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

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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.

46

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

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81 **1. Introduction**

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

91

92 Human beings had utilized living whole-cell microorganisms for making numerous fermentative
93 products for thousands of years. Living whole-cell fermentation is the predominant
94 biomanufacturing platform (**Fig. 1a**). However, the primary goal of microorganisms is their
95 proliferation while bioconversions are side effects. Recent advances in synthetic biology and
96 systems biology present numerous breakthroughs, such as the production of non-natural
97 products (e.g., 1,4-butanediol (Yim et al. 2011), isobutanol (Atsumi et al. 2008), etc.). However,
98 some inherent constraints of living microorganisms (e.g., net ATP generation, intact cellular
99 membrane, etc.) prevent them from implementing some important chemical reactions, for
100 example, 12 H₂ production from one glucose and water.

101

102 Whole cell lysates (**Fig. 1b**) have been used as an important scientific tool to investigate
103 complicated biological reactions for more than 100 years. For example, Eduard Buchner
104 discovered that the yeast lysate converted glucose to ethanol (Buchner 1897). Due to his
105 paradigm-shifting discovery, he won the Nobel Chemistry Prize in 1907. Later, numerous
106 scientists applied this tool to discover and study natural metabolic pathways. For example,
107 Harden et al. discovered key enzymes in glycolysis (Nobel Chemistry Prize 1929), Calvin
108 elucidated the CO₂ assimilation in plants (Nobel Chemistry Prize 1961), Nirenberg and
109 Matthaei interpreted the genetic code and its function in protein synthesis (Nobel Physiology
110 Prize 1968) (Matthaei et al. 1962). Recently, cell-free protein synthesis has been suggested to
111 be the fastest way to make recombinant proteins, even for membrane or complicated proteins
112 (Carlson et al. 2012; Swartz 2013). Cell-free protein synthesis has been scaled up to 100 liter
113 levels recently (Hodgman and Jewett 2012).

114

115 In vitro synthetic biosystems emerge as a manufacturing platform by the assembly of numerous
116 enzymes and enzyme complexes from different sources and/or (biomimetic) coenzymes (**Fig.**
117 **1c**). Such systems could surpass the constraints of whole-cells and cell lysates for
118 implementing some biological reactions that microbe cannot do, for example, high yield
119 production of hydrogen (Martín del Campo et al. 2013), or enzymatic transformation of cellulose
120 to starch (You et al. 2013). Although in vitro synthetic biosystems are on their early stage, they
121 have a great potential to becoming a disruptive biomanufacturing platform, especially for low-
122 cost production of biofuels and biochemicals. Table 1 presents the comparison of
123 biomanufacturing advantages between whole cell-based fermentations and in vitro (cell-free)
124 synthetic systems, from product yield, volumetric productivity, reaction condition, product
125 separation, product titer, product purity to process control and optimization. Among them, three
126 key criteria of biomanufacturing are (i) product yield mainly related to feedstock cost, (ii)
127 volumetric productivity (or reaction rate) mainly related to capital investment, and (iii) product
128 separation (or product titer) mainly related to processing costs.

129

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

140

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

149 Schoffelen and van Hest 2012). For example, the pharmaceutical and fine chemistry industries
150 adopt this platform to produce high-value chiral alcohols, α -hydroxy acids, and α -amino acids,
151 such as, (S)-2-butanol, L-*tert*-leucine, (S)-ethyl-4-chloro-3-hydroxybutyrate, atorvastatin, and so
152 on (Born scheuer et al. 2012; Huisman et al. 2010; Wildeman et al. 2007). In the organic
153 chemistry field, the synthesis of monosaccharides, activated monosaccharides,
154 oligosaccharides, and glycopeptides by using multi-enzyme one pot has been intensively
155 investigated, such as, L-fructose, 5-deoxy-5-ethyl-D-xylulose, amylose, and so on (Endo and
156 Koizumi 2000; Fessner 1998; Fessner and Helaine 2001; Franke et al. 2003; Huang et al. 2006;
157 Qi et al. 2014; Schoevaart et al. 2000; Zhang et al. 2005). In this century, a few researchers
158 propose to put more than four biocatalytic components in one vessel to implement very
159 complicated reactions comparable to microbial cell factories. Because in vitro synthetic
160 biosystems for future biomanufacturing are on their early stage, different names have been
161 suggested in the literature, such as, synthetic pathway biotransformation (SyPaB) (Zhang 2010a;
162 Zhang 2011a), synthetic cascade biomanufacturing (Steffler and Sieber 2013), cell-free
163 bioprocessing (Swartz 2013), synthetic biochemistry (Korman et al. 2014; Opgenorth et al.
164 2014), cell-free biosystems for biomanufacturing or cell-free biomanufacturing (You and Zhang
165 2013), in vitro metabolic engineering (Honda et al. 2004), cascade enzyme factories (Zhang and
166 Huang 2012), and so on.

167
168 Here I present pathway design principles of in vitro synthetic biosystems for biomanufacturing of
169 biofuels and biochemicals (called biocommodities) with eight examples, provide a detailed
170 economic analysis for enzymatic hydrogen production for identifying remaining challenges, and
171 suggest respective solutions. The other applications of in vitro synthetic systems or cell extracts
172 are not covered here, for example, the synthesis of fine chemicals (Bruggink et al. 2003; Bujara
173 et al. 2011), of radiolabelled products (Rodriguez and Leyh 2014; Schultheisz et al. 2008;
174 Schultheisz et al. 2009), of special compounds (Schultheisz et al. 2008; Scism and Bachmann
175 2010), of proteins (Carlson et al. 2012; Hodgman and Jewett 2012; Swartz 2013), and of drugs
176 (Boltje et al. 2009; Xu et al. 2014; Xu et al. 2011), in vitro genetic or protein circuits and
177 computation (Hockenberry and Jewett 2012; Katz and Privman 2010; Kim and Winfree 2011;
178 Noireaux 2003; Yordanov et al. 2014), in-depth understanding of in vivo biosystems (Bogorad et
179 al. 2013; Keller et al. 2013), and so on.

180

181 **2. Opportunities of in vitro Synthetic Biosystems**

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

194

195 **2.1. General Design Principles**

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

209

210 In vitro synthetic enzymatic pathways must have a balance in ATP production and consumption
211 while in vivo synthetic pathways in whole cells can obtain or depose extra ATP from or to
212 cellular metabolisms. If net ATP is generated for the case of in vitro ethanol fermentation via the
213 glycolysis pathway, the accumulation of ATPs stops systems from running for a long time
214 (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

249 and Liese 2004; Wong et al. 1981), and phosphite/phosphite dehydrogenase (Johannes et al.
250 2007). Single-enzyme NAD(P)H regeneration systems are widely used in the synthesis of high-
251 value of chiral compounds in the pharmaceutical industry. A 12-enzyme system is used to
252 produce nearly 12 NADPH from one glucose unit of cellobiose (Wang et al. 2011). Among all
253 hydrogen-donor compounds, renewable sugars have the lowest substrate costs, but this 12-
254 enzyme system requires more enzymes and increases system complexity (Wang et al. 2011).
255 The utilization of electrochemistry to generate reduced coenzymes is low-cost and clean, but
256 the instability of NAD(P)H under high over-potential must be solved before this technique
257 becomes industrially feasible (Kohlmann et al. 2008; Paul et al. 2014; Zheng et al. 2009).

258

259 Thermodynamics should be calculated between substrates and desired products, especially for
260 the cases of non-natural biochemical reactions designed. An online calculator for biochemical
261 thermodynamics – eQuilibrator is available (<http://equilibrator.weizmann.ac.il/>) (Jankowski et al.
262 2008). Also, it is important to identify reduced power sources as substrates, which may be
263 carbohydrates, glycerol, H₂, CO, methane, methanol, electricity and so on. For example, butanol
264 production based on glucose is spontaneous (i.e., delta G < 0) mainly due to a negative
265 enthalpy (Li et al. 2010). In contrast, methane generation from acetate occurs spontaneously
266 due to a gain of entropy but a positive enthalpy (Thauer et al. 1977; Zhang 2011a). Synthetic
267 pathways or special enzymatic reactions cannot be accomplished against thermodynamics laws
268 (Bornscheuer et al. 2012). To ensure to drive the reaction towards the desired direction, Gibbs
269 free energy under standard or custom conditions need be checked. To drive the reactions
270 towards desired products, we could apply a push-pull strategy sometimes. In situ product
271 removal (for example, hydrogen removal by vacuum or gas flush (Martin del Campo et al. 2013a;
272 Myung et al. 2014), butanol stripping (Smith et al. 2010)) provides more pull forces, while
273 increasing the pressure or concentration of gaseous substrates could generate more push
274 forces for desired reactions (Keller et al. 2013; Rieckenberg et al. 2014; Zhang and Huang
275 2012).

276

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

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

285

286 **2.2. Examples of Biofuels and Bioelectricity**

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

292

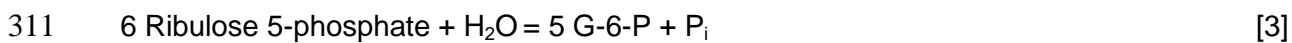
293 **2.2.1. Hydrogen**

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

301

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

308



312

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



317 G-1-P = G-6-P [5]
318
319 As a result, the whole stoichiometric reaction can be written as equation 6, that is, neither costly
320 G-6-P nor ATP is needed for high-yield hydrogen production, suggesting a great potential of
321 low-cost green hydrogen production from polysaccharides.
322
323 $C_6H_{10}O_5 \text{ (aq)} + 7H_2O \text{ (l)} = 12H_2 \text{ (g)} + 6CO_2 \text{ (g)}$ [6]
324
325 The proof-of-concept experiment was conducted by using 13 enzymes from five different
326 sources (e.g., bacterium, yeast, plant, animal, and archaea) (Zhang et al. 2007). Furthermore,
327 oligosaccharides (e.g., cellobiose, cellopentaose and sucrose) along with their respective
328 phosphorylases (e.g., cellobiose phosphorylase, celldextrin phosphorylase, and sucrose
329 phosphorlyase) can be used to produce high-yield hydrogen (Myung et al. 2014; Ye et al. 2009).
330 However, one glucose unit per mole of polysaccharide or oligosaccharide cannot be converted
331 to G-1-P. To completely utilize all hexose units, G-6-P can be produced from low-cost
332 polyphosphate instead of ATP mediated by polyphosphate glucokinase (Liao et al. 2012).
333 Therefore, high-yield hydrogen can be produced from all hexose units of sucrose (Myung et al.
334 2014) and glucose (Rollin et al. 2014). To utilize xylose, the most abundant pentose for the
335 production of low-cost hydrogen, a novel polyphosphate xylulokinase was discovered (Martin
336 del Campo et al. 2013b). By using this enzyme plus polyphosphate, nearly 10 mol of hydrogen
337 was produced from one xylose and water (Martín del Campo et al. 2013).
338
339 The overall reaction (Eq. 6) is spontaneous and endothermic (i.e., $\Delta G^\circ = \sim -50 \text{ kJ/mol}$ and $\Delta H^\circ =$
340 $+ 598 \text{ kJ/mol}$) (Zhang et al. 2007). This entropy-driven reaction can generate the chemical
341 energy output in the form of hydrogen more than the chemical energy of polysaccharides by
342 absorbing low temperature waste heat, which is widely available from refrigerators, air
343 conditioners, cooling towers, and fuel cells. These overall reactions are spontaneous due to the
344 phase changes from more orderly liquid to less orderly gas, resulting in a large positive ΔS (Ye
345 et al. 2009; Zhang et al. 2007).
346
347 High-yield enzymatic hydrogen production from a variety of carbohydrates from starch,
348 celldextrins, sucrose, glucose, fructose, and xylose (**Figure 3**) brings multiple implications: (i)
349 low-cost green hydrogen production for hydrogen fuel cell vehicles, (ii) a new hydrogen carrier –

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

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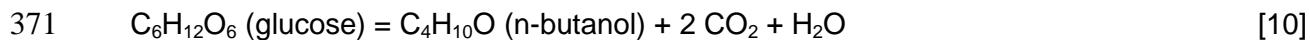
353 **2.2.2. n-Butanol**

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

359

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

367



372

373 This synthetic pathway has three features: (i) ATP balance, where the ATP consumption during
374 the conversion of glucose to fructose-1,6-bisphosphate matches the ATP regeneration from
375 phosphoenolpyruvate to pyruvate mediated by pyruvate kinase; (ii) NADH balance, where
376 NADH regeneration by non-phosphorylating GAP dehydrogenase and CoA-acylating aldehyde
377 dehydrogenase (ADDH) matches its consumption by 3-hydroxyacyl-CoA dehydrogenase,
378 ADDH, NADH-dependent flavinoxidoreductase, and hydroxybutyryl-CoA dehydrogenase; and
379 (iii) CoA balance, where CoA is needed by ADDH and is released by acetyl-CoA
380 acetyltransferase and ADDH. The overall reaction is an enthalpy-driven reaction, meaning a
381 slight chemical energy loss (Huang and Zhang 2011). By compromising reaction temperatures
382 and optimizing enzyme loading, the maximum n-butanol yield was 82% (Krutsakorn et al.
383 2013a), comparable with best product yields in ABE fermentation (Ezeji et al. 2007).

384

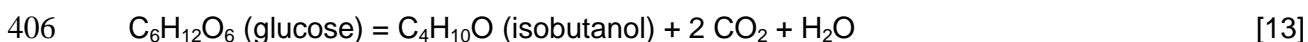
385 **2.2.3. Isobutanol**

386 Isobutanol, a branched C4 alcohol, has a similar energy density to n-butanol, limited miscibility
387 with water, and is completely miscible with gasoline (Peralta-Yahya et al. 2012). Isobutanol's
388 branching gives it a better octane number than n-butanol. Isobutanol can be produced through
389 the Ehrlich pathway, also called the 2-keto-acid pathway (Hazelwood et al. 2008; Peralta-Yahya
390 et al. 2012). Heterologous pathway for isobutanol production from carbohydrates have been
391 introduced to a number of microorganisms, such as *E. coli* (Atsumi et al. 2008), *Bacillus subtilis*
392 (Li et al. 2011), *Corynebacterium glutamicum* (Smith et al. 2010), *S. cerevisiae* (Chen et al.
393 2011), and *Clostridium acetobutylicum* (Higashide et al. 2011). However, its hydrophobic effect
394 is toxic to cellular membranes, resulting on low isobutanol titers (e.g., 1-2%) (Atsumi et al.
395 2010).

396

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

403



407

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

414

415 **2.2.4. Bioelectricity**

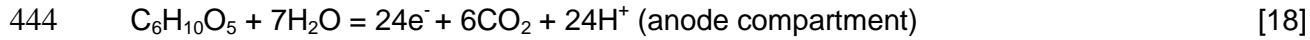
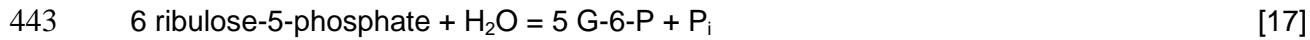
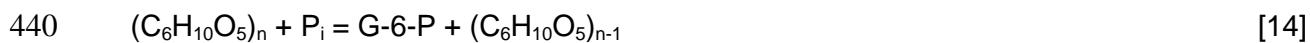
416 Enzymatic fuel cells (EFCs) are an emerging electrobiochemical device that directly converts
417 chemical energy of a variety of fuels to electricity through enzymes (Calabrese Barton et al.

418 2004; Moehlenbrock and Minteer 2008; Zhu et al. 2011). Inspired by living cells that can utilize
419 complex organic compounds (e.g., starch, glycogen) as energy sources, sugar-powered EFCs
420 could be next generation of biodegradable, highly safe, metal-free biobatteries. Compared to
421 microbial fuel cells, EFCs usually generate much higher power density in terms of mW cm^{-2} of
422 membrane or electrode, suggesting their great potentials in powering portable electronics
423 (Cooney et al. 2008; Sakai et al. 2009; Zebda et al. 2011).

424

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

439



445

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

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

454

455 **2.3. Examples of Biochemicals**

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

460

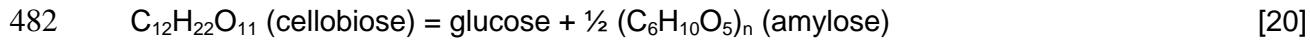
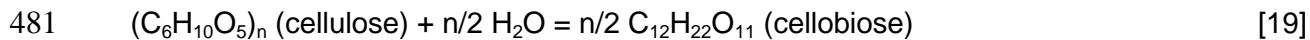
461 **2.3.1. Synthetic Amylose**

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

470

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

480



485

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

499

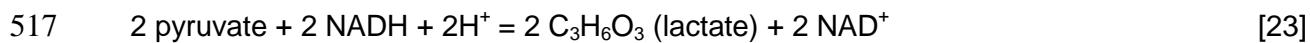
500 **2.3.2. Lactate**

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

509

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

516



519

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

530

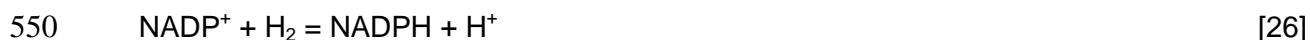
531 **2.3.3. 1,3-Propanediol**

532 1,3-propanediol (1,3-PDO) is a colorless viscous liquid, which can be used as monomer in the
533 production of polymers such as polytrimethylene terephthalate. 1,3-PDO can be produced from
534 glucose (Nakamura and Whited 2003) or glycerol (Dietz and Zeng 2014) by whole-cell
535 fermentation. However, its yields based on glycerol are approximately 0.50-0.76 mol 1,3-
536 PDO/mol glycerol (Celińska 2010; Dietz and Zeng 2014; Zeng and Sabra 2011) due to the
537 synthesis of cell mass, the generation of extra reducing power by branching glycerol utilization
538 pathways under micro-aerobic conditions, and the production of undesired side-products.

539

540 Zeng and his coworkers developed a straightforward in vitro pathway for converting glycerol to
541 1,3-PDO under strictly anaerobic condition (**Figure 9**) (Rieckenberg et al. 2014). This pathway
542 includes two modules: (i) generation of 1,3-PDO from glycerol by consuming one NADPH
543 catalyzed by glycerol dehydratase and NADPH-dependent propanediol oxidoreductase-
544 isoenzyme (equation 25); and (ii) NADPH regeneration from H₂ by hydrogenase (equation 26).
545 As a result, one mole of 1,3-PDO was produced from one mole of glycerol and one mole of H₂
546 (equation 27). Via this pathway, a very high 1,3-PDO yield of about 0.95 mol/mol has been
547 accomplished (Rieckenberg et al. 2014).

548



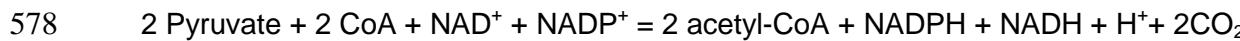
552

553 **2.3.4. Poly-3-hydroxybutyrate**

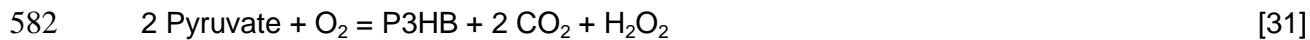
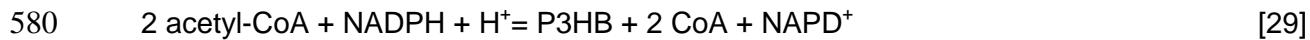
554 Poly-3-hydroxybutyrate (P3HB) consisting of 1,000 to 30,000 hydroxy fatty acid monomers is
555 the simplest and most commonly occurring form of polyhydroxyalkanoates, which can be either
556 thermoplastic or elastomeric biodegradable plastics. P3HAB is usually produced by microbial
557 fermentation. Its synthesis is usually caused by certain deficiency conditions (e.g., a lack of
558 phosphorous, nitrogen, trace elements, or oxygen) and the excess supply of carbon sources.
559 The intracellular accumulated polyhydroxyalkanoates can be as high as 80% of the dry cell
560 weight. However, its purification is costly and its intracellular polymerization synthesis is difficult
561 to control (Gerngross and Slater 2003).

562

563 Munekata and his coworkers (Satoh et al. 2003) demonstrate an in vitro pathway to produce
564 P3HB from acetate, where one extra NADPH input is generated from glucose mediated by
565 glucose dehydrogenase. The weight-average molecular weight and polydispersity of in vitro
566 synthesized P3HAB are 6.64×10^6 and 1.36, respectively (Satoh et al. 2003). Recently, Bowie
567 and his coworkers (Opgenorth et al. 2014) design an in vitro pathway for the production of
568 P3HB from pyruvate without an external NAD(P)H input (**Figure 10**). This in vitro pathway
569 contains three modules: (i) acetyl-CoA generation from pyruvate, co-producing NADH and
570 NADPH by using two NADP-dependent and NAD-dependent pyruvate dehydrogenase,
571 respectively (equation 28); (ii) addition of one unit of P3HB3 from 2 acetyl-CoA, accompanies
572 with the consumption of NADPH (equation 29); and (iii) oxidation of NADH by using NADH
573 oxidase (NOX). As a result, the whole system has a reaction of equation 31 with balanced
574 coenzymes. This delicate purge valve system based on PDH-1/PDH-2/NOX relieves extra
575 NADPH accumulation and allows the carbon flux to drive forward the desired product. In the
576 optimized system, the maximum yield of P3HB was approximately 94% (Opgenorth et al. 2014).
577



579 [28]



583

584 **3. Economic Analysis of the Hydrogen Case**

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

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

592

593 In vitro synthetic biosystems for biomanufacturing can be regarded as two-step
594 biotransformation: (i) the production of numerous enzymes by whole-cell fermentation followed
595 by their purification and (ii) in vitro biotransformation mediated by enzyme cocktails (Zhang
596 2011a). Therefore, it is very important to estimate a carbohydrate fraction of hydrogen
597 production to overall sugar consumption for both production of enzymes and hydrogen. All
598 enzymes except hydrogenase (**Figure 3**) can be produced in *E. coli* under aerobic conditions
599 (Myung et al. 2014), where recombinant enzyme yields (Y_{rE}) could range from 0.05 to 0.1 kg
600 protein/kg glucose (Huang and Zhang 2011; Wang et al. 2011; Zhang et al. 2010). In contrast,
601 recombinant active hydrogenase must be produced under anaerobic conditions, and its yields
602 (Y_{H2ase}) could range from 0.001 to 0.01 kg hydrogenase/kg glucose (Chandrayan et al. 2012;
603 Sun et al. 2010).

604

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

613

614 The overall production cost of hydrogen from carbohydrates before tax include (i) feedstock
615 (mainly, carbohydrates) used for enzyme synthesis and hydrogen production, (ii) biocatalysts --
616 enzymes, (iii) coenzymes, and (iv) capital investment and processing costs. Three key
617 inputs/assumptions are (i) carbohydrate price = \$0.30/kg glucose equivalent (or $6 * \text{CH}_2\text{O}$), (ii)
618 coenzyme costs are \$4,500/kg NADP, \$1,500/kg NAD, and \$50/kg benzyl nicotinamide (BNA);
619 and (iii) enzyme costs are \$20/kg recombinant protein and \$100/kg hydrogenase (Rollin et al.
620 2013).

621

622 **Figure 11b** shows the effects of enzyme stability on hydrogen production costs, when TTN of
623 coenzyme is 10^6 . Clearly, hydrogen production costs decrease exponentially from more than
624 \$100,000 to several US dollars/kg H₂ where TTN of all enzymes increases from 10^3 to 10^8 ,
625 suggesting that enhancing enzyme stability is the most important factor for low-cost
626 biomanufacturing. When TTN of enzymes are more than 10^9 , further improvement of enzyme
627 stability has nearly no impact on lowering hydrogen production costs. Similar to this case, no
628 R&D efforts are being made to improve stability of industrial glucose isomerase. When enzymes
629 are stable enough (e.g., TTN = 10^9), the cost of coenzyme greatly determines final hydrogen
630 production cost (e.g., \$8.43/kg H₂ using NADP, \$4.64/kg H₂ using NAD, and \$2.61/kg H₂ using
631 BNA), suggesting the importance of the replacement of high-cost and labile natural coenzymes
632 with low-cost and stable biomimetic coenzymes (Paul et al. 2014; Rollin et al. 2013).

633

634 **Figure 11c** shows the effects of coenzymes' TTN and costs on hydrogen production costs,
635 when TTN of all enzymes is 10^9 . Clearly, increasing TTN of coenzymes exponentially decrease
636 hydrogen production costs, when TTNs are less than 10^5 . When TTN of coenzymes are larger
637 than 10^8 , hydrogen production cost could be as low as \$2.38/kg H₂. However, the highest TTN
638 for NAD reported is 10^6 (Kazandjian and Klibanov 1985), it is essentially important to replace
639 natural coenzymes with biomimics (Rollin et al. 2013) by considering an increasing stability
640 order of coenzymes from NADP to NAD to BNA (Paul et al. 2014).

641

642 **Figure 11d** shows the effects of carbohydrate costs from \$0.05/kg to \$0.5/kg on the overall
643 hydrogen production cost, where the sum of capital expenses and operation expense is
644 \$0.35/kg H₂, overall enzyme cost is \$0.13/kg H₂ (i.e., TTN of enzymes = 10^9), and coenzyme
645 cost is \$0.01/kg H₂ (i.e., TTN of BNA = 10^8). It is noted that hydrogen separation costs from the
646 aqueous enzymatic solution are very low; hydrogen and CO₂ can be separated by membrane
647 technology, pressure swing adsorption, or a hybrid of both (Zhang 2009); high-purity hydrogen
648 without CO can be used by proton exchange membrane fuel cells easily (Huang and Zhang
649 2011). **Figure 11d** shows that the overall hydrogen production costs are proportional to
650 carbohydrate cost. When carbohydrate cost is \$0.2/kg and \$0.3/kg, the hydrogen production
651 costs could be \$1.99/kg and \$2.74/kg of H₂, respectively. These data are very cost-competitively
652 with hydrogen produced from natural gas without considering two extra benefits: carbon-neutral
653 hydrogen production and distributed hydrogen production systems based on scattered biomass
654 resources. Also, the fraction of carbohydrate cost to overall hydrogen production cost ranges

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

659

660 Distributed hydrogen production from local biomass carbohydrates has multiple advantages
661 over centered hydrogen production from fossil fuels. High capital costs of fossil fuel-to-hydrogen
662 result from its technologically complex, multistep process involving high-pressure and high-
663 temperature gasification, and steam shifting and separation of hydrogen from other products.
664 Critical challenges associated with a lack of high density hydrogen storage approaches, a lack
665 of infrastructure for compressed hydrogen distribution, and the high cost of hydrogen
666 compression lead to serious doubt about the feasibility of the hydrogen economy. Beyond the
667 low-cost and green hydrogen production, the production of high-yield and high-purity hydrogen
668 from local carbohydrate resources via in vitro synthetic biosystems is an out-of-the-box solution
669 to address numerous challenges of the hydrogen economy (Zhang 2009; Zhang 2010b; Zhang
670 2013; Zhang et al. 2013).

671

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

679

680 **4. Challenges and Solutions**

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

684

685 **4.1. Stable Enzymes as Standardized Building Blocks**

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

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

702

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

714

715 **4.2. Synthetic Multiple-Enzyme Complexes (Metabolons) as Building Modules**

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

722

723 The simplest way to obtain multi-function enzymes is the creation of chimeric proteins, in which
724 two or more cascade enzymes are combined by a linker to form a multi-functional single
725 polypeptide (Bulow et al. 1985; Conrado et al. 2008). For example, Bulow et al. constructed a
726 bifunctional enzyme containing cascade reactions mediated by *E. coli* β-galactosidase and
727 galactokinase (Bulow et al. 1985). This synthetic fusion enzyme displayed kinetic advantages
728 (1.5-2.4 fold) over free enzyme mixtures. Similar effects have been reported in other fusion
729 enzymatic systems (Agapakis et al. 2010; Meynil-Salles et al. 2007; Orita et al. 2007; Riedel
730 and Bronnenmeier 1998). However, the misfolding of large multi-domain proteins often happens
731 and decreases their apparent activities (Chang et al. 2005), resulting in a large uncertainty of
732 this strategy.

733

734 Scaffolding molecules, includes proteins and nucleic acids, can recruit enzymes to form
735 multienzyme complexes (Aldaye et al. 2008; You et al. 2012) (Wei et al. 2012). Inspired by
736 cellulosomes (Bayer et al. 1994), a synthetic scaffold containing different cohesins from different
737 microorganisms is used for the assembly of three metabolic enzymes (You et al. 2012).
738 Triosephosphate isomerase (TIM), aldolase (ALD), and fructose 1,6-bisphosphatase (FBP) are
739 engineered to have a dockerin at their C-terminals. These three dockerin-containing enzymes
740 can be self-assembled into a static trifunctional enzyme complex through the interaction with a
741 mini-scaffold protein consisting of three different matching cohesins. The synthetic metabolon
742 showed more than one order of magnitude enhancements in reaction rates compared to the
743 non-complexed TIM, ALD, and FBP mixture (You et al. 2012). Furthermore, it is found that the
744 annexation of more high-activity ALD in the synthetic enzyme complexes drastically decreases
745 the degree of substrate channeling by five times (You and Zhang 2014). Either DNA or RNA can
746 be designed to fold into various structures in vitro, forming simple structures such as sheets to
747 very more complicated structures such as tubes and capsules (Aldaye et al. 2008; Wei et al.
748 2012). It is more convenient to in vitro synthesize oligonucleotide as DNA or RNA scaffolds by
749 using automate oligonucleotide synthesis machines than protein scaffolds. However, the costs
750 of DNA and RNA is far more expensive than that of protein scaffolds produced by whole cells,
751 which may impair their applications on a large scale, especially for the production of
752 biocommodities.

753

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

757 another enzyme (Mansson et al. 1983) on solid supports. The enhanced reaction rates among
758 co-immobilization of cascade enzymes have been observed for several systems (Mateo et al.
759 2006; Myung et al. 2013; Zhang 2011b), but direct cross linking could lead to loss of enzyme
760 activity (Sheldon and van Pelt 2013).

761
762 Regardless of fusion proteins, the use of scaffolds, and co-immobilization, the general rules of
763 constructing such synthetic enzyme complexes need further investigation for their optimal ratios
764 and their orientation.

765
766 **4.3. Bulk Enzyme Production and Separation**

767 A wide range of bulk enzyme production methods have been explored in recent years (Demain
768 and Vaishnav 2009). Desired enzymes are produced in recombinant forms, using bacteria,
769 fungal, yeast and plant platforms, preferred due to in-depth knowledge of the organisms, ease
770 of genetic manipulation, and low production cost (Demain and Vaishnav 2009). For example,
771 protease is produced at a cost of \$~10 per kg by *B. subtilis*, cellulase is produced at a cost of
772 \$~10 per kg by *Trichoderma* spp. and *A. niger* spp., and recombinant proteins produced at
773 costs of tens of dollars per kg in *E. coli* (Taylor et al. 2008; Zhang and Zhang 2010; Zhang et al.
774 2006). The DOE Biomass Program even anticipated industrial enzyme production costs as low
775 as \$0.7 per kg of dry enzyme in the future, equaling that of soybean protein (Rollin et al. 2013).
776 To increase recombinant protein expression levels, systematic efforts have been conducted by
777 optimization of codon usage, expression plasmid and host, inducer type, concentration, addition
778 time, and so on. It resulted in ~500-fold enhancement of a hyperthermophilic *T. maritima* 6-
779 phosphogluconate dehydrogenase in *E. coli* (Wang and Zhang 2009b). The other option is
780 overexpression of the target protein in its native organism, such as hydrogenase by *Pyrococcus*
781 *furiosus* (Chandrayan et al. 2012).

782
783 Instead of costly chromatographic separation techniques, several low-cost, scalable enzyme
784 purification approaches have been developed, for example, simple centrifugation for secretory
785 enzymes (e.g., cellulase, amylase, protease), one-step cellulose binding module-tagged
786 enzyme purification and immobilization on low-cost cellulosic materials (Liao et al. 2012; Myung
787 et al. 2011), heat precipitation for thermostable enzymes (Sun et al. 2012; Ye et al. 2012a), and
788 ammonia precipitation (Myung and Zhang 2013). It is noted that the protein of thermostable
789 enzymes in mesophilic hosts can decrease the enzyme purification cost because enzymes
790 cloned from thermophilic microbes can be stable at high temperature (60-80 °C). Therefore,

791 heat treatment can deactivate *E. coli* endogenous proteins; after centrifugation, only soluble
792 thermostable proteins remain in the supernatant. This heat precipitation provides a simple and
793 less-costly purification method for numerous thermostable enzymes (Krutsakorn et al. 2013a;
794 Krutsakorn et al. 2013b; Sun et al. 2012; Wang and Zhang 2009b; Ye et al. 2012a). Honda and
795 his coworkers attempted to co-expressing nine thermoenzymes in one *E. coli* cell (Ninh et al.
796 2014). After heat precipitation, they harvested the thermoenzyme cocktail suitable for one-pot
797 biotransformation (Ninh et al. 2014).

798

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

801

802 **4.4. NAD(P)-Dependent Enzyme Engineering**

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

812

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

825

826 **4.5. System Optimization and Modeling**

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

834

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

857

858 **4.6. Stabilization of Thermolabile Metabolites at Elevated Temperatures**

859 Elevating reaction temperature usually increases reaction rates but may cause the degradation
860 of substrates, products or intermediates. High product yields of in vitro synthetic biosystems are
861 usually accomplished at modest temperatures, when the degradation of metabolites are minimal.
862 High reaction temperatures may decrease product yields. For example, high yield of lactate was
863 obtained at a temperature of 50°C, while 60°C lead to the degradation of glyceraldehyde 3-
864 phosphate, dihydroxacetone phosphate, phosphoenolpyruvate, NADH, etc. (Ye et al. 2012a).
865 Similarly, nearly theoretical yields of hydrogen were obtained at 30-50°C (Martin del Campo et
866 al. 2013a; Myung et al. 2014; Ye et al. 2009), but the yields were lower at 60°C (Rollin et al.
867 2014).

868

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

888

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

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

895

896 **4.7. Scale-Up by Integrative Innovation**

897 The implementation of in vitro synthetic biosystems for biomanufacturing is like making of
898 iPhone by integrating numerous well-known parts and creating a seemingly-new system.
899 Recent advances in DNA sequencing, bioinformatics, recombinant DNA technology, enzyme
900 engineering and immobilization, as well as bulk enzyme production and purification allow to
901 develop highly efficient, tunable enzymes tailored for large-scale industrial production
902 (Bornscheuer et al. 2012). The integration of numerous stable enzymes in one bioreactor that
903 can last a very long reaction time (e.g., several months or even years) would become a
904 disruptive technology for low-cost biomanufacturing.

905

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

913

914 **4.8. Other Issues**

915 A few in vitro synthetic pathways have been investigated to help understand in-depth natural
916 pathways that may be used to produce advanced biofuels and biochemicals in vivo. For
917 example, Khosla and his coworkers investigated the detailed kinetics of the fatty acid synthesis
918 using the reconstitution of the purified *E. coli* fatty acid synthase components (Yu et al. 2011).
919 Liu and his co-workers investigated the synthesis of farnesene (a precursor of new jet fuel)
920 through the mevalonate pathway by using purified enzymes (Zhu et al. 2014a). Adams and his
921 coworkers demonstrated the feasibility of production of 3-hydroxypropionic acid, one of the top
922 12 industrial chemical building blocks, from hydrogen and carbon dioxide by using *P. furiosus*
923 cell-free extract (Keller et al. 2013). However, these in vitro synthetic biosystems have more
924 challenges in future biomanufacturing, such as NAD(P)H imbalance, ATP imbalance, or CoA
925 involvement. For example, when fatty acid ethyl ester is produced from glucose, this pathway

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

936

937 **5. Conclusions**

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

950

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1383

1384 **Figure Legends**
1385

1386 **Figure 1.** Schemes of biotransformation catalyzed by whole cell (A), cell extract (B), and in vitro
1387 synthetic biosystem (C).

1389 **Figure 2.** History of biomanufacturing catalyzed by whole cells and in vitro (cell-free)
1390 biosystems associated with key milestones.

1391
1392 **Figure 3.** Scheme of in vitro synthetic enzymatic pathways for the production of high-yield
1393 hydrogen from a variety of carbohydrates -- starch, celldextrin, sucrose, glucose, fructose and
1394 xylose as well as water. The pathways are compiled and modified from References (Martín del
1395 Campo et al. 2013; Myung et al. 2014; Rollin et al. 2014; Ye et al. 2009; Zhang et al. 2007). The
1396 enzymes are α GP, alpha-glucan phosphorylase; CDP, celldextrin phosphorylase; CBP,
1397 cellobiose phosphorylase; SP, sucrose phosphorlyase; GI, glucose isomerase; XI, xylose
1398 isomerase; PPGK, polyphosphate glucokinase; PPXK, polyphosphate xylulokinase; PGM,
1399 phosphoglucomutase; G6PDH, glucose 6-phosphate dehydrogenase; 6PGDH, 6-
1400 phosphogluconate dehydrogenase; RPI, ribose 5-phosphate isomerase; RPE, ribulose-5-
1401 phosphate 3-epimerase; TK, transketolase; TAL, transaldolase; TIM, triose phosphate
1402 isomerase; ALD, (fructose-bisphosphate) aldolase; FBP, fructose bisphosphatase; PGI,
1403 phosphoglucose isomerase; and H₂ase. P_i and (P_i)_n are inorganic phosphate and polyphosphate
1404 with a degree of polymerization of n. The metabolites are: g1p, glucose-1-phosphate; g6p,
1405 glucose-6-phosphate; ru5p, ribulose 5-phosphate; x5p, xylulose 5-phosphate; r5p, ribose 5-
1406 phosphate; s7p, sedoheptulose 7-phosphate; g3p, glyceraldehyde 3-phosphate; e4p, erythrose
1407 4-phosphate; dhap, dihydroxacetone phosphate; fdp, fructose-1,6-diphosphate; f6p, fructose 6-
1408 phosphate.
1409

1410 **Figure 4.** Scheme of the in vitro synthetic enzymatic pathway for the production of n-butanol
1411 from glucose, modified from Ref. (Krutsakorn et al. 2013a). The enzymes are HK, hexokinase;
1412 PGI, phosphoglucose isomerase; PFK, 6-phosphofructokinase; FBA, fructose-bisphosphate
1413 aldolase; TIM, triosephosphate isomerase; GAPN, non-phosphorylating GAP dehydrogenase;
1414 PGM, cofactor-independent phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase;
1415 PDC, pyruvate decarboxylase; ADDH, CoA-acylating aldehyde dehydrogenase; HBD,
1416 hydroxybutyryl-CoA dehydrogenase; HPD, 3-hydroxypropionyl-CoA dehydratase; NFO, NADH-
1417 dependent flavinoxidoreductase; and HAD, 3-hydroxyacyl-CoA dehydrogenase. Metabolites are

1418 g6p, glucose-6-phosphate; f6p, fructose 6-phosphate; fdp, fructose-1,6-diphosphate; g3p,
1419 glyceraldehyde 3-phosphate; dhap, dihydroxacetone phosphate; 3pg, 3-phosphoglycerate; 2pg,
1420 2-phosphoglycerate; and pep, phosphoenolpyruvate.

1421
1422 **Figure 5.** Scheme of the in vitro ATP-free synthetic enzymatic pathway for the production of
1423 isobutanol from glucose, modified from Ref. (Guterl et al. 2012). The enzymes are GDH,
1424 glucose dehydrogenase; DHAD, dihydroxy acid dehydratase; AIDH, glyceraldehyde
1425 dehydrogenase; KDGA, 2-keto-3-desoxygluconate aldolase; ALS, acetolactate synthase; KARI,
1426 ketolacid reductoisomerase; KDC, 2-ketoacid decarboxylase; and ALD, alcohol dehydrogenase.
1427

1428 **Figure 6.** Scheme of the in vitro ATP-free synthetic enzymatic pathway for the production of
1429 electricity from maltodextrin, modified from Ref. (Zhu et al. 2014b). Most enzymes and
1430 metabolites are the same as them in Figure 2, except the enzyme DI, diaphorase.
1431

1432 **Figure 7.** Scheme of the in vitro ATP-free synthetic enzymatic pathway for the production of
1433 synthetic amylose and ethanol from cellulose, modified from Ref. (You et al. 2013). The
1434 enzymes are EG, endoglucanase; CBH, cellobiohydrolase; CBP, cellobiose phosphorylase; and
1435 PGP, potato alpha-glucan phosphorylase.
1436

1437 **Figure 8.** Scheme of the in vitro ATP-neutral synthetic enzymatic pathway for the production of
1438 lactate from glucose, modified from Ref. (Ye et al. 2012a). Enzymes are HK, hexokinase; PGI,
1439 g6p isomerase; PFK, 6-phosphofructokinase; FBA, fructose-bisphosphate aldolase; TIM,
1440 triosephosphate isomerase; GAPN, non-phosphorylating GAP dehydrogenase; PGM, cofactor-
1441 independent phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; and MLDH,
1442 malate/lactate dehydrogenase.
1443

1444 **Figure 9.** Scheme of the in vitro synthetic enzymatic pathway for the production of 1,3-
1445 propanediol from glycerol, modified from Ref. (Rieckenberg et al. 2014). Enzymes are GDH,
1446 glycerol dehydratase; PDORI, NADPH-dependent propanediol oxidoreductase-isoenzyme; and
1447 H₂ase, hydrogenase. VB12, which a key coenzyme for GDH, may be repaired in vitro for a long
1448 time running.
1449

1450 **Figure 10.** Scheme of the in vitro synthetic enzymatic pathway for the production of poly-(R)-3-
1451 hydroxybutyrate from pyruvate, modified from Ref. (Opogenorth et al. 2014). Enzymes are PDH,

1452 pyruvate dehydrogenase; ACC, acetyl-CoA acetyltransferase; PhaB, 3-hydroxybutyryl-CoA
1453 reductase; PhaC, polyhydroxybutyrate synthase; and NOX, NADH oxidase.

1454

1455 **Figure 11.** Economic analysis of enzymatic hydrogen production from carbohydrates. (a) The
1456 carbohydrate allocation to hydrogen production relative to overall carbohydrate consumption in
1457 terms of enzyme TTN. $Y(rE)$ is the protein yield based on carbohydrate consumption by aerobic
1458 fermentation in terms of kg recombinant protein per kg of carbohydrate, $Y(H_2\text{ase})$ is the
1459 hydrogenase yield based on carbohydrate consumption by anaerobic fermentation in terms of
1460 kg recombinant protein per kg of carbohydrate. (b) Effects of enzyme TTN and coenzyme costs.
1461 (c) Effects of coenzyme TTN and coenzyme type. (d) Effects of carbohydrate costs on final
1462 hydrogen production costs.

1463

Table 1.

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

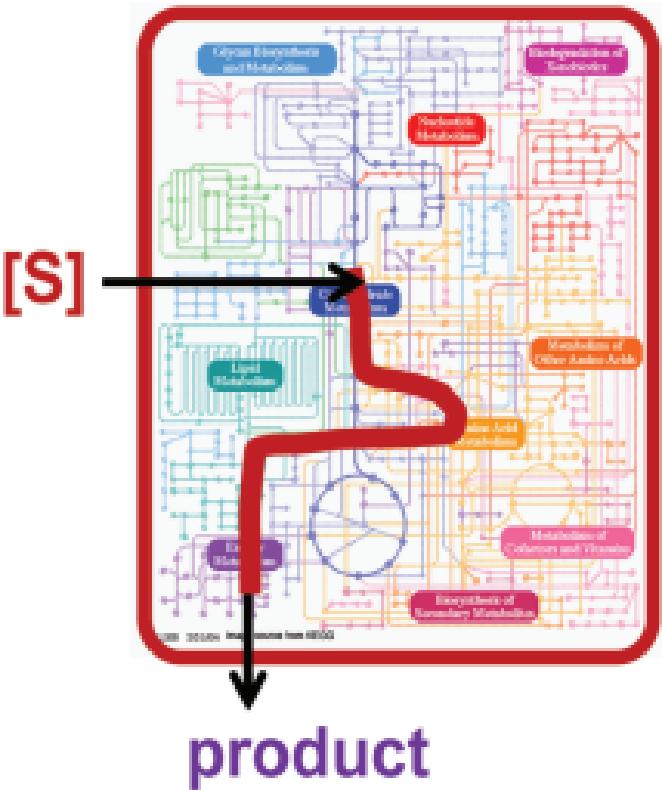
Feature	Microbial cells	In vitro synthetic biosystems	Comparative examples	Ref.
Product yield	Low-modest	Very high	Hydrogen	(Martín del Campo et al. 2013; Myung et al. 2014)
			1,3-propanediol	(Rieckenberg et al. 2014)
			Electricity	(Zhu et al. 2014b)
			Lactate	(Ye et al. 2012a)
			Poly-3-hydroxybutyrate	(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	(Satoh 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)

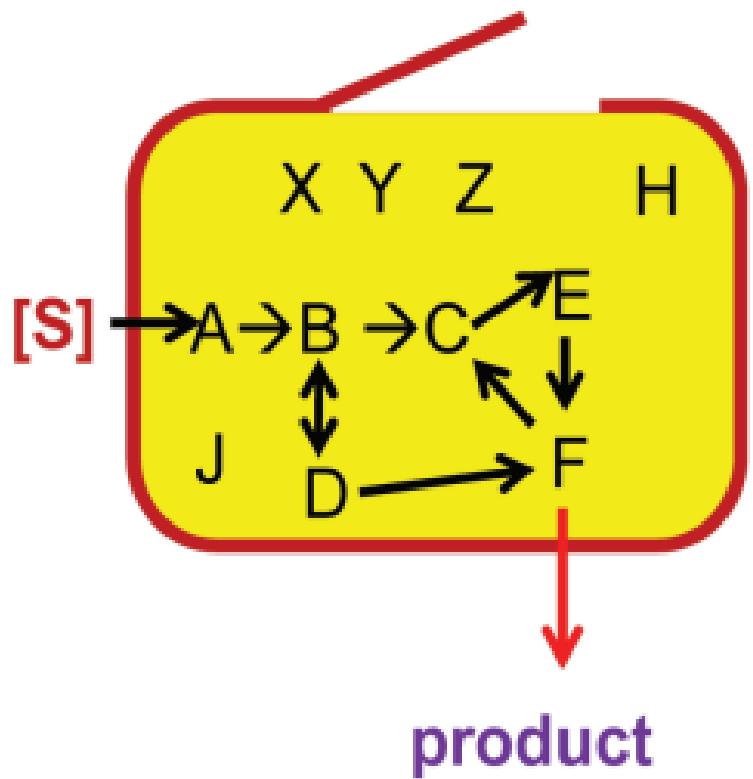
1, high product yield due to co-production of ethanol, resulting in no sugar losses.

2, cell-free protein synthesis.

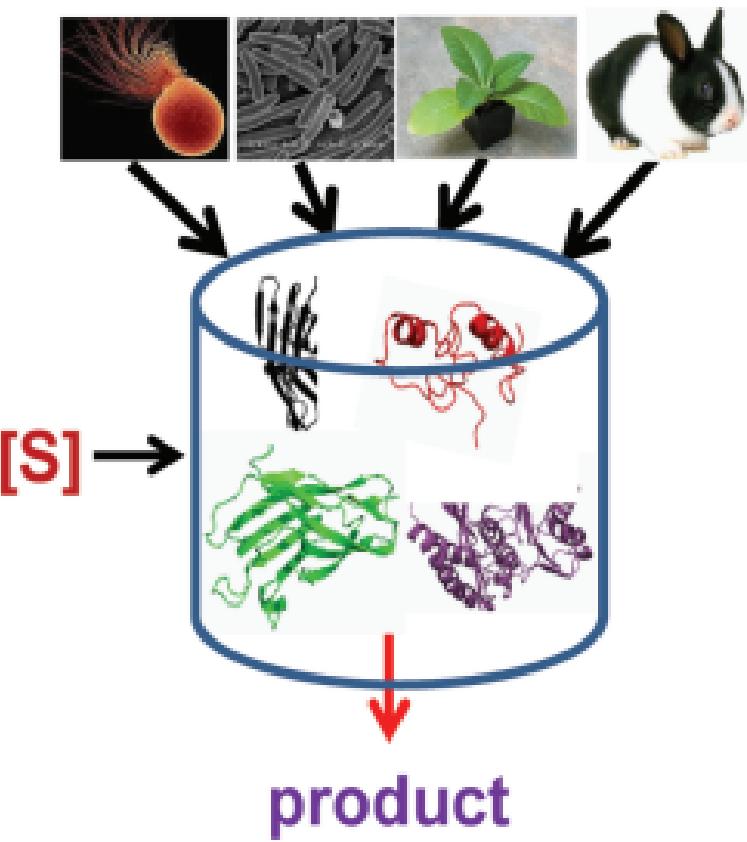
(a) whole cell



(b) cell extract



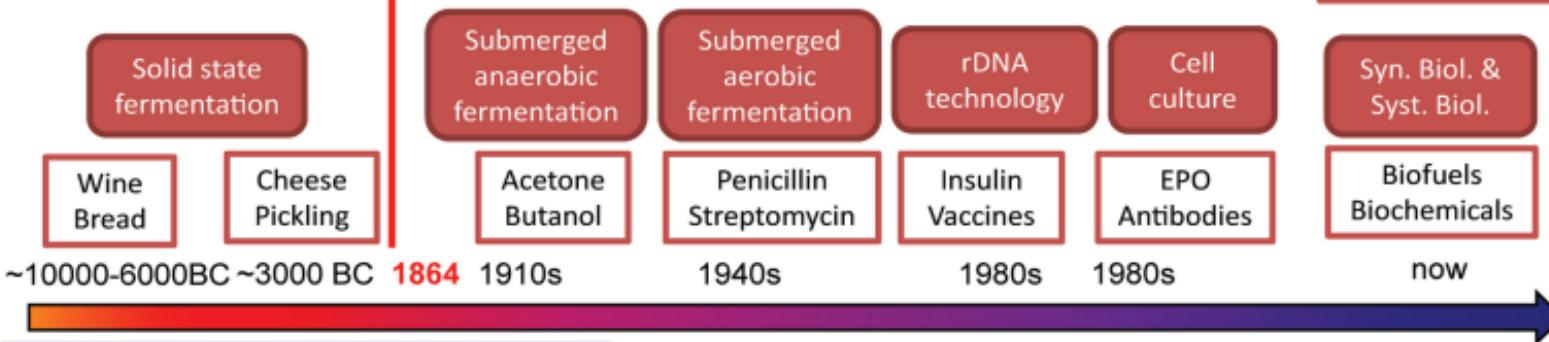
(c) in vitro synthetic biosystem



Whole cell-based biomanufacturing



Biogenesis



in vitro (cell-free) biomanufacturing

1897



Eduard Buchner



Nobel Prize
Chem 1907

Cell-free ethanol fermentation

1970-1980s

Fructose
Cephalosporins

Immobilized
enzyme

1990s

Drugs
(precursors)

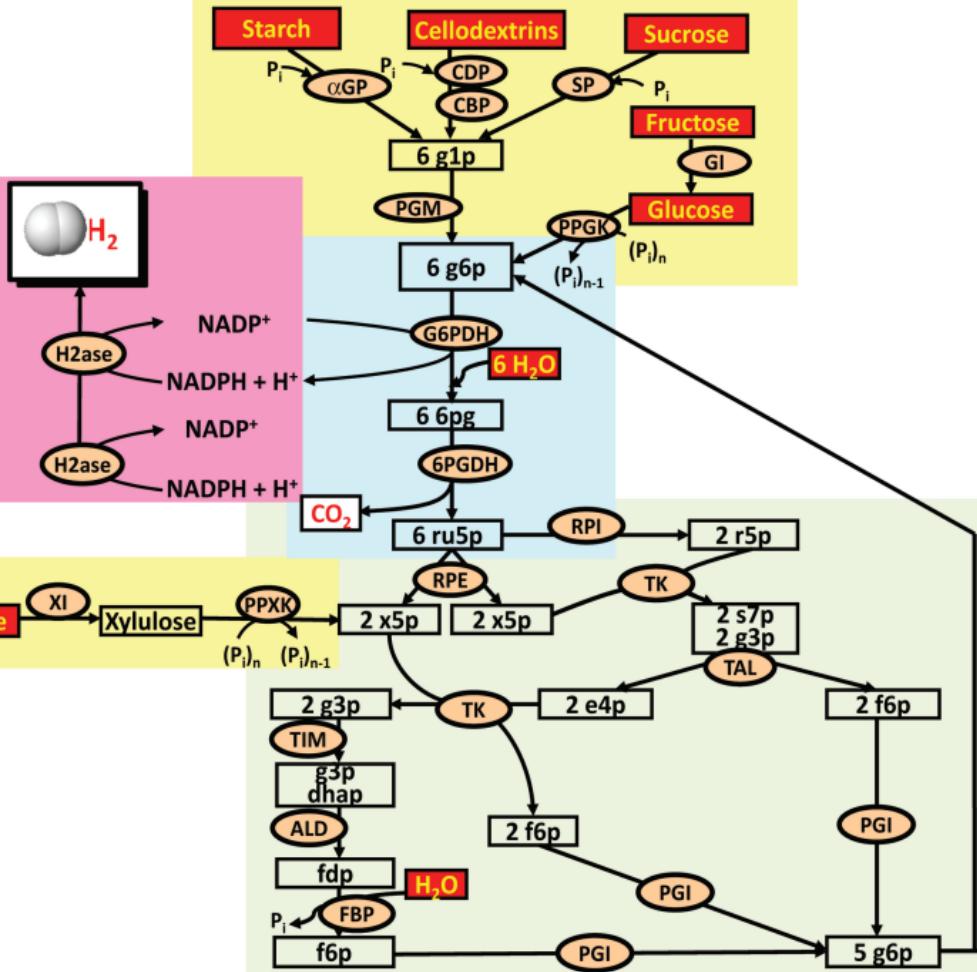
Multi-enzyme
one pot

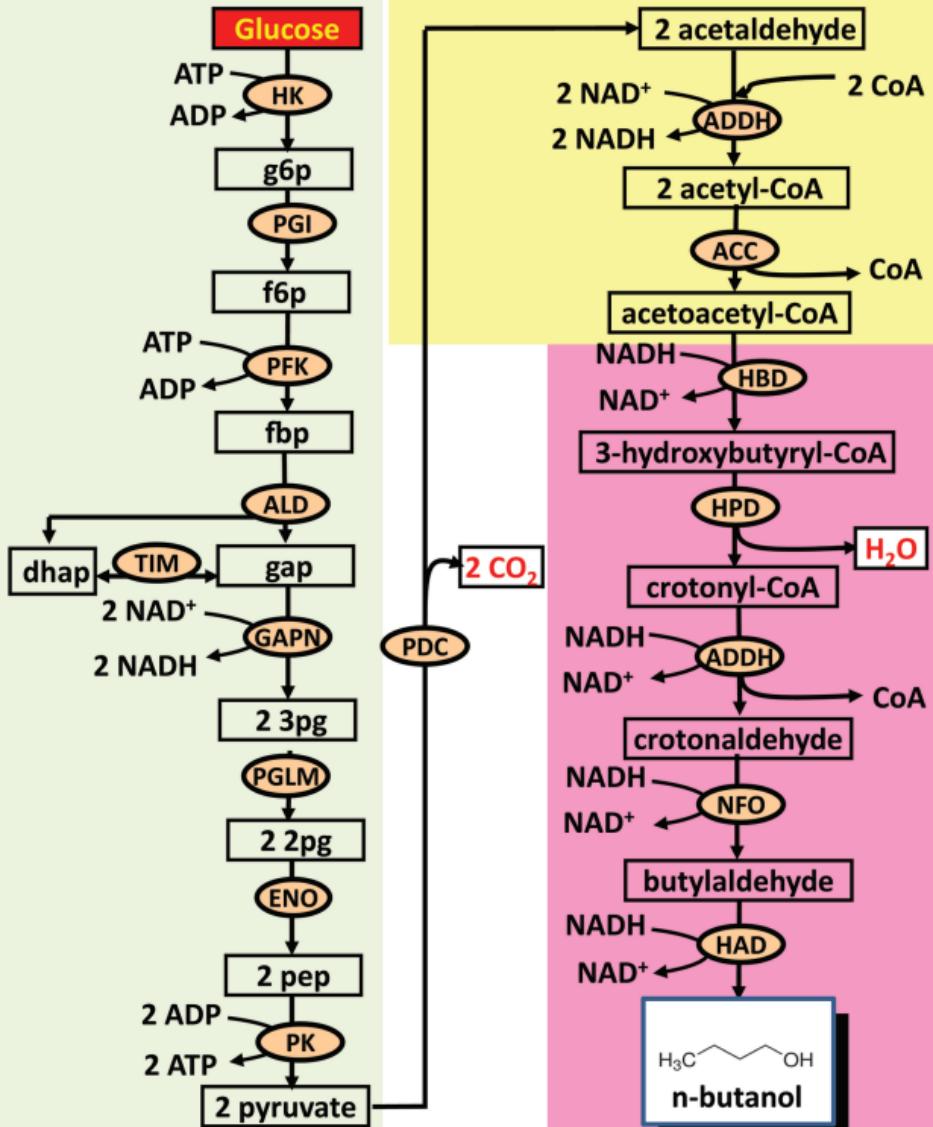
now

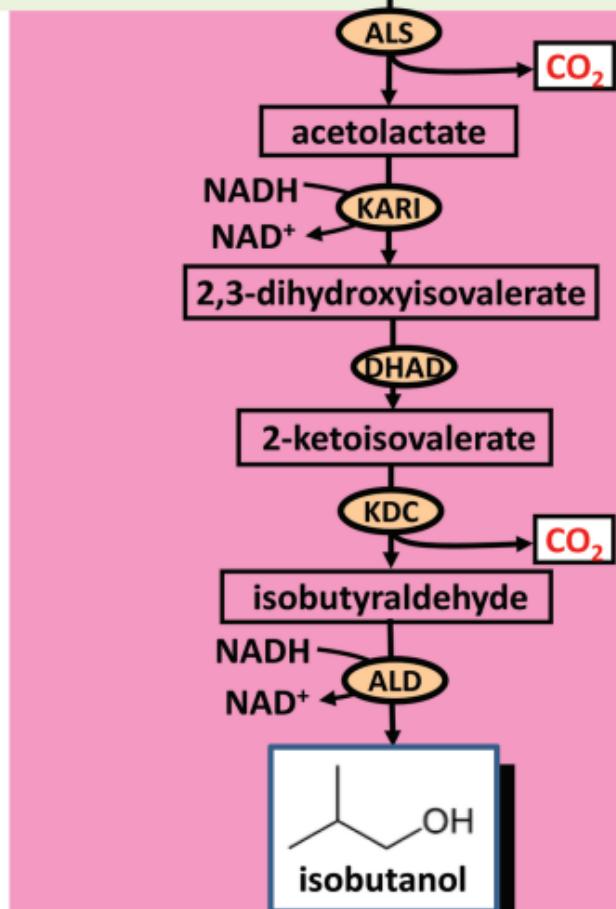
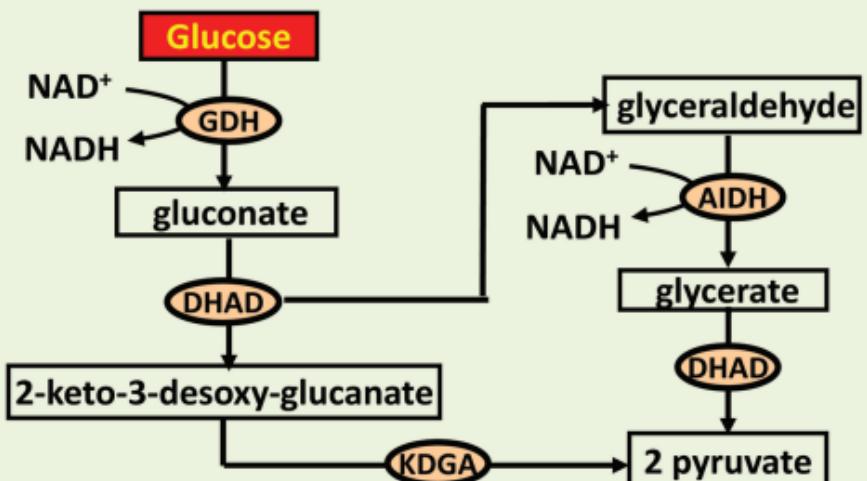
Biofuels
Biochemicals

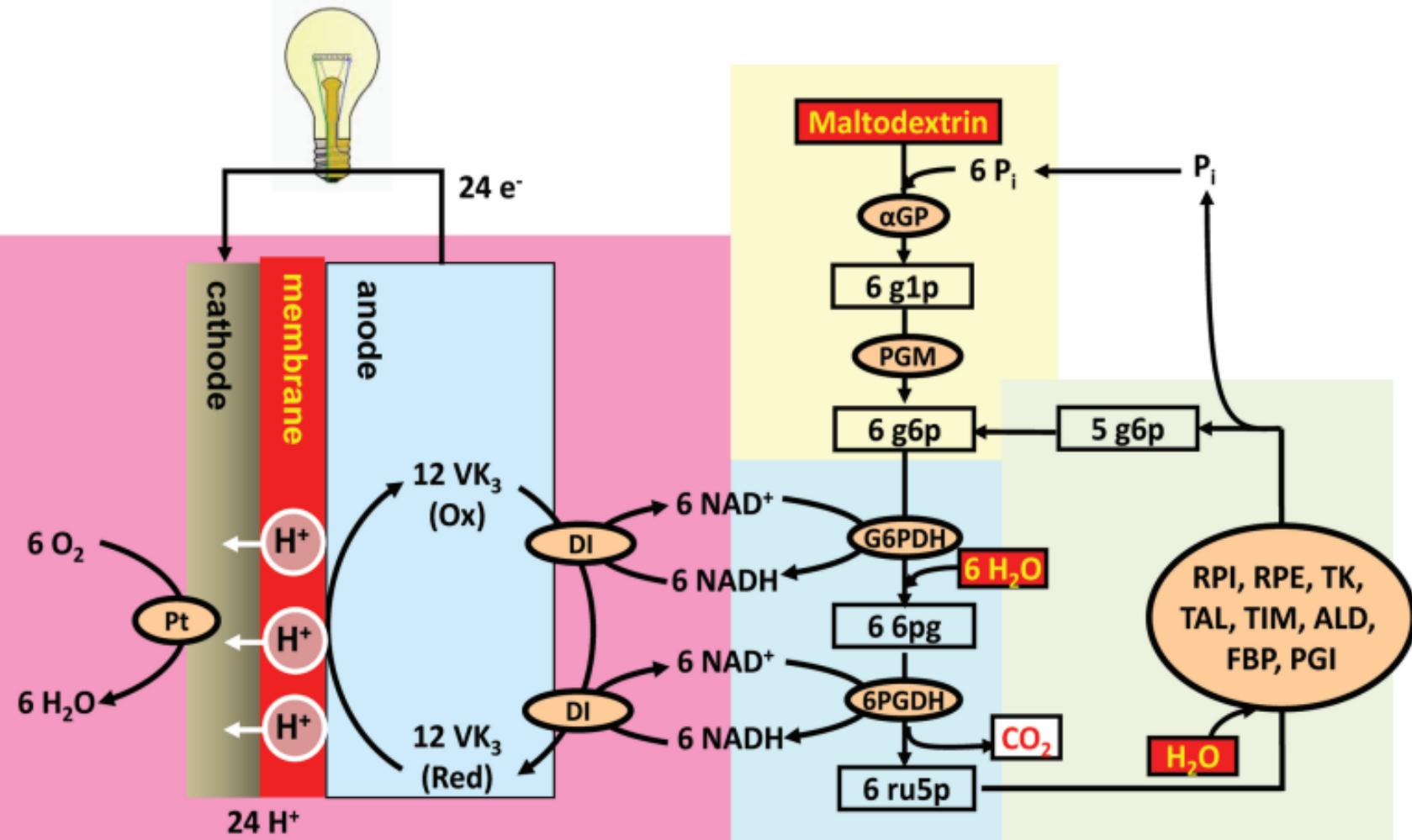
Feed &
food

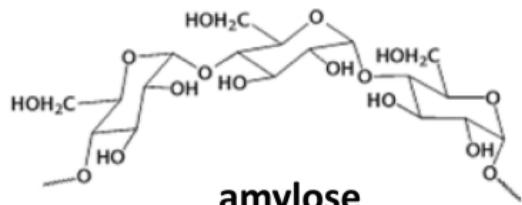
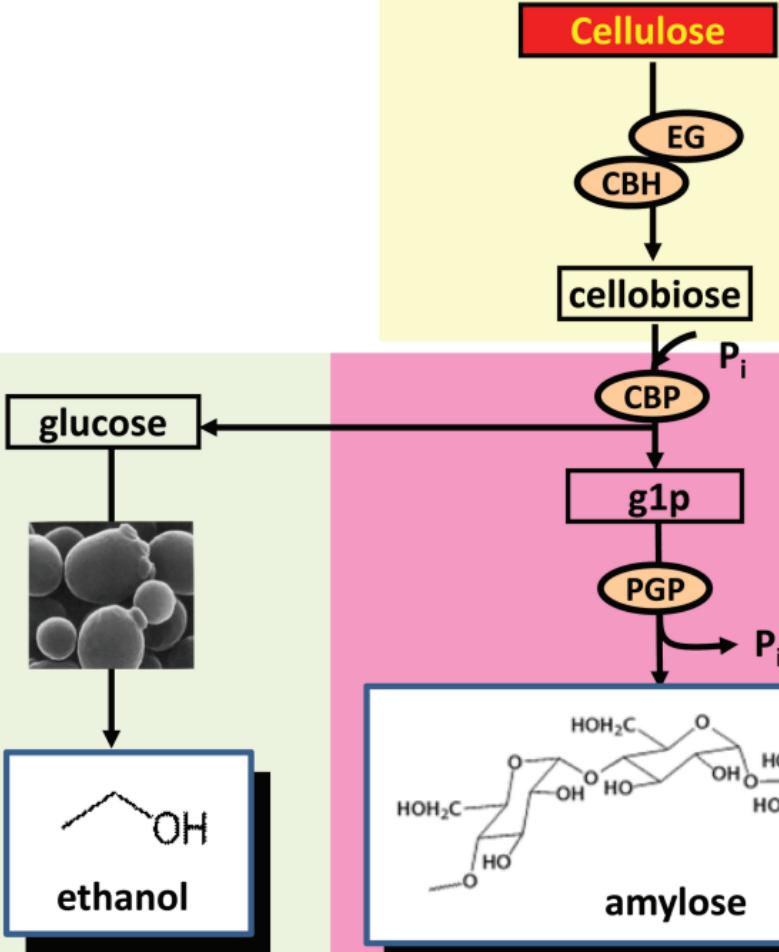
In vitro syn.
biosystems

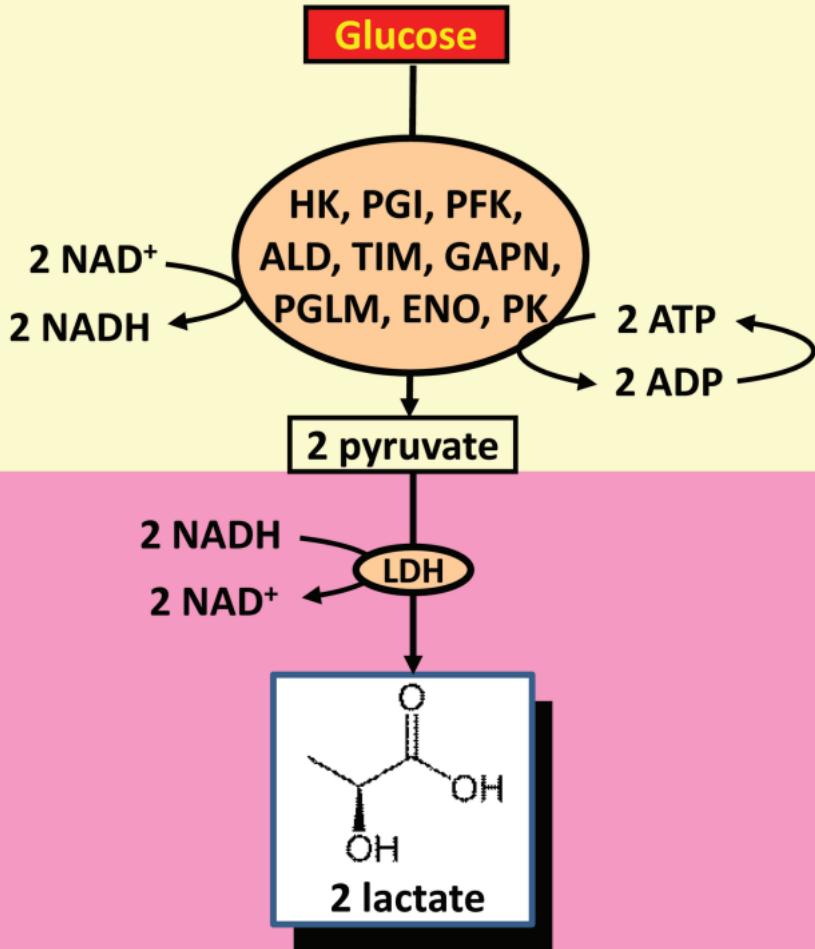


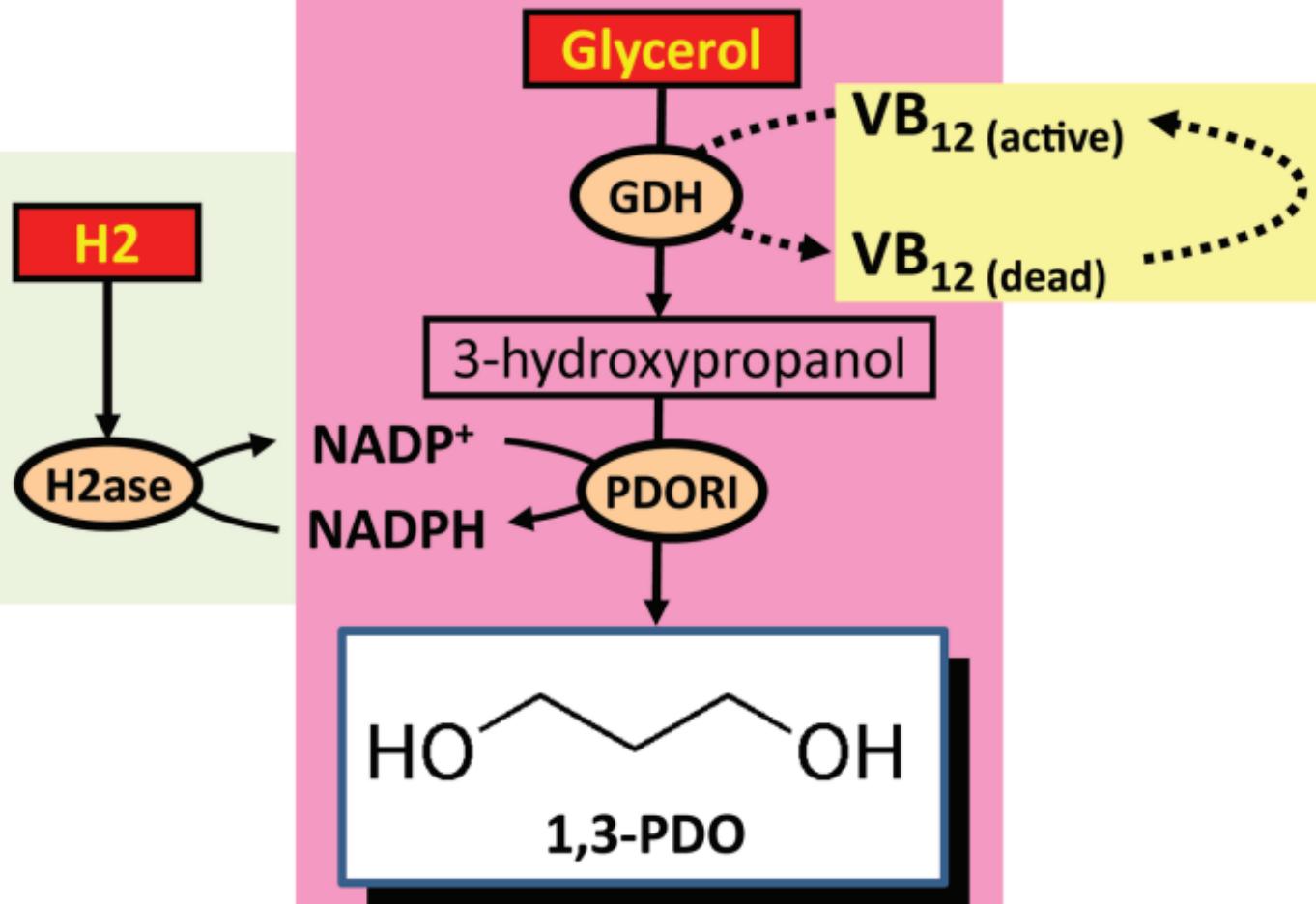


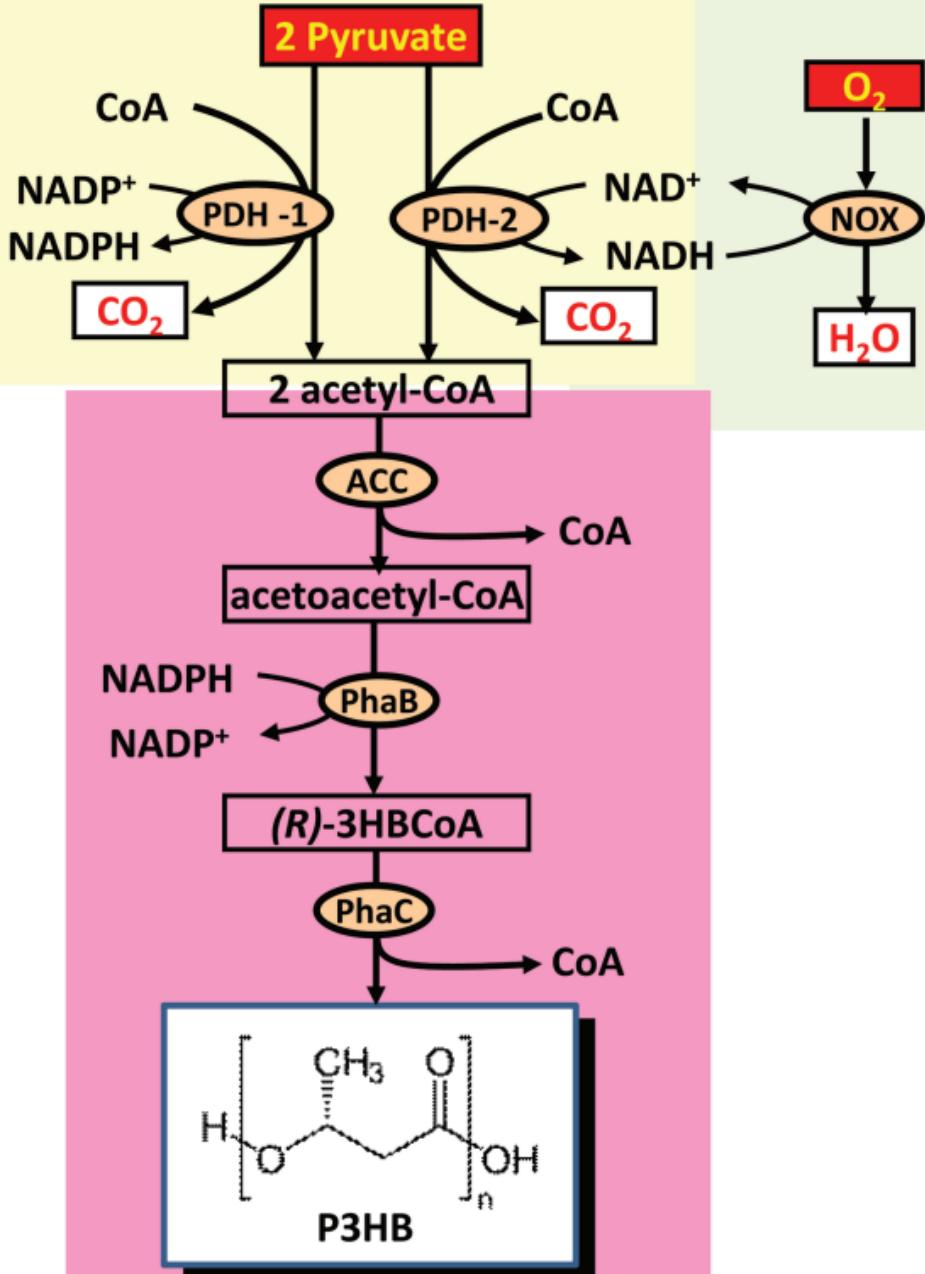




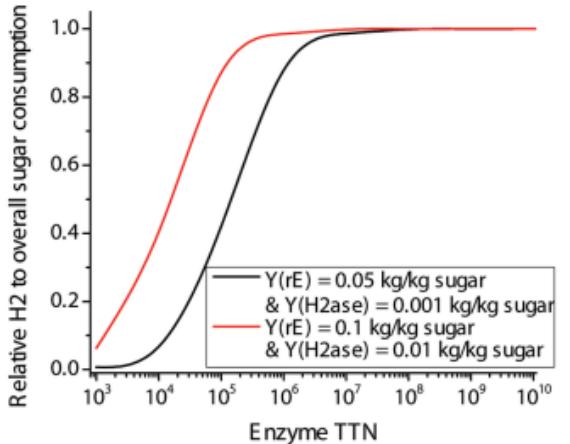




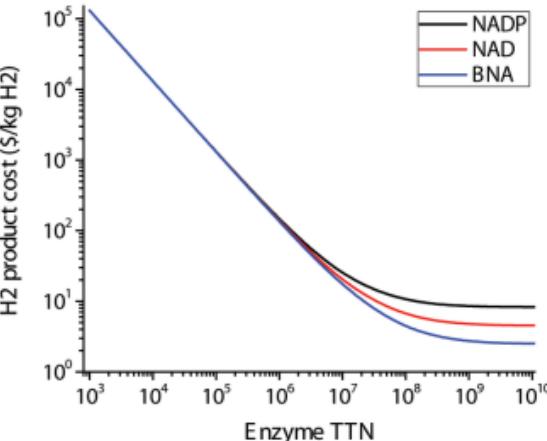




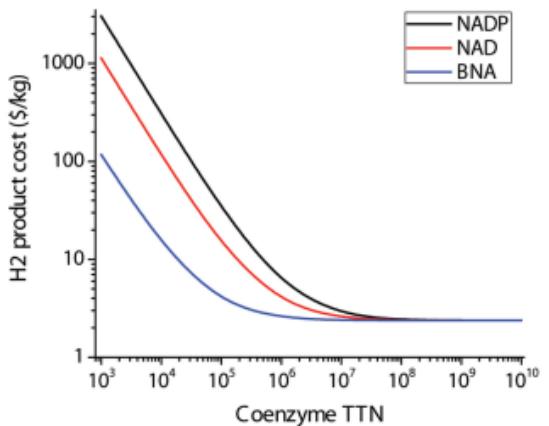
(a) Allocation to H₂



(b) Effects of TTN of enzyme and coenzyme



(c) Effects of TTN and type of coenzyme



(d) Effects of carbohydrate cost

