Biohydrogenation from Biomass Sugar Mediated by *in vitro* Synthetic Enzymatic Pathways

Running title: Biohydrogenation by Enzyme Cocktail

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Revised manuscript for **Chemistry and Biology**

ABSTRACT

Different from NAD(P)H regeneration approaches mediated by a single enzyme or a whole-cell microorganism, we demonstrate high-yield generation of NAD(P)H from a renewable biomass sugar – cellobiose through *in vitro* synthetic enzymatic pathways consisting of 12 purified enzymes and coenzymes. When the NAD(P)H generation system was coupled with its consumption reaction mediated by xylose reductase, the NADPH yield was as high as 11.4 mol NADPH per cellobiose (i.e., 95% of theoretical yield – 12 NADPH per glucose unit) in a batch reaction. Consolidation of endothermic reactions and exothermic reactions in one pot results in a very high energy-retaining efficiency of 99.6% from xylose and cellobiose to xylitol. The combination of this high-yield and projected low-cost biohydrogenation and aqueous phase reforming may be important for the production of sulfur-free liquid jet fuel in the future.

KEYWORDS: biohydrogenation, biomass, cell-free synthetic pathway biotransformation, cofactor regeneration, sulfur-free jet fuel

Highlight

- A combination of 12 enzymes *in vitro* produces nearly 12 NADPH per glucose unit
- Consolidating endo- and exo-thermic reactions leads to high energy efficiency.
- Enzyme cocktails work in the presence of microbe-toxic hydrolysate and impurities
- A novel hybrid of biocatalysis and catalysis could produce jet fuel.

Graphical Abstract

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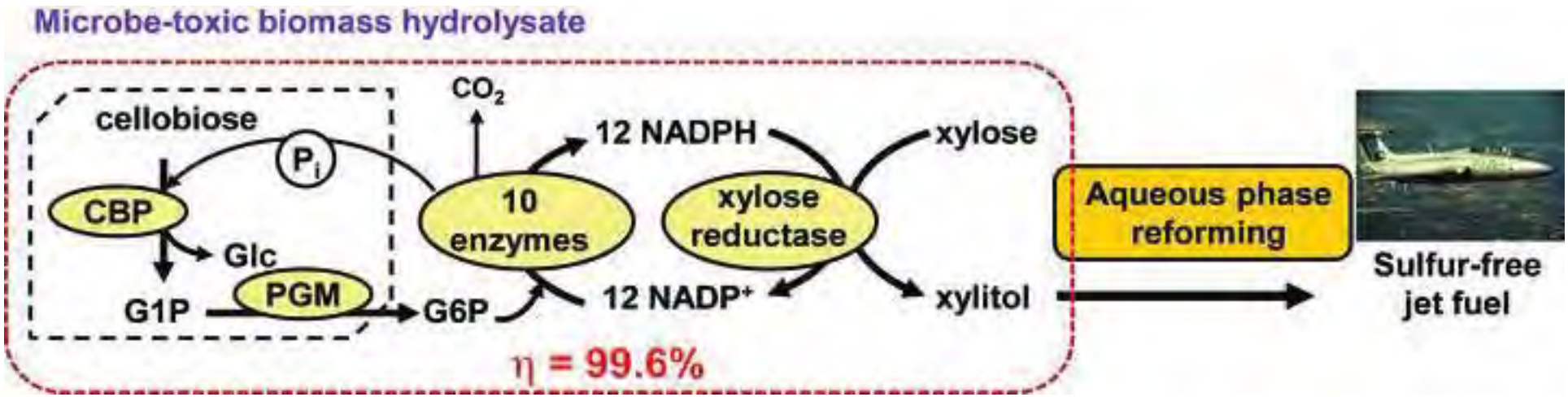

INTRODUCTION

Hydrogenation by using dihydrogen has been widely applied in the food, pharmaceutical, and energy industries. Because most dihydrogen is produced from non-renewable carbon-containing fossil fuels, such as natural gas, oil, and coal, net greenhouse gas CO_2 is released to the atmosphere in the process of dihydrogen generation (Navarro et al., 2009; Zhang, 2010b). NAD(P)H-based biohydrogenation catalyzed by enzymes is becoming more and more accepted, especially for the synthesis of high-value chiral compounds in the pharmaceutical industry (Wichmann and Vasic-Racki, 2005; Wildeman et al., 2007). NAD(P)H is usually generated by using a pair of a hydrogen-donor substrate and a single enzyme, including 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 (G6P)/G6P dehydrogenase (Wong and Whitesides, 1981), dihydrogen/hydrogenase (Mertens and Liese, 2004; Wong et al., 1981), and phosphite/phosphite dehydrogenase (Johannes et al., 2007; Vrtis et al., 2002). The relatively high costs of hydrogen donors (e.g., formic acid, alcohols, phosphite) and non-renewable hydrogen donor resources for NAD(P)H regeneration enable their applications to the synthesis of high-value chiral products while prohibit economically viable biohydrogenation for the production of low-value biofuel precursors, such as xylitol (Huber et al., 2005; Metzger, 2006).

Lignocellulosic biomass, the most abundant renewable bioresource, is a natural recalcitrant composite mainly composed of cellulose, hemicellulose, and lignin (Zhang, 2008). Biomass is believed to be a major renewable bioresource for the production of low-cost biofuels. Numerous biofuels can be made from biomass, including ethanol (Shaw et al., 2008), butanol and long

chain alcohols (Atsumi et al., 2008; Zhang et al., 2008), fatty acid esters (Lu et al., 2008; Steen et al., 2010), hydrogen (Chou et al., 2008; Zhang et al., 2007), hydrocarbons (Kunkes et al., 2008; Schirmer et al., 2010), waxes (Rude and Schirmer, 2009; Steen et al., 2010), and so on. Future transportation fuels might mainly consist of hydrogen for most vehicles, electricity for short-distance vehicles, and high-energy density liquid biofuels for jet planes (Huang and Zhang, 2011; Zhang, 2010b). Therefore, it would be important to efficiently produce high-energy density liquid jet fuel (e.g., hydrocarbons, fatty acid esters or butanol) suitable for jet planes from less costly biomass sugars, because energy application usually decides its production (Smil, 2008).

Dilute (sulfuric) acid pretreatment (DA), the most investigated biomass pretreatment method, has been tested in several pilot or demonstration biorefineries. After DA pretreatment and solid/liquid separation, the solid phase containing most cellulosic materials, minor hemicellulose, and lignin is hydrolyzed by cellulase and other hydrolytic enzymes for the generation of soluble sugars followed by further bioconversion (Zhu et al., 2009b). Biomass hydrolysate, a liquid supernatant, contains xylose (mainly) and small amounts of glucose, cellobiose, furfural, acetic acid, and phenolic compounds (Lau et al., 2009). Since biomass hydrolysate is highly toxic to most microorganisms, it must be detoxified before microbial fermentation (Mussatto and Roberto, 2004; Palmqvist and Hahn-Hagerdal, 2000). One of the common pre-detoxification approaches is neutralization by $CaCO_3$ so that the precipitate $CaSO_4$ can selectively absorb some inhibitors (Mussatto and Roberto, 2004; Palmqvist and Hahn-Hagerdal, 2000). Since thermodynamics decides economics for commodity production in a long term (Smil, 2008), high-energy efficiency production of liquid biofuels or their precursors (e.g., polyols) from the sugars

in the biomass hydrolysate may be very important for cost-effective production of biofuels eventually.

Cell-free synthetic pathway biotransformation (SyPaB) is the *in vitro* assembly of a number of enzymes and coenzymes for implementation of complicated biochemical reactions that a single enzyme cannot do (Zhang, 2010a; Zhang et al., 2010). This technology originates from cell-free ethanol fermentation discovered by the Nobel Prize Winner Eduard Buchner (1907 Chemistry). Later, i*n vitro* assembly of numerous purified enzymes for mimicking natural pathways has been used for the study of natural metabolisms (Brown et al., 2008; Kresge et al., 2005) and the synthesis of high-value products (Bujara et al., 2010; Meyer et al., 2007; Schultheisz et al., 2008; Wang and Zhang, 2009a). Recently, this technology has been demonstrated for the production of very high yields of low-value hydrogen through the non-natural synthetic pathways (Ye et al., 2009; Zhang et al., 2007), i.e., breaking the Thauer limit of anaerobic hydrogen-producing fermentation (i.e., 4 H_2/glucose) (Thauer et al., 1977). Furthermore, the possibility of SyPaB for the production of low-value biocommodities has been discussed and analyzed because of its obvious biomanufacturing advantages, such as high product yield, fast reaction rate, broad reaction condition, easy control, and so on (Zhang, 2010a; Zhang et al., 2010).

In this study, we demonstrated to generate ~12 moles of NAD(P)H per one glucose unit of oligosaccharides by the *in vitro* new synthetic enzymatic pathways. To avoid using any costly starch or its derived oligosaccharides, a biomass sugar – cellobiose – was used as a hydrogen donor because it is a primary product of the enzymatic hydrolysis of cellulose (Lynd et al., 2002; Zhang et al., 2006b) and a ready component of the dilute-acid pretreated biomass hydrolysate

(Zhu et al., 2009a). Also, the enzyme mixture was shown to work in the presence of microorganism-toxic biomass hydrolysate, where the *E. coli* strain cannot grow at all.

RESULTS

NAD(P)H Generation Pathway Design

One-enzyme reactions starting from glucose or glucose-6-phosphate (G6P) mediated by respective glucose dehydrogenase or G6P dehydrogenase usually generate only one reduced NAD(P)H factor (Fig. 1a) (Wichmann and Vasic-Racki, 2005). A two-enzyme reaction from G6P mediated by G6P dehydrogenase and 6-phosphogluconate dehydrogenase can produce two NADPH and one ribulose-5-phosphate (Ru5P) as well as release CO_2 (Fig. 1b) (Wang and Zhang, 2009b; Wong and Whitesides, 1981). It is expected that six molecules of Ru5P can be generated back to five molecules of G6P and one phosphate by using four enzymes in the non-oxidative pentose phosphate pathway (PPP) [i.e., phosphoribose isomerase (R5PI); ribulose 5-phosphate epimerase (Ru5PE); transketolase (TKL); and transaldolase (TAL)], and four enzyme in the glycolysis and gluconeogenesis pathways [i.e., triose phosphate isomerase (TIM); aldolase (ALD); fructose-1, 6-bisphosphatase (FBP); and PGI, phosphoglucose isomerase (PGI)] (Fig. 1c). Therefore, one molecule of G6P can generate 12 NADPH, six CO_2, and one phosphate (Eq. 1).

$$G6P + 12\ NADP^+ + 7\ H_2O \rightarrow 12\ NADPH + 12\ H^+ + P_i + 6\ CO_2\ (g) \qquad [1]$$

To avoid using costly G6P, a substrate phosphorolysis reaction can be used to generate G6P from polysaccharides and phosphate by glucan phosphorylase and phosphoglucomutase (PGM). The

integrated 12-enzyme cocktail can generate 12 NAD(P)H by consuming an anhydrous glucose unit of polysaccharide without the use of costly ATP (Eq. 2) (Fig. 1d).

$$(C_6H_{10}O_5)_n + 12\ NADP^+ + 7\ H_2O \rightarrow (C_6H_{10}O_5)_{n-1} + 12\ NADPH + 12H^+ + 6\ CO_2\ (g) \quad [2]$$

Proof-of-Concept Experiments

A non-natural synthetic pathway (Fig. 2) was designed to contain cellobiose phosphorylase (CBP) and phosphoglucomutase (PGM) for producing glucose-6-phosphate (G6P) from cellobiose (CB) (Eq. 3), followed by the 6-enzyme pentose phosphate pathway (PPP) and 4-enzymes in the glycolysis and gluconeogenesis pathways for generating 12 NADPH per G6P (Eq. 1).

$$C_{12}H_{22}O_{11} + P_i \rightarrow G6P + C_6H_{12}O_6 \quad [3]$$

By coupling NADPH generation from cellobiose with a NADPH consumption reaction mediated by xylose reductase (XR) that can convert xylose to xylitol (Woodyer et al., 2005) in Eq. 4, the proof-of-principle reaction contained 13 enzymes in one pot for implementing Eq. 5. In it, nine enzymes of them were produced heterologously in *E. coli*, including CBP, PGM, 6PGDH, R5PI, Ru5PE, TK, TIM, FBP, and XR, and were purified with nearly homogeneity (Fig. S1), along with the other commercial enzymes (Table 1).

$$C_5H_{10}O_5 + NADPH + H^+ \rightarrow C_5H_{12}O_5 + NADP^+ \quad [4]$$

$$C_{12}H_{22}O_{11} + 12\ C_5H_{10}O_5 + 7\ H_2O \rightarrow C_6H_{12}O_6 + 12\ C_5H_{12}O_5 + 6\ CO_2\ (g) \quad [5]$$

The xylitol synthesis profile of four cases: CB/12-E, G6P/10-E, G6P/1-E (Control 1), and G6P/2-E (Control 2) were examined (Fig. 3). Our previous study showed that the reactions mediated by two dehydrogenases (G6PDH and 6PGDH) were rate-limiting step when each enzyme loading was the same (i.e., one unit per reaction) (Ye et al., 2009). In the following experiments, two dehydrogenases loadings were increased to 10 units per reaction. When G6P was a hydrogen donor, xylitol was generated fast regardless of whether one, two or ten enzymes were used for NADPH generation. The initial xylose-synthesis reaction rate based on cellobiose (2.8 mmol/h/L, 12-E) was slower than those based on G6P (11.0 mmol/h/L, G6P/10-E; and 3.2 mmol/h/L, G6P/2-E), in consistence with the mechanism that the 12-E system needed two extra steps for generation of low concentration G6P, followed by NADHP generation. The initial xylose-synthesis reaction in the G6P/2-E case was lower than that in the G6P/10-E case because more enzymes in the following cascade pathways can help decrease levels of glucose-6-phosphate and 6-phosphogluconate that strongly inhibited the activities of G6PDH and 6PGDH. The highest xylitol level obtained was 26.2 mM at hour 54 for the case of the G6P/10-E, higher than that of the CB/12-E. The yields of NADPH regenerated were 6.66, 8.74, 1.97, and 0.98 mole/mole of glucose equivalent for the CB/12-E, G6P/10-E, G6P/2-E and G6P/1-E, respectively (Table 2). The relatively low NADPH yields for the CB/12-E and G6P/10-E reactions in the proof-of-concept experiments were attributed to incomplete conversion of intermediate metabolites, including NADPH, glyceraldehyde-3-phosphate, ribulose-5-phosphate, fructose bisphosphate, etc., and substrate G6P in the batch reactions. For example, approximately 0.22 and 0.60 mM G6P were detected at the end of the batch reactions of the G6P/10-E and CB/12-E, respectively.

NADH can be regenerated based on the same pathway with modifications by using the NAD^+-specific *Leuconostoc mesenteroides* G6PDH and *Moorella thermoacetica* 6PGDH (Table 1). But the NADH yield of the G6P/10-E was relatively low, ca. 5.40 per glucose unit (Table 2) because xylose reductase cannot as efficiently utilize NADH as NADPH (Woodyer et al., 2005).

Biomass hydrolysate is a liquid fraction after dilute sulfuric acid lignocelluloses pretreatment. It is highly toxic to microbial fermentation (Lau et al., 2009). For example, a 20% (v/v) of pH-neutral hydrolysate from dilute acid-pretreated corn stover completely inhibited ethanol fermentation of an ethanol-producing *E. coli* KO11 (data not shown). But the enzyme cocktails were able to produce 5.60 and 6.20 mol of NADPH/mol of cellobiose consumed based on NaOH- and $CaCO_3$-neutralized diluted acid pretreated corn stover hydrolysate, respectively (Table 2). The higher NADPH yield for the $CaCO_3$-neutralized sample may be due to partial removal of toxic compounds through simple adsorption by solid $CaSO_4$. These NADPH yields based on the hydrolysate were slightly lower than the value of the CB/12-E (6.66), suggesting that the enzyme cocktails can work in the presence of other microorganism-toxic compounds.

Optimization of NADPH yields

To further improve NADPH yield, the enzyme ratios in the synthetic pathways had to be optimized because of incomplete utilization of G6P and accumulated other metabolites at the end of the proof-of-principle experiments. Our previous metabolic engineering kinetics models suggested that NADPH regeneration mediated by two dehydrogenases (G6PDH and 6PDDH) were rate-limiting when each enzyme have the same loading (Ye et al., 2009). Therefore, their enzyme loadings were increased to 10-fold higher than the others in the pathway for accelerating

192 the overall reaction rates (Table 1). The updated model in metabolite pathway flux with the new
193 settings (e.g., new enzyme parameters and loadings) suggested that the reaction linking
194 glyceraldehyde-3-phosphate and dihydroxacetone phosphate for forming fructose 1,6-
195 bisphosphate might be enzyme-limited. When we increased fructose 1,6-bisphosphate aldolase
196 from 1 to 10 U/mL (Table 1), the NADPH yields were enhanced to 11.5 and 11.4 for the
197 G6P/10-E and the cellobiose/12-E, respectively (Table 2). The levels of G6P, other metabolites,
198 and NADPH by the end of the reactions (data not shown) were not detectable (e.g., below 0.1
199 mM). For $CaCO_3$-neutralized biomass hydrolysate, the NADPH yield was 10.9. In the future,
200 enzymatic conversion of toxic biomass hydrolysate to polyols would be very important for direct
201 utilization of the microorganism-toxic biomass hydrolysate without significant detoxification.
202 The decreases in an initial $NADP^+$ concentration to 0.02 mM resulted in nearly two orders of
203 magnitude increases in coenzyme TTN from 23 and 23 to 1440 and 1665 based on G6P and CB,
204 respectively, but these attempts drastically decreased NADPH yields to 8.30 and 7.20,
205 respectively (Table 2).
206
207 **DISCUSSION**
208 This study shows that a biomass sugar – cellobiose -- can be used as a hydrogen donor for high-
209 yield biohydrogenation. As compared to other enzyme-mediated biohydrogenation approaches,
210 complete oxidation of biomass sugars for biohydrogenation has the lowest substrate cost
211 ($1.35/kg H_2 added), much lower than the costs of hydrogen generated from natural gas (e.g., ~
212 $2.5/kg H_2) or other substrates, such as alcohols, formic acid, and phosphite (Fig. 4a). Although
213 nearly 12 moles of hydrogen has been produced per mole of glucose unit by SyPaB as described
214 before (Ye et al., 2009; Zhang et al., 2007), direct biohydrogenation through NAD(P)H but not

215 through hydrogen is more advantageous because of (i) consolidating hydrogen generation and
216 hydrogen consumption in one reactor, (ii) avoiding use of costly hydrogenase (Mertens and
217 Liese, 2004; Wong et al., 1981), (iii) lowering safety risk for hydrogen-containing bioreactors,
218 and (iv) no strictly anaerobic conditions needed for enzymatic biohydrogen reaction.
219
220 In addition to substrate costs, there are two cost factors influencing biohydrogenation –
221 coenzyme and enzymes. When TTN values of the coenzyme are the same for all enzymatic
222 biohydrogenation approaches, coenzyme expenditures are the same regardless of enzyme
223 number in the enzymatic reactions. Both increasing total turn-over number of enzymes (i.e.,
224 enzyme stability) and decreasing their production costs can drastically decrease expenditures in
225 enzymes (Fig. 4b). When enzyme costs are $400 per kg, TTN values must be higher than $3 \times$
226 10^7 mol reaction catalyzed by enzyme per mol of enzyme so to decrease the enzyme costs to a
227 critical low level (e.g., $1.00/per kg of hydrogen added). When the enzyme production costs
228 can be reduced to $40/kg or as low levels as the bulk (recombinant non-membrane) industrial
229 enzymes at $5-20 per kg, the expenditure of the enzyme mixture would be lower than $1.00/kg
230 of hydrogen added at TTN values of each enzyme $\geq 3 \times 10^6$ or $0.10/kg of hydrogen added at
231 TTN values of each enzyme $\geq 3 \times 10^7$ (Fig. 4b). In practice, it is relatively easy to discover the
232 thermoenzymes with the TTN values of more than 10^7 from (hyper-)thermophilic
233 microorganisms by utilizing their available genomic DNA sequences and bioinformatics analysis
234 (Blumer-Schuette et al., 2008; Nelson et al., 1999), for example, *Clostridium thermocellum*
235 phosphoglucomutase (Wang and Zhang, 2010), *Thermotoga maritima* 6-phosphogluconate
236 dehydrogenase (Wang and Zhang, 2009b), and so on. In the food industry, immobilized
237 thermostable glucose isomerase has reached a TTN value of more than 250 million (Vasic-Racki,

238 2006). In addition, the TTN value of the immobilized *C. thermocellum* phosphoglucose
239 isomerase by simple adsorption on a low-cost cellulosic material is as high as 10^9 (submitted for
240 publication). Considering the cost contributions from the sugar feedstock (\$1.35/kg H_2 added),
241 the enzyme mixture (\$0.10-1.00/kg H_2 added), and future use of less costly and more stable
242 biomimetic coenzyme (Ryan et al., 2008; Zhang, 2010a; Zhang et al., 2010), biohydrogenation
243 would be very cost competitive with hydrogenation where hydrogen is made from natural gas
244 (\$2.0-2.7/kg H_2).
245
246 It was vital to analyze the carbohydrate allocation based on glucose between the product and
247 biocatalyst (e.g., microbial cell and purified enzyme) since bulk enzyme in the SyPaB system
248 must be produced through microbial fermentation, which consumes carbohydrate. Typical
249 values of recombinant enzyme yield based on glucose ($Y_{P/S}$) ranges from ~0.005 to 0.175 g of
250 protein/g of glucose consumed, based on several well-known facts of (i) cell mass yield based on
251 glucose in aerobic fermentation ($Y_{X/S}$) = ~0.5 g cell mass/g glucose (Huang and Zhang, 2011;
252 Shuler and Kargi, 2001; Zhang et al., 2010), (ii) typical intracellular cellular protein content =
253 ~0.5 g protein/g cell mass (Zhang and Lynd, 2003), and (iii) typical recombinant enzyme
254 percentage in total cellular protein = 2-70% (Myung et al., 2010; Wang and Zhang, 2010; Wang
255 and Zhang, 2009b; Ye et al., 2010). When each enzyme in the SyPaB systems has TTN values
256 of $\geq 3 \times 10^7$ and $Y_{P/S}$ values of ~0.03 (a very conservative value for recombinant protein
257 production in *E. coli*), the carbohydrate allocation to the production of the enzyme mixtures was
258 ~0.01, i.e., ~99% of carbohydrate would be used to produce the desired product. The calculation
259 of carbohydrate allocation to the enzyme mixture is presented as below. When $Y_{P/S}$ is 0.03 g of
260 protein/g of glucose, one kg of glucose can produce 0.03 kg of enzyme (i.e., 0.6×10^{-3} mol of

261 enzyme, MW = 50,000). So 35 kg of glucose is needed to produce $18 \times 10^{-3} = 35 * 0.6 \times 10^{-3}$ mol
262 of the enzyme mixture, where an elemental enzyme use in the 12-E system is 0.6×10^{-3} mole and
263 the lumped stoichiometric coefficient for the 12-E system is 35. In this system, the exact use for
264 each enzyme is 0.6×10^{-3} mole multiplied by its stoichiometric coefficient (Fig. 2), for example,
265 0.6×10^{-3} mole of PGM and 3.6×10^{-3} mole of G6PDH. When each enzyme has TTN values of
266 more than 3×10^7, a 12-E cocktail made from 35 kg of glucose can produce 432 kg of H
267 equivalent = 24 H equivalent (i.e., 12 NAD(P)H) per anhydroglucose ($C_6H_{10}O_5$) and seven
268 water (or per glucose, $C_6H_{12}O_6$, and six water) * 3×10^7 mol reaction/mol enzyme * 0.6×10^{-3}
269 mol enzyme * 10^{-6} kg/mol H equivalent. So, 432 kg of H equivalent is made from 3,240 kg of
270 glucose and 1,944 kg of water. Therefore, the allocation of carbohydrate to the enzyme cocktail
271 is 35/(35 + 3,240) * 100% = 1.07%. The higher $Y_{P/S}$ values, the lower allocation of
272 carbohydrate to the synthesis of the enzyme cocktails, the more allocation to the desired product.
273
274 We suggest a new high-energy efficiency way for producing liquid hydrocarbons from the
275 biomass hydrolysate through this biohydrogenation followed by aqueous phase reforming (APR)
276 because the overall energy conversion efficiency mainly decides the economics of biofuel
277 production. This hybrid process has a very high energy retaining efficiency (94.6%) involving
278 nearly no loss in combustion energy through biohydