degraded under some conditions, such as oxygen presence, low or high pH, high temperature, and so on. Their costs allow their use for the synthesis of high-value chiral compounds (Wildeman et al. 2007) but prevent them from the production of biocommodities (**Figure 11c**). The best solution is the replacement of NAD(P) with low-cost and high stability biomimetic cofactors (mNADs) (Paul et al. 2014; Rollin et al. 2013). However, most wild-type NAD(P)-dependent redox enzymes cannot work on mNADs, except flavin-containing monooxygenase (Lutz et al. 2004), enoate reductase (Paul et al. 2013), and diaphorase.

Coenzyme engineering from natural to biomimetic coenzymes remains in the early stage (Campbell et al. 2012; Paul et al. 2014; Rollin et al. 2013). Three approaches are rational design, directed evolution, or swapping modules (Rollin et al. 2013). Clark and Fish demonstrate that an engineered P450 mutant with two amino acid changes can utilize BNA (Ryan et al. 2008). Because of NAD(P)-dependent redox enzymes that have highly conserved cofactor-binding motifs – Rossmann motif, the module swap strategy could be useful for coenzyme specificity switch. For example, Yaoi et al. demonstrate the replacement of the NADP-binding domain of an isocitrate dehydrogenase by an NAD-binding domain, resulting in a change of coenzyme preference (Yaoi et al. 1996). The success of the use of biomimetic coenzymes along with engineered NAD(P)-dependent redox enzymes could greatly expand enzyme-based biocatalysis, whose applications are not restricted by reactions catalyzed by hydrolases and isomerases.

4.5. System Optimization and Modeling

In vitro synthetic biosystems can be modeled at multiple levels from molecules to modules to systems (see a review (Jandt et al. 2013)). The relative simplicity of in vitro biosystems compared to whole cells makes them far easier to simulate processes and predict optimal enzyme ratios for maximizing product yield and accelerating volumetric productivity. Different from great challenges in assays of thousands of intracellular metabolites (Bennett et al. 2009), it is relatively easy to measure tens of metabolites in in vitro biosystems. Also, protein chips allow better pathway construction and optimization in vitro (Jung and Stephanopoulos 2004).

A nonlinear kinetic model was developed to simulate enzymatic hydrogen production and predict rate-limiting steps (Ye et al. 2009). This model suggests that hydrogenase and two dehydrogenases were responsible for catalyzing rate-limiting steps when the same unit of enzymes were used (Ye et al. 2009). Later, Ardao and Zeng (Ardao and Zeng 2013) further improved this in silico model by using a genetic algorithm to solve a multi-objective optimization. As a result, when using yield and rate as objective functions in this model, one set of solutions predicted the ability for this system to retain 90% yield while increasing the reaction rate to 355 mmol/L/h (Ardao and Zeng 2013). Furthermore, parameter setting of this model were fit with experimental data under an elevated temperature of 50°C by using a genetic algorithm, and then a global sensitivity analysis was used to identify the most important enzymes to determine reaction rate and yield improvements (in revision for publication). Via an optimal set of enzyme concentrations predicted by the model, volumetric productivity of hydrogen was increased to 32 mmol H₂/L/h by more than 10 fold. The maximum productivity was further enhanced to 54 mmol H₂/L/h by elevating reaction temperature to 60°C and increasing substrate level. The reaction rate of 54 mmol of H₂ per hour per liter equals 3.7 W/L, approximately 21-time the maximum algal photohydrogen rate. This productivity is comparative to industrial biogas and hydrogen production (Argun and Kargi 2011). In the past decade, enzymatic hydrogen generation has been enhanced from 0.21 (Woodward et al. 2000) to 54 mmol of H₂/L/h by a factor of more than 250. Further increases in volumetric productivity could decrease capital investment for bioreactors. The most ambitious goal is the production of on-demand hydrogen from carbohydrates and water in a small-size hydrogen fuel cell vehicle (Zhang 2009). It means that we would increase hydrogen productivity to 5-10 moles H₂/L/h, by another 100-200 fold.

4.6. Stabilization of Thermolabile Metabolites at Elevated Temperatures

Elevating reaction temperature usually increases reaction rates but may cause the degradation of substrates, products or intermediates. High product yields of in vitro synthetic biosystems are usually accomplished at modest temperatures, when the degradation of metabolites are minimal. High reaction temperatures may decrease product yields. For example, high yield of lactate was obtained at a temperature of 50°C, while 60°C lead to the degradation of glyceraldehyde 3-phosphate, dihydroxacetone phosphate, phosphoenolpyruvate, NADH, etc. (Ye et al. 2012a). Similarly, nearly theoretical yields of hydrogen were obtained at 30-50°C (Martin del Campo et al. 2013a; Myung et al. 2014; Ye et al. 2009), but the yields were lower at 60°C (Rollin et al. 2014).

Hyperthermophiles, whose optimal temperatures are more than 80°C, have evolved several mechanisms for protecting thermo-labile small molecules -- metabolites and coenzymes: rapid turnover, substrate channeling, and local stabilization (Daniel 2000). For example, labile NADPH must be recycled efficiently for an extreme thermoacidophile *Picrophilus torridus* though its halftime is 1.7 min at pH 4.5 and 65 °C (Angelov et al. 2005). To mimic the rapid turnover strategy in vitro, it is suggested that the enzymes responsible for converting easily-degraded metabolites is over-added (Myung et al. 2010) or high catalytic efficiency enzymes are used. Triosephosphate isomerase which is responsible for interconversion of two thermolabile metabolites – glyceraldehyde 3-phosphate and dihydroxacetone phosphate evolves to be kinetically-perfect and keep their levels very low (Zhang 2011b). Inspired to natural enzyme complexes -- tryptophan synthase (Hyde et al. 1988), carbamoyl-phosphate synthetase (Thoden et al. 1997), fatty acid synthase (Maier et al. 2008), the construction of synthetic enzyme complexes that channels labile metabolites among cascade enzymes is a powerful strategy (Cheng et al. 2008; Zhang 2011b). Because it is known that decreasing water activity or dehydration can stabilize a large number of biomolecules, creating desolvating environments for labile metabolites, or binding them around respective enzymes could help decrease their degradation odds. For example, the highest TTN of NAD was achieved in an organic solvent (Kazandjian and Klivanov 1985) and the stability of NAD can be enhanced by its immobilization or binding with its enzymes (Liu and Wang 2007).

Hyperthermophiles generally accumulate very unusual compatible solutes, such as di-myo-inositol-phosphate, di-glycerol-phosphate, ectoines, which have not been identified in mesophiles (Lentzen and Schwarz 2006; Santos and da Costa 2002). These solutes can protect

thermal degradation of cell components and enzymes (Lentzen and Schwarz 2006; Santos and da Costa 2002). Therefore, it is speculated that such compatible solutes may protect metabolites by decreasing water activity. But this hypothesis needs further in vitro testing.

4.7. Scale-Up by Integrative Innovation

The implementation of in vitro synthetic biosystems for biomanufacturing is like making of iPhone by integrating numerous well-known parts and creating a seemingly-new system. Recent advances in DNA sequencing, bioinformatics, recombinant DNA technology, enzyme engineering and immobilization, as well as bulk enzyme production and purification allow to develop highly efficient, tunable enzymes tailored for large-scale industrial production (Bornscheuer et al. 2012). The integration of numerous 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.

The successful implementation of this game-changing platform requires a teamwork of academic researchers, industrial developers, and even policy makers. To our limited knowledge, the only successful example is 100 liter cell-free protein synthesis (Hodgman and Jewett 2012). This case is possible because of relatively easy preparation of the *E. coli* cell lysate instead of their purification and high-value products. It is believed that the success of the first in vitro synthetic biosystem for large-scale biomanufacturing in next several years will erase doubt of its industrial feasibility.

4.8. Other Issues

A few in vitro synthetic pathways have been investigated to help understand in-depth natural pathways that may be used to produce advanced biofuels and biochemicals in vivo. For example, Khosla and his coworkers investigated the detailed kinetics of the fatty acid synthesis using the reconstitution of the purified *E. coli* fatty acid synthase components (Yu et al. 2011). Liu and his co-workers investigated the synthesis of farnesene (a precursor of new jet fuel) through the mevalonate pathway by using purified enzymes (Zhu et al. 2014a). Adams and his coworkers demonstrated the feasibility of production of 3-hydroxypropionic acid, one of the top 12 industrial chemical building blocks, from hydrogen and carbon dioxide by using *P. furiosus* cell-free extract (Keller et al. 2013). However, these in vitro synthetic biosystems have more challenges in future biomanufacturing, such as NAD(P)H imbalance, ATP imbalance, or CoA involvement. For example, when fatty acid ethyl ester is produced from glucose, this pathway

always generates extra NAD(P)H (Huang and Zhang 2011). For microbial cells, they can get rid of extra NADPH easily under aerobic conditions (Steen et al. 2010), but leading to decreased energy conversion efficiencies (Huang and Zhang 2011). To balance extra NADPH in vitro, the possible solutions may co-produce hydrogen from NADPH via hydrogenase under anaerobic conditions, like the case of enzymatic hydrogen production (Martin del Campo et al. 2013a; Myung et al. 2014), or oxidize NADPH oxidization via NADPH oxidase under aerobic conditions, like the case of P3HB synthesis (Opgenorth et al. 2014). Another challenge is the use of and stability of CoA. Similar to the case of NAD(P), small-size biomimetic CoA molecules may be used to replace CoA. If possible, the design of CoA-free pathways may be the first choice as occurred in the synthetic isobutanol pathway (**Figure 5**).

5. Conclusions

Different from whole cell biocatalysis that has been exploited for thousands of years, in vitro synthetic biosystems is on their early stage. The primary goal of any living entities is to duplicate themselves instead of the production of desired products, while the primary goal of in vitro synthetic biosystems is to produce desired products. Although in vitro biosystems could not be used to produce a few mature industrial products, such as ethanol, penicillin, bulk enzymes, living products (e.g., stem cells and tissues), they could be a disruptive biomanufacturing platform for the cost-competitive production of biocommodities due to their unique biomanufacturing advantages (**Table 1**). Great potential markets of biocommodities (e.g., up to trillions of dollars for biofuels, at least tens of billions dollars for biochemicals) will motivate to address remaining obstacles pertaining to cost and stability of enzymes and coenzymes, low-cost production of numerous bulk enzymes, standardization of building parts and modules, biomimetic coenzymes, biosystem optimization and scale-up, with next several decades.

Acknowledgements.

This work was supported by the Shell GameChanger Program and DOE ARPA-E PETRO to PZ as well as NSF STTR I (IIP - 1321528) to Cell Free Bioinnovations Inc. In addition, funding for this work was provided in part, by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture.

958

959 **References**

- 960 Agapakis C, Ducat D, Boyle P, Wintermute E, Way J, Silver P. 2010. Insulation of a synthetic hydrogen
961 metabolism circuit in bacteria. *J. Biol. Eng.* 4(1):3.
- 962 Aldaye FA, Palmer AL, Sleiman HF. 2008. Assembling Materials with DNA as the Guide. *Science*
963 321(5897):1795-1799.
- 964 Angelov A, Fütterer O, Valerius O, Braus GH, Liebl W. 2005. Properties of the recombinant
965 glucose/galactose dehydrogenase from the extreme thermoacidophile, *Picrophilus torridus*. *FEBS*
966 *J.* 272(4):1054-1062.
- 967 Ardao I, Zeng A-P. 2013. *In silico* evaluation of a complex multi-enzymatic system using one-pot and
968 modular approaches: Application to the high-yield production of hydrogen from a synthetic
969 metabolic pathway. *Chem. Eng. Sci.* 87:183–193.
- 970 Argun H, Kargi F. 2011. Bio-hydrogen production by different operational modes of dark and photo-
971 fermentation: an overview. *Int. J. Hydrogen Energy* 36(13):7443-7459.
- 972 Arnold FH. 1998. Enzyme engineering reaches the boiling point. *Proc. Natl. Acad. Sci. USA* 95:2035-
973 2036.
- 974 Atsumi S, Hanai T, Liao JC. 2008. Non-fermentative pathways for synthesis of branched-chain higher
975 alcohols as biofuels. *Nature* 451(7174):86-89.
- 976 Atsumi S, Wu T-Y, Machado IMP, Huang W-C, Chen P-Y, Pellegrini M, Liao JC. 2010. Evolution,
977 genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. *Mol. Syst. Biol.*
978 6(1):449.
- 979 Bayer EA, Morag E, Lamed R. 1994. The cellulosome--a treasure-trove for biotechnology. *Trends*
980 *Biotechnol.* 12:379-386.
- 981 Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD. 2009. Absolute metabolite
982 concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.*
983 5(8):593-599.
- 984 Betancor L, Berne C, Luckarift HR, Spain JC. 2006. Coimmobilization of a redox enzyme and a cofactor
985 regeneration system. *Chem. Commun.*(34):3640-3642.
- 986 Bogorad IW, Lin T-S, Liao JC. 2013. Synthetic non-oxidative glycolysis enables complete carbon
987 conservation. *Nature advance online publication.*
- 988 Boltje TJ, Buskas T, Boons G-J. 2009. Opportunities and challenges in synthetic oligosaccharide and
989 glycoconjugate research. *Nat. Chem.* 1(8):611-622.
- 990 Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC, Robins K. 2012. Engineering the third
991 wave of biocatalysis. *Nature* 485(7397):185-194.
- 992 Bozic M, Pricelius S, Guebitz GM, Kokol V. 2010. Enzymatic reduction of complex redox dyes using
993 NADH-dependent reductase from *Bacillus subtilis* coupled with cofactor regeneration. *Appl.*
994 *Microbiol. Biotechnol.* 85:563-571.
- 995 Bruggink A, Schoevaart R, Kieboom T. 2003. Concepts of nature in organic synthesis: cascade catalysis
996 and multistep conversions in concert. *Org. Proc. Res. Dev.* 7(5):622-640.
- 997 Buchner E. 1897. Alkoholische Gärung ohne Hefezellen (Vorläufige Mitteilung). *Berichte der Deutschen*
998 *Chemischen Gesellschaft* 30:117-124.
- 999 Bujara M, Schümperli M, Pellaux R, Heinemann M, Panke S. 2011. Optimization of a blueprint for in
1000 vitro glycolysis by metabolic real-time analysis. *Nat. Chem. Biol.* 7(5):271-277.
- 1001 Bulow L, Ljungcrantz P, Mosbach K. 1985. Preparation of a soluble bifunctional enzyme by gene fusion.
1002 *Nat. Biotechnol.* 3(9):821-823.
- 1003 Calabrese Barton S, Gallaway J, Atanassov P. 2004. Enzymatic biofuel cells for implantable and
1004 microscale devices. *Chem. Rev.* 104(10):4867-4886.
- 1005 Campbell E, Meredith M, Minter SD, Banta S. 2012. Enzymatic biofuel cells utilizing a biomimetic
1006 cofactor. *Chem. Commun.* 48(13):1898-1900.

1007 Carlson ED, Gan R, Hodgman CE, Jewett MC. 2012. Cell-free protein synthesis: Applications come of
1008 age. *Biotechnol. Adv.* 30:1185-1194.

1009 Casillas CE, Kammen DM. 2010. The Energy-Poverty-Climate Nexus. *Science* 330(6008):1181-1182.

1010 Celińska E. 2010. Debottlenecking the 1,3-propanediol pathway by metabolic engineering. *Biotechnol.*
1011 *Adv.* 28(4):519-530.

1012 Chandrayan SK, McTernan PM, Hopkins RC, Sun JS, Jenney FE, Adams MWW. 2012. Engineering
1013 Hyperthermophilic Archaeon *Pyrococcus furiosus* to Overproduce Its Cytoplasmic NiFe -
1014 Hydrogenase. *J. Biol. Chem.* 287(5):3257-3264.

1015 Chang H-C, Kaiser CM, Hartl FU, Barral JM. 2005. De novo Folding of GFP Fusion Proteins: High
1016 Efficiency in Eukaryotes but Not in Bacteria. *J. Mol. Biol.* 353(2):397-409.

1017 Chaudhuri SK, Lovley DR. 2003. Electricity generation by direct oxidation of glucose in mediatorless
1018 microbial fuel cells. *Nat. Biotechnol.* 21(10):1229-1232.

1019 Chen K, Arnold FH. 1993. Turning the activity of an enzyme for unusual environments: sequential
1020 random mutagenesis of subtilisin E for catalysis in dimethylformamide. *Proc. Natl. Acad. Sci.*
1021 *USA* 90:5618-5622.

1022 Chen X, Nielsen K, Borodina I, Kielland-Brandt M, Karhumaa K. 2011. Increased isobutanol production
1023 in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism. *Biotechnol.*
1024 *Biofuels* 4(1):21.

1025 Cheng Y, Chang C-eA, Yu Z, Zhang Y, Sun M, Leyh TS, Holst MJ, McCammon JA. 2008. Diffusional
1026 channeling in the sulfate-activating complex: Combined continuum modeling and coarse-grained
1027 Brownian dynamics studies. *Biophys. J.* 95(10):4659-4667.

1028 Conrado RJ, Varner JD, DeLisa MP. 2008. Engineering the spatial organization of metabolic enzymes:
1029 Mimicking nature's synergy. *Curr. Opin. Biotechnol.* 19(5):492-499.

1030 Cooney MJ, Svoboda V, Lau C, Martin G, Minteer SD. 2008. Enzyme catalysed biofuel cells. *Energy*
1031 *Environ. Sci.* 1:320-337.

1032 Daniel RM. 2000. Biomolecular stability and life at high temperatures. *Cell. Mol. Life Sci.* 57(2):250-264.

1033 Datta R, Henry M. 2006. Lactic acid: recent advances in products, processes and technologies — a review.
1034 *J. Chem. Tech. Biotechnol.* 81(7):1119-1129.

1035 Demain AL. 2004. Pickles, pectin, and penicillin. *Annu. Rev. Microbiol.* 58:1-42.

1036 Demain AL, Vaishnav P. 2009. Production of recombinant proteins by microbes and higher organisms.
1037 *Biotechnol. Adv.* 27(3):297-306.

1038 Dietz D, Zeng A-P. 2014. Efficient production of 1,3-propanediol from fermentation of crude glycerol
1039 with mixed cultures in a simple medium. *Bioprocess and Biosystems Engineering* 37(2):225-233.

1040 El-Zahab B, Jia H, Wang P. 2004. Enabling multienzyme biocatalysis using nanoporous materials.
1041 *Biotechnol. Bioeng.* 87(2):178-183.

1042 Endo T, Koizumi S. 2000. Large-scale production of oligosaccharides using engineered bacteria. *Curr.*
1043 *Opin. Struct. Biol.* 10(5):536-541.

1044 Ezeji TC, Qureshi N, Blaschek HP. 2007. Bioproduction of butanol from biomass: from genes to
1045 bioreactors. *Curr. Opin. Biotechnol.* 18(3):220-227.

1046 Fessner W-D. 1998. Enzyme mediated C---C bond formation. *Curr. Opin. Chem. Biol.* 2(1):85-97.

1047 Fessner W-D, Helaine V. 2001. Biocatalytic synthesis of hydroxylated natural products using aldolases
1048 and related enzymes. *Curr. Opin. Biotechnol.* 12(6):574-586.

1049 Franke D, Machajewski T, Hsu C-C, Wong C-H. 2003. One-pot synthesis of L-Fructose using coupled
1050 multienzyme systems based on rhamnulose-1-phosphate aldolase. *J. Org. Chem.* 68(17):6828-
1051 6831.

1052 Ge J, Lei J, Zare RN. 2012. Protein-inorganic hybrid nanoflowers. *Nat. Nanotech.* 7(7):428-432.

1053 Gerngross T, Slater S. 2003. Biopolymers and the environment. *Science* 299:822-825.

1054 Goerke AR, Swartz JR. 2008. Development of cell-free protein synthesis platforms for disulfide bonded
1055 proteins. *Biotechnol. Bioeng.* 99(2):351-367.

- Guterl J-K, Garbe D, Carsten J, Steffler F, Sommer B, Reiß S, Philipp A, Haack M, Rühmann B, Ketting U and others. 2012. Cell-free metabolic engineering - Production of chemicals via minimized reaction cascades. *ChemSusChem* 5:2165-2172.
- Hazelwood LA, Daran J-M, van Maris AJA, Pronk JT, Dickinson JR. 2008. The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on *Saccharomyces cerevisiae* Metabolism. *Appl. Environ. Microbiol.* 74(8):2259-2266.
- Higashide W, Li Y, Yang Y, Liao JC. 2011. Metabolic Engineering of *Clostridium cellulolyticum* for Production of Isobutanol from Cellulose. *Appl. Environ. Microbiol.* 77(8):2727-2733.
- Hockenberry A, Jewett MC. 2012. Synthetic in vitro circuits. *Curr. Opin. Chem. Biol.* 16:253-259.
- Hodgman CE, Jewett MC. 2012. Cell-free synthetic biology: Thinking outside the cell. *Metab. Eng.* 14:261-269.
- Honda Y, Kitaoka M, Hayashi K. 2004. Reaction mechanism of chitobiose phosphorylase from *Vibrio proteolyticus*: identification of family 36 glycosyltransferase in *Vibrio*. *Biochem. J.* 377(1):225-232.
- Huang K-T, Wu B-C, Lin C-C, Luo S-C, Chen C, Wong C-H, Lin C-C. 2006. Multi-enzyme one-pot strategy for the synthesis of sialyl Lewis X-containing PSGL-1 glycopeptide. *Carbohydr. Res.* 341(12):2151-2155.
- Huang WD, Zhang Y-HP. 2011. Analysis of biofuels production from sugar based on three criteria: Thermodynamics, bioenergetics, and product separation *Energy Environ. Sci.* 4:784-792.
- Huisman GW, Liang J, Krebber A. 2010. Practical chiral alcohol manufacture using ketoreductases. *Curr. Opin. Chem. Biol.* 14(2):122-129.
- Hyde CC, Ahmed SA, Padlan EA, Miles EW, Davies DR. 1988. Three-dimensional structure of the tryptophan synthase alpha 2 beta 2 multienzyme complex from *Salmonella typhimurium*. *J. Biol. Chem.* 263(33):17857-17871.
- Jandt U, You C, Zhang Y-HP, Zeng AP. 2013. Compartmentalization and Metabolic Channeling for Multienzymatic Biosynthesis: Practical Strategies and Modeling Approaches. *Adv. Biochem. Eng. Biotechnol.* 137:1-25.
- Jankowski MD, Henry CS, Broadbelt LJ, Hatzimanikatis V. 2008. Group contribution method for thermodynamic analysis of complex metabolic networks. *Biophys. J.* 95(3):1487-1499.
- Jewett MC, Calhoun KA, Voloshin A, Wu JJ, Swartz JR. 2008. An integrated cell-free metabolic platform for protein production and synthetic biology. *Mol. Syst. Biol.* 4:57.
- Joglekar HG, Rahman I, Babu S, Kulkarni BD, Joshi A. 2006. Comparative assessment of downstream processing options for lactic acid. *Separation and Purification Technology* 52(1):1-17.
- Johannes TW, Woodyer RD, Zhao H. 2007. Efficient regeneration of NADPH using an engineered phosphite dehydrogenase. *Biotechnol. Bioeng.* 96(1):18-26.
- Jung GY, Stephanopoulos G. 2004. A functional protein chip for pathway optimization and *in vitro* metabolic engineering. *Science* 304(5669):428-431.
- Kanter G, Yang J, Voloshin A, Levy S, Swartz JR, Levy R. 2007. Cell-free production of scFv fusion proteins: an efficient approach for personalized lymphoma vaccines. *Blood* 109(8):3393-3399.
- Katz E, Privman V. 2010. Enzyme-based logic systems for information processing. *Chem. Soc. Rev.* 39(5):1835-1857.
- Kazandjian R, Klibanov A. 1985. Regioselective oxidation of phenols catalyzed by polyphenol oxidase in chloroform. *J. Am. Chem. Soc.* 107:5448-5450.
- Keller MW, Schut GJ, Lipscomb GL, Menon AL, Iwuchukwu IJ, Leuko TT, Thorgersen MP, Nixon WJ, Hawkins AS, Kelly RM and others. 2013. Exploiting microbial hyperthermophilicity to produce an industrial chemical, using hydrogen and carbon dioxide. *Proc. Nat. Acad. Sci. USA* 110:5840-5845.
- Kerfeld CA, Heinhorst S, Cannon GC. 2010. Bacterial Microcompartments. *Annual Review of Microbiology* 64(1):391-408.
- Kim J, Winfree E. 2011. Synthetic *in vitro* transcriptional oscillators. *Mol. Syst. Biol.* 7:1-15.

- 1106 Kohlmann C, Märkle W, Lütz S. 2008. Electroenzymatic synthesis. *J. Mol. Catal. B: Enzymatic* 51(3-
1107 4):57-72.
- 1108 Korman TP, Sahachartsiri B, Li D, Vinokur JM, Eisenberg D, Bowie JU. 2014. A synthetic biochemistry
1109 system for the in vitro production of isoprene from glycolysis intermediates. *Protein Science*
1110 25:576–585.
- 1111 Kragl U, Eckstein M, Kaftzik N. 2002. Enzyme catalysis in ionic liquids. *Curr. Opin. Biotechnol.*
1112 13(6):565-571.
- 1113 Krutsakorn B, Honda K, Ye X, Imagawa T, Bei X, Okano K, Ohtake H. 2013a. In vitro production of n-
1114 butanol from glucose. *Metab. Eng.* 20:84-91.
- 1115 Krutsakorn B, Imagawa T, Honda K, Okano K, Ohtake H. 2013b. Construction of an in vitro bypassed
1116 pyruvate decarboxylation pathway using thermostable enzyme modules and its application to N-
1117 acetylglutamate production. *Microb. Cell Fact.* 12(1):91.
- 1118 Lentzen G, Schwarz T. 2006. Extremolytes: natural compounds from extremophiles for versatile
1119 applications. *Appl. Microbiol. Biotechnol.* 72(4):623-634.
- 1120 Li H, Cann AF, Liao JC. 2010. Biofuels: Biomolecular Engineering Fundamentals and Advances. *Annu.*
1121 *Rev. Chem. Biomol. Eng.* 1(1):19-36.
- 1122 Li S, Wen J, Jia X. 2011. Engineering *Bacillus subtilis* for isobutanol production by heterologous *Ehrlich*
1123 pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression.
1124 *Appl. Microbiol. Biotechnol.*:1-13.
- 1125 Liao HH, Myung S, Zhang Y-HP. 2012. One-step purification and immobilization of thermophilic
1126 polyphosphate glucokinase from *Thermobifida fusca* YX: glucose-6-phosphate generation
1127 without ATP *Appl. Microbiol. Biotechnol.* 93:1109-1117.
- 1128 Liu W, Wang P. 2007. Cofactor regeneration for sustainable enzymatic biosynthesis. *Biotechnol. Adv.*
1129 25(4):369-384.
- 1130 Logan BE. 2009. Exoelectrogenic bacteria that power microbial fuel cells. *Nat. Rev. Microbiol.* 7(5):375-
1131 381.
- 1132 Lutz J, Hollmann F, Ho TV, Schnyder A, Fish RH, Schmid A. 2004. Bioorganometallic chemistry:
1133 biocatalytic oxidation reactions with biomimetic NAD⁺/NADH co-factors and [Cp*Rh(bpy)H]⁺
1134 for selective organic synthesis. *J. Organomet. Chem.* 689(25):4783-4790.
- 1135 Lynd LR, Wyman CE, Gerngross TU. 1999. Biocommodity engineering. *Biotechnol. Prog.* 15:777-793.
- 1136 Maier T, Leibundgut M, Ban N. 2008. The crystal structure of a mammalian fatty acid synthase. *Science*
1137 321(5894):1315-1322.
- 1138 Mansson MO, Siegbahn N, Mosbach K. 1983. Site-to-site directed immobilization of enzymes with bis-
1139 NAD analogues. *Proc. Nat. Acad. Sci. USA* 80(6):1487-1491.
- 1140 Martín del Campo JS, Rollin J, Myung S, Chun Y, Chandrayan S, Patiño R, Adams MWW, Zhang Y-HP.
1141 2013. High-Yield Production of Dihydrogen from Xylose by Using a Synthetic Enzyme Cascade
1142 in a Cell-Free System. *Angew. Chem. Int. Ed.* 52(17):4587-4590.
- 1143 Martín del Campo JS, Rollin J, Myung S, You C, Chandrayan S, Patiño R, Adams MWW, Zhang YHP.
1144 2013a. High-yield production of dihydrogen from xylose by using a synthetic enzyme cascade in
1145 a cell-free system. *Angew. Chem. Int. Ed.* 52(17):4587-4590.
- 1146 Martín del Campo JS, You C, Kim J-E, Patiño R, Zhang Y-HP. 2013b. Discovery and Characterization of
1147 a Novel ATP/Polyphosphate Xylulokinase from a Hyperthermophilic Bacterium *Thermotoga*
1148 *maritima*. *J. Ind. Microbiol. Biotechnol.* 40:661-669.
- 1149 Mateo C, Chmura A, Rustler S, van Rantwijk F, Stolz A, Sheldon RA. 2006. Synthesis of
1150 enantiomerically pure (S)-mandelic acid using an oxynitrilase-nitrilase bienzymatic cascade: a
1151 nitrilase surprisingly shows nitrile hydratase activity. *Tetrahedron: Asymmetry* 17(3):320-323.
- 1152 Matthaei JH, Jones OW, Martin RG, Nirenberg MW. 1962. Characteristics and composition of RNA
1153 coding units. *Proc. Nat. Acad. Sci. USA* 48(4):666-677.
- 1154 Mertens R, Liese A. 2004. Biotechnological applications of hydrogenases. *Curr. Opin. Biotechnol.*
1155 15(4):343-348.

1156 Meynial-Salles I, Forchhammer N, Croux C, Girbal L, Soucaille P. 2007. Evolution of a *Saccharomyces*
1157 *cerevisiae* metabolic pathway in *Escherichia coli*. *Metab. Eng.* 9(2):152-159.

1158 Moehlenbrock M, Minter S. 2008. Extended lifetime biofuel cells. *Chem. Soc. Rev.* 37:1188-1196.

1159 Moradian A, Benner SA. 1992. A biomimetic biotechnological process for converting starch to fructose:
1160 thermodynamic and evolutionary considerations in applied enzymology. *J. Am. Chem. Soc.*
1161 114(18):6980-6987.

1162 Myung S, Rollin J, You C, Sun F, Chandrayan S, Adams MWW, Zhang Y-HP. 2014. In vitro metabolic
1163 engineering of hydrogen production at theoretical yield from sucrose. *Metab. Eng.* 24(1):70-77.

1164 Myung S, Wang YR, Zhang Y-HP. 2010. Fructose-1,6-bisphosphatase from a hyper-thermophilic
1165 bacterium *Thermotoga maritima*: Characterization, metabolite stability and its implications. *Proc.*
1166 *Biochem.* 45:1882-1887.

1167 Myung S, You C, Zhang Y-HP. 2013. Recyclable Cellulose-Containing Magnetic Nanoparticles:
1168 Immobilization of Cellulose-Binding Module-Tagged Proteins and Synthetic Metabolon
1169 Featuring Substrate Channeling. *J. Mater. Chem. B* 1:4419-4427.

1170 Myung S, Zhang X-Z, Zhang Y-HP. 2011. Ultra-stable phosphoglucose isomerase through
1171 immobilization of cellulose-binding module-tagged thermophilic enzyme on low-cost high-
1172 capacity cellulosic adsorbent. *Biotechnol. Prog.* 27:969-975.

1173 Myung S, Zhang Y-HP. 2013. Non-complexed four cascade enzyme mixture: simple purification and
1174 synergetic co-stabilization. *PLoS One* 8:e61500.

1175 Nakamura CE, Whited GM. 2003. Metabolic engineering for the microbial production of 1,3-propanediol.
1176 *Current Opinion in Biotechnology* 14(5):454-459.

1177 Ninh PH, Honda K, Sakai T, Okano K, Ohtake H. 2014. Assembly and Multiple Gene Expression of
1178 Thermophilic Enzymes in *Escherichia coli* for In Vitro Metabolic Engineering. *Biotechnol.*
1179 *Bioeng.*:10.1002/bit.25338.

1180 Noireaux V. 2003. Principles of cell-free genetic circuit assembly. *Proc. Natl. Acad. Sci. USA*
1181 100:12672-12677.

1182 Opgenorth PH, Korman TP, Bowie JU. 2014. A synthetic biochemistry molecular purge valve module
1183 that maintains redox balance. *Nat. Commun.* 5:4113.

1184 Orita I, Sakamoto N, Kato N, Yurimoto H, Sakai Y. 2007. Bifunctional enzyme fusion of 3-hexulose-6-
1185 phosphate synthase and 6-phospho-3-hexuloisomerase. *Appl. Microbiol. Biotechnol.* 76(2):439-
1186 445.

1187 Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, del Cardayre S. 2002. Genome
1188 shuffling of *Lactobacillus* for improved acid tolerance. *Nat. Biotechnol.* 20(7):707-712.

1189 Paul CE, Arends IWCE, Hollmann F. 2014. Is simpler better? synthetic nicotinamide cofactor analogues
1190 for redox chemistry. *ACS Catal.* 4:788-797.

1191 Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD. 2012. Microbial engineering for the
1192 production of advanced biofuels. *Nature* 488(7411):320-328.

1193 Qi P, You C, Zhang YHP. 2014. One-Pot Enzymatic Conversion of Sucrose to Synthetic Amylose by
1194 using Enzyme Cascades. *ACS Catalysis* 4:1311-1317.

1195 Resnick SM, Zehnder AJB. 2000. *In vitro* ATP regeneration from polyphosphate and AMP by
1196 polyphosphate:AMP phosphotransferase and adenylate kinase from *Acinetobacter johnsonii*
1197 210A. *Appl. Environ. Microbiol.* 66(5):2045-2051.

1198 Rieckenberg F, Ardao I, Rujananon R, Zeng A-P. 2014. Cell-free synthesis of 1,3-propanediol from
1199 glycerol with a high yield. *Eng. Life Sci.* 14:380-386.

1200 Riedel K, Bronnenmeier K. 1998. Intramolecular synergism in an engineered exo-endo-1,4- β -glucanase
1201 fusion protein. *Mol. Microbiol.* 28:767-775.

1202 Rodriguez SB, Leyh TS. 2014. An Enzymatic Platform for the Synthesis of Isoprenoid Precursors. *PLoS*
1203 *ONE* 9(8):e105594.

1204 Rollin JA, Tam W, Zhang Y-HP. 2013. New biotechnology paradigm: cell-free biosystems for
1205 biomanufacturing. *Green Chemistry* 15:1708-1719.

1206 Rollin JA, Ye XH, Martin dCJS, Adams MWW, Zhang Y-HP. 2014. Novel hydrogen detection apparatus
 1207 along with bioreactor Systems. Adv. Biochem. Eng. Biotechnol.:DOI: 10.1007/10_2014_274.
 1208 Rupp S. 2013. New Bioproduction Systems: From Molecular Circuits to Novel Reactor Concepts in Cell-
 1209 Free Biotechnology. Springer Berlin Heidelberg. p 1-21.
 1210 Ryan JD, Fish RH, Clark DS. 2008. Engineering cytochrome P450 enzymes for improved activity
 1211 towards biomimetic 1,4-NADH cofactors. ChemBioChem 9(16):2579-2582.
 1212 Sakai H, Nakagawa T, Tokita Y, Hatazawa T, Ikeda T, Tsujimura S, Kano K. 2009. A high-power
 1213 glucose/oxygen biofuel cell operating under quiescent conditions. Energy Environ. Sci. 2:133-
 1214 138
 1215 Santacoloma PA, Sin Gr, Gernaey KV, Woodley JM. 2010. Multienzyme-catalyzed processes: next-
 1216 generation biocatalysis. Org. Proc. Res. Dev. 15(1):203-212.
 1217 Santos H, da Costa MS. 2002. Compatible solutes of organisms that live in hot saline environments.
 1218 Environ. Microbiol. 4(9):501-509.
 1219 Satoh Y, Tajima K, Tannai H, Munekata M. 2003. Enzyme-catalyzed poly(3-hydroxybutyrate) synthesis
 1220 from acetate with CoA recycling and NADPH regeneration in Vitro. J. Biosci. Bioeng. 95(4):335-
 1221 341.
 1222 Schoevaart R, van Rantwijk F, Sheldon RA. 2000. A four-step enzymatic cascade for the one-pot
 1223 synthesis of non-natural carbohydrates from glycerol. J. Org. Chem. 65(21):6940-6943.
 1224 Schoffelen S, van Hest JCM. 2012. Multi-enzyme systems: bringing enzymes together in vitro. Soft
 1225 Matter 8(6):1736-1746.
 1226 Schultheisz HL, Szymczyna BR, Scott LG, Williamson JR. 2008. Pathway engineered enzymatic de
 1227 Novo purine nucleotide synthesis. ACS Chem. Biol. 3(8):499-511.
 1228 Schultheisz HL, Szymczyna BR, Williamson JR. 2009. Enzymatic synthesis and structural
 1229 characterization of ¹³C, ¹⁵N-poly(ADP-ribose). J. Am. Chem. Soc. 131(40):14571-14578.
 1230 Scism RA, Bachmann BO. 2010. Five-component cascade synthesis of nucleotide analogues in an
 1231 engineered self-immobilized enzyme aggregate. ChemBioChem 11(1):67-70.
 1232 Serdakowski A, Dordick J. 2008. Enzyme activation for organic solvents made easy. Trends Biotechnol
 1233 26:48-54.
 1234 Shaw A, Bott R, Day AG. 1999. Protein engineering of α -amylase for low pH performance. Curr. Opin.
 1235 Biotechnol. 10:349-352.
 1236 Sheldon RA, van Pelt S. 2013. Enzyme immobilisation in biocatalysis: why, what and how. Chemical
 1237 Society Reviews.
 1238 Sheppard AW, Gillespie I, Hirsch M, Begley C. 2011. Biosecurity and sustainability within the growing
 1239 global bioeconomy. Curr. Opin. Environ. Sustain. 3(1-2):4-10.
 1240 Smith K, Cho K-M, Liao J. 2010. Engineering *Corynebacterium glutamicum* for isobutanol production.
 1241 Appl. Microbiol. Biotechnol. 87(3):1045-1055.
 1242 Sokic-Lazic D, Minteer SD. 2008. Citric acid cycle biomimic on a carbon electrode. Biosens. Bioelectron.
 1243 24(4):939-944.
 1244 Somerville C, Youngs H, Taylor C, Davis SC, Long SP. 2010. Feedstocks for Lignocellulosic Biofuels.
 1245 Science 329(5993):790-792.
 1246 Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, del Cardayre SB, Keasling JD. 2010.
 1247 Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature
 1248 463(7280):559-562.
 1249 Steffler F, Sieber V. 2013. Refolding of a Thermostable Glyceraldehyde Dehydrogenase for Application
 1250 in Synthetic Cascade Biomanufacturing. PLoS ONE 8(7):e70592.
 1251 Sun FF, Zhang XZ, Myung S, Zhang Y-HP. 2012. Thermophilic *Thermotoga maritima* ribose-5-
 1252 phosphate isomerase RpiB: Optimized heat treatment purification and basic characterization.
 1253 Protein Expr. Purif. 82:302-307.
 1254 Sun J, Hopkins RC, Jenney FE, McTernan PM, Adams MWW. 2010. Heterologous expression and
 1255 maturation of an NADP-dependent [NiFe]-hydrogenase: a key enzyme in biofuel production.
 1256 PLoS One 5(5):e10526.

1257 Swartz JR. 2013. Cell-free bioprocessing. *Chem. Eng. Prog.* 2013(11):40-45.

1258 Taylor LEI, Dai Z, Decker SR, Brunecky R, Adney WS, Ding S-Y, Himmel ME. 2008. Heterologous
1259 expression of glycosyl hydrolases in planta: a new departure for biofuels. *Trends Biotechnol.*
1260 26:413-424

1261 Thauer K, Jungermann K, Decker K. 1977. Energy conservation in chemotrophic anaerobic bacteria.
1262 *Bacteriol. Rev.* 41:100-180.

1263 Thauer RK, Kaster AK, Seedorf H, Buckel W, Hedderich R. 2008. Methanogenic archaea: ecologically
1264 relevant differences in energy conservation. *Nat. Rev. Microbiol.* 6:579-591.

1265 Thoden JB, Holden HM, Wesenberg G, Raushel FM, Rayment I. 1997. Structure of carbamoyl phosphate
1266 synthetase: A Journey of 96 Å from substrate to product. *Biochemistry* 36(21):6305-6316.

1267 Vasic-Racki D. 2006. History of industrial biotransformations -- Dreams and realities. In: Liese A,
1268 Seebald S, Wandrey C, editors. *Industrial Biotransformations*. KGaA, Weinheim: Wiley-VCH. p
1269 1-37.

1270 Wang Y, Huang W, Sathitsuksanoh N, Zhu Z, Zhang Y-HP. 2011. Biohydrogenation from biomass sugar
1271 mediated by *in vitro* synthetic enzymatic pathways. *Chem. Biol.* 18:372-380.

1272 Wang Y, Zhang Y-HP. 2009a. Cell-free protein synthesis energized by slowly-metabolized maltodextrin.
1273 *BMC Biotechnol.* 9:58.

1274 Wang Y, Zhang Y-HP. 2009b. Overexpression and simple purification of the *Thermotoga maritima* 6-
1275 phosphogluconate dehydrogenase in *Escherichia coli* and its application for NADPH regeneration.
1276 *Microb. Cell Fact.* 8:30.

1277 Wei B, Dai M, Yin P. 2012. Complex shapes self-assembled from single-stranded DNA tiles. *Nature*
1278 485(7400):623-626.

1279 Welch P, Scopes RK. 1985. Studies on cell-free metabolism: Ethanol production by a yeast glycolytic
1280 system reconstituted from purified enzymes. *J Biotechnol* 2(5):257-273.

1281 Wichmann R, Vasic-Racki D. 2005. Cofactor regeneration at the lab scale. *Adv. Biochem. Eng.*
1282 *Biotechnol.* 92:225-260.

1283 Wildeman SMAD, Sonke T, Schoemaker HE, May O. 2007. Biocatalytic reductions: From lab curiosity
1284 to "first choice". *Acc. Chem. Res.* 40:1260-1266.

1285 Wong CH, Daniels L, Ormejohnson WH, Whitesides GM. 1981. Enzyme-catalyzed organic-synthesis -
1286 NAD(P)H regeneration using dihydrogen and the hydrogenase from *Methanobacterium*
1287 *thermoautotrophicum*. *J Am Chem Soc* 103(20):6227-6228.

1288 Wong CH, Whitesides GM. 1981. Enzyme-catalyzed organic-synthesis - NAD(P)H cofactor regeneration
1289 by using glucose-6-phosphate and the glucose-6-phosphate-dehydrogenase from *Leuconostoc-*
1290 *Mesenteroides*. *J Am Chem Soc* 103(16):4890-4899.

1291 Woodward J, Orr M, Cordray K, Greenbaum E. 2000. Enzymatic production of biohydrogen. *Nature*
1292 405:1014-1015.

1293 Wyman CE. 1999. Biomass ethanol: technical progress, opportunities, and commercial challenges. *Annu.*
1294 *Rev. Energy Environ.* 24:189-226.

1295 Xu Y, Cai C, Chandarajoti K, Hsieh P-H, Li L, Pham TQ, Sparkenbaugh EM, Sheng J, Key NS,
1296 Pawlinski R and others. 2014. Homogeneous low-molecular-weight heparins with reversible
1297 anticoagulant activity. *Nat. Chem. Biol.* advance online publication.

1298 Xu Y, Masuko S, Takieddin M, Xu H, Liu R, Jing J, Mousa SA, Linhardt RJ, Liu J. 2011.
1299 Chemoenzymatic synthesis of homogeneous ultralow molecular weight heparins. *Science*
1300 334(6055):498-501.

1301 Xu Z, Jing K, Liu Y, Cen P. 2007. High-level expression of recombinant glucose dehydrogenase and its
1302 application in NADPH regeneration. *J Ind. Microbiol. Biotechnol.* 34(1):83-90.

1303 Yaoi T, Miyazaki K, Oshima T, Komukai Y, Go M. 1996. Conversion of the coenzyme specificity of
1304 isocitrate dehydrogenase by module replacement. *J. Biochem.* 119(5):1014-1018.

1305 Ye X, Honda K, Sakai T, Okano K, Omasa T, Hirota R, Kuroda A, Ohtake H. 2012a. Synthetic metabolic
1306 engineering-a novel, simple technology for designing a chimeric metabolic pathway. *Microb. Cell*
1307 *Fact.* 11(1):120.

- Ye X, Wang Y, Hopkins RC, Adams MWW, Evans BR, Mielenz JR, Zhang Y-HP. 2009. Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails. *ChemSusChem* 2(2):149-152.
- Ye X, Zhang C, Zhang Y-HP. 2012b. Engineering a large protein by combined rational and random approaches: stabilizing the *Clostridium thermocellum* cellobiose phosphorylase. *Mol. BioSyst.* 8:1815-1823.
- Yim H, Haselbeck R, Niu W, Pujol-Baxley C, Burgard A, Boldt J, Khandurina J, Trawick JD, Osterhout RE, Stephen R and others. 2011. Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat. Chem. Biol.* 7(7):445-452.
- Yordanov B, Kim J, Petersen RL, Shudy A, Kulkarni VV, Phillips A. 2014. Computational Design of Nucleic Acid Feedback Control Circuits. *ACS Syn. Biol.* 3(8):600-616.
- You C, Chen H, Myung S, Sathitsuksanoh N, Ma H, Zhang X-Z, Li J, Zhang Y-HP. 2013. Enzymatic transformation of nonfood biomass to starch. *Proc. Nat. Acad. Sci. USA* 110:7182-7187.
- You C, Myung S, Zhang Y-HP. 2012. Facilitated substrate channeling in a self-assembled trifunctional enzyme complex. *Angew. Chem. Int. Ed.* 51:8787-8790.
- You C, Zhang Y-HP. 2013. Cell-free biosystems for biomanufacturing. *Adv. Biochem. Eng. Biotechnol.* 131:89-119.
- You C, Zhang YHP. 2014. Annexation of a high-activity enzyme in a synthetic three-enzyme complex greatly decreases the degree of substrate channeling. *ACS Syn. Biol.* 3:380-386.
- Yu X, Liu T, Zhu F, Khosla C. 2011. In vitro reconstitution and steady-state analysis of the fatty acid synthase from *Escherichia coli*. *Proc. Nat. Acad. Sci. USA* 108(46):18643-18648.
- Zebda A, Gondran C, Le Goff A, Holzinger M, Cinquin P, Cosnier S. 2011. Mediatorless high-power glucose biofuel cells based on compressed carbon nanotube-enzyme electrodes. *Nat. Commun.* 2:370.
- Zeng A-P, Sabra W. 2011. Microbial production of diols as platform chemicals: Recent progresses. *Curr. Opin. Biotechnol.* 22(6):749-757.
- Zhang J, Shao J, Kowal P, Wang PG. 2005. *Enzymatic Synthesis of Oligosaccharides*: Wiley-VCH Verlag GmbH & Co. KGaA. 137-167 p.
- Zhang X-Z, Sathitsuksanoh N, Zhu Z, Zhang Y-HP. 2011a. One-step production of lactate from cellulose as sole carbon source without any other organic nutrient by recombinant cellulolytic *Bacillus subtilis*. *Metab. Eng.* 13:364-372.
- Zhang X-Z, Zhang Y-HP. 2010. One-step production of biocommodities from lignocellulosic biomass by recombinant cellulolytic *Bacillus subtilis*: Opportunities and challenges. *Eng. Life Sci.* 10:398-406.
- Zhang Y-HP. 2008. Reviving the carbohydrate economy via multi-product biorefineries. *J. Ind. Microbiol. Biotechnol.* 35(5):367-375.
- Zhang Y-HP. 2009. A sweet out-of-the-box solution to the hydrogen economy: is the sugar-powered car science fiction? *Energy Environ. Sci.* 2(2):272-282.
- Zhang Y-HP. 2010a. Production of biocommodities and bioelectricity by cell-free synthetic enzymatic pathway biotransformations: Challenges and opportunities. *Biotechnol. Bioeng.* 105:663-677.
- Zhang Y-HP. 2010b. Renewable carbohydrates are a potential high density hydrogen carrier. *Int. J. Hydrogen Energy* 35:10334-10342.
- Zhang Y-HP. 2011a. Simpler is better: high-yield and potential low-cost biofuels production through cell-free synthetic pathway biotransformation (SyPaB). *ACS Catal.* 1:998-1009.
- Zhang Y-HP. 2011b. Substrate channeling and enzyme complexes for biotechnological applications. *Biotechnol. Adv.* 29:715-725.
- Zhang Y-HP. 2011c. What is vital (and not vital) to advance economically-competitive biofuels production. *Proc. Biochem.* 46:2091-2110.
- Zhang Y-HP. 2013. Next generation biorefineries will solve the food, biofuels, and environmental trilemma in the energy-food-water nexus. *Energy Sci. Eng.* 1:27-41.

1358 Zhang Y-HP, Evans BR, Mielenz JR, Hopkins RC, Adams MWW. 2007. High-yield hydrogen production
1359 from starch and water by a synthetic enzymatic pathway. PLoS One 2(5):e456.
1360 Zhang Y-HP, Himmel M, Mielenz JR. 2006. Outlook for cellulase improvement: Screening and selection
1361 strategies. Biotechnol. Adv. 24(5):452-481.
1362 Zhang Y-HP, Huang W-D. 2012. Constructing the electricity-carbohydrate-hydrogen cycle for a
1363 sustainability revolution. Trends Biotechnol. 30(6):301-306.
1364 Zhang Y-HP, Myung S, You C, Zhu Z, Rollin JA. 2011b. Toward low-cost biomanufacturing through in
1365 vitro synthetic biology: bottom-up design. J. Mater. Chem. 21(47):18877-18886.
1366 Zhang Y-HP, Sun J-B, Zhong J-J. 2010. Biofuel production by *in vitro* synthetic pathway transformation.
1367 Curr. Opin. Biotechnol. 21:663-669.
1368 Zhang Y-HP, Xu J-H, Zhong JJ. 2013. A new high-energy density hydrogen carrier - carbohydrate -
1369 might be better than methanol. Int. J. Energy Res. 37:769-779.
1370 Zheng H, Ohno Y, Nakamori T, Suye S-I. 2009. Production of L-malic acid with fixation of HCO_3^- by
1371 malic enzyme-catalyzed reaction based on regeneration of coenzyme on electrode modified by
1372 layer-by-layer self-assembly method. J. Biosci. Bioeng. 107(1):16-20.
1373 Zhu F, Zhong X, Hu M, Lu L, Deng Z, Liu T. 2014a. In vitro reconstitution of mevalonate pathway and
1374 targeted engineering of farnesene overproduction in *Escherichia coli*. Biotechnol. Bioeng.
1375 111:1396-1405.
1376 Zhu Z-G, Kin Tam T, Sun F, You C, Zhang Y-HP. 2014b. A high-energy-density sugar biobattery based
1377 on a synthetic enzymatic pathway. Nat. Commun. 5:3026.
1378 Zhu ZG, Sun F, Zhang X, Zhang Y-HP. 2012. Deep oxidation of glucose in enzymatic fuel cells through
1379 a synthetic enzymatic pathway containing a cascade of two thermostable dehydrogenases.
1380 Biosens. Bioelectron. 36:110-115.
1381 Zhu ZG, Wang YR, Minteer S, Zhang Y-HP. 2011. Maltodextrin-powered enzymatic fuel cell through a
1382 non-natural enzymatic pathway. J. Power Sources 196:7505-7509.

1383

Figure Legends

Figure 1. Schemes of biotransformation catalyzed by whole cell (A), cell extract (B), and in vitro synthetic biosystem (C).

Figure 2. History of biomanufacturing catalyzed by whole cells and in vitro (cell-free) biosystems associated with key milestones.

Figure 3. Scheme of in vitro synthetic enzymatic pathways for the production of high-yield hydrogen from a variety of carbohydrates -- starch, cellodextrin, sucrose, glucose, fructose and xylose as well as water. The pathways are compiled and modified from References (Martín del Campo et al. 2013; Myung et al. 2014; Rollin et al. 2014; Ye et al. 2009; Zhang et al. 2007). The enzymes are α GP, alpha-glucan phosphorylase; CDP, cellodextrin phosphorylase; CBP, cellobiose phosphorylase; SP, sucrose phosphorylase; GI, glucose isomerase; XI, xylose isomerase; PPGK, polyphosphate glucokinase; PPXK, polyphosphate xylulokinase; PGM, phosphoglucomutase; G6PDH, glucose 6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; RPI, ribose 5-phosphate isomerase; RPE, ribulose-5-phosphate 3-epimerase; TK, transketolase; TAL, transaldolase; TIM, triose phosphate isomerase; ALD, (fructose-bisphosphate) aldolase; FBP, fructose bisphosphatase; PGI, phosphoglucose isomerase; and H_2 ase. P_i and $(P_i)_n$ are inorganic phosphate and polyphosphate with a degree of polymerization of n. The metabolites are: g1p, glucose-1-phosphate; g6p, glucose-6-phosphate; 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, dihydroxacetone phosphate; fdp, fructose-1,6-diphosphate; f6p, fructose 6-phosphate.

Figure 4. Scheme of the in vitro synthetic enzymatic pathway for the production of n-butanol from glucose, modified from Ref. (Krutsakorn et al. 2013a). The enzymes are HK, hexokinase; PGI, phosphoglucose isomerase; PFK, 6-phosphofructokinase; FBA, fructose-bisphosphate aldolase; TIM, triosephosphate isomerase; GAPN, non-phosphorylating GAP dehydrogenase; PGM, cofactor-independent phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; PDC, pyruvate decarboxylase; ADDH, CoA-acylating aldehyde dehydrogenase; HBD, hydroxybutyryl-CoA dehydrogenase; HPD, 3-hydroxypropionyl-CoA dehydratase; NFO, NADH-dependent flavinoxidoreductase; and HAD, 3-hydroxyacyl-CoA dehydrogenase. Metabolites are

g6p, glucose-6-phosphate; f6p, fructose 6-phosphate; fdp, fructose-1,6-diphosphate; g3p, glyceraldehyde 3-phosphate; dhap, dihydroxyacetone phosphate; 3pg, 3-phosphoglycerate; 2pg, 2-phosphoglycerate; and pep, phosphoenolpyruvate.

Figure 5. Scheme of the in vitro ATP-free synthetic enzymatic pathway for the production of isobutanol from glucose, modified from Ref. (Guterl et al. 2012). The enzymes are GDH, glucose dehydrogenase; DHAD, dihydroxy acid dehydratase; AIDH, glyceraldehyde dehydrogenase; KDGA, 2-keto-3-desoxygluconate aldolase; ALS, acetolactate synthase; KARI, ketolacid reductoisomerase; KDC, 2-ketoacid decarboxylase; and ALD, alcohol dehydrogenase.

Figure 6. Scheme of the in vitro ATP-free synthetic enzymatic pathway for the production of electricity from maltodextrin, modified from Ref. (Zhu et al. 2014b). Most enzymes and metabolites are the same as them in Figure 2, except the enzyme DI, diaphorase.

Figure 7. Scheme of the in vitro ATP-free synthetic enzymatic pathway for the production of synthetic amylose and ethanol from cellulose, modified from Ref. (You et al. 2013). The enzymes are EG, endoglucanase; CBH, cellobiohydrolase; CBP, cellobiose phosphorylase; and PGP, potato alpha-glucan phosphorylase.

Figure 8. Scheme of the in vitro ATP-neutral synthetic enzymatic pathway for the production of lactate from glucose, modified from Ref. (Ye et al. 2012a). Enzymes are HK, hexokinase; PGI, g6p isomerase; PFK, 6-phosphofructokinase; FBA, fructose-bisphosphate aldolase; TIM, triosephosphate isomerase; GAPN, non-phosphorylating GAP dehydrogenase; PGM, cofactor-independent phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; and MLDH, malate/lactate dehydrogenase.

Figure 9. Scheme of the in vitro synthetic enzymatic pathway for the production of 1,3-propanediol from glycerol, modified from Ref. (Rieckenberg et al. 2014). Enzymes are GDH, glycerol dehydratase; PDORI, NADPH-dependent propanediol xidoreductase-isoenzyme; and H₂ase, hydrogenase. VB12, which a key coenzyme for GDH, may be repaired in vitro for a long time running.

Figure 10. Scheme of the in vitro synthetic enzymatic pathway for the production of poly-(R)-3-hydroxybutyrate from pyruvate, modified from Ref. (Opgenorth et al. 2014). Enzymes are PDH,

pyruvate dehydrogenase; ACC, acetyl-CoA acetyltransferase; PhaB, 3-hydroxybutyryl-CoA reductase; PhaC, polyhydroxybutyrate synthase; and NOX, NADH oxidase.

Figure 11. Economic analysis of enzymatic hydrogen production from carbohydrates. (a) The carbohydrate allocation to hydrogen production relative to overall carbohydrate consumption in terms of enzyme TTN. $Y(rE)$ is the protein yield based on carbohydrate consumption by aerobic fermentation in terms of kg recombinant protein per kg of carbohydrate, $Y(H_2ase)$ is the hydrogenase yield based on carbohydrate consumption by anaerobic fermentation in terms of kg recombinant protein per kg of carbohydrate. (b) Effects of enzyme TTN and coenzyme costs. (c) Effects of coenzyme TTN and coenzyme type. (d) Effects of carbohydrate costs on final hydrogen production costs.

Table 1.

Comparison of biomanufacturing features by microbial cells and in vitro synthetic biosystems.

Feature	Microbial cells	In vitro synthetic biosystems	Comparative examples	Ref.
Product yield	Low-modest	Very high	Hydrogen	(Martín del Campo et al. 2013; Myung et al. 2014)
			1,3-propanediol	(Rieckenberg et al. 2014)
			Electricity	(Zhu et al. 2014b)
			Lactate	(Ye et al. 2012a)
			Poly-3-hydroxybutyrate	(Opgenorth et al. 2014)
			Synthetic starch ¹	(You et al. 2013)
Volumetric productivity	Low-modest	Modest-high	Electricity	(Sakai et al. 2009; Zhu et al. 2014b)
			Recombinant proteins ²	(Carlson et al. 2012)
Reaction conditions	Narrow	Broad	Toxic products (e.g., isobutanol)	(Guterl et al. 2012)
			Toxic compounds (e.g., biomass hydrolysate)	(Wang et al. 2011)
			Presence of organic solvents	(Chen and Arnold 1993; Serdakowski and Dordick 2008)
			Extreme pH or temperature	(Arnold 1998; Shaw et al. 1999)
			Presence of ionic liquids	(Kragl et al. 2002)
Product separation	Cellular membrane	No membrane	Hydrogen	(Martín del Campo et al. 2013; Myung et al. 2014)
			Poly-3-hydroxybutyrate	(Opgenorth et al. 2014)
			Starch	(You et al. 2013)
			Recombinant proteins ²	(Carlson et al. 2012)
Product titer	Low-high	Low → high	isobutanol	(Guterl et al. 2012)
Product purity /quality	Low-modest	Very high	Recombinant antibodies ²	(Goerke and Swartz 2008; Kanter et al. 2007)
			Synthetic starch	(Qi et al. 2014; You et al. 2013)
			Poly-3-hydroxybutyrate	(Sato et al. 2003)
			Electricity	(Zhu et al. 2014b)
			Hydrogen	(Martín del Campo et al. 2013; Myung et al. 2014)
Reaction control and optimization	Hard	Easy	Recombinant proteins ²	(Goerke and Swartz 2008; Kanter et al. 2007; Wang and

				Zhang 2009a)
			Hydrogen	(Ardao and Zeng 2013; Rollin et al. 2014; Ye et al. 2009)
			Synthetic starch	(Qi et al. 2014; You et al. 2013)
			n-butanol	(Krutsakorn et al. 2013a)

1, high product yield due to co-production of ethanol, resulting in no sugar losses.

2, cell-free protein synthesis.

Figure 1
[Click here to download high resolution image](#)

(a) whole cell

(b) cell extract

(c) in vitro synthetic biosystem

Figure 2
[Click here to download high resolution image](#)

Whole cell-based biomanufacturing

in vitro (cell-free) biomanufacturing

Figure 3
[Click here to download high resolution image](#)

Figure 4
[Click here to download high resolution image](#)

Figure 5
[Click here to download high resolution image](#)

Figure 6
[Click here to download high resolution image](#)

Figure 7
[Click here to download high resolution image](#)

Figure 9
[Click here to download high resolution image](#)

Figure 8
[Click here to download high resolution image](#)

Figure 10
[Click here to download high resolution image](#)

Figure 11

[Click here to download high resolution image](#)

(a) Allocation to H₂

(b) Effects of TTN of enzyme and coenzyme

(c) Effects of TTN and type of coenzyme

(d) Effects of carbohydrate cost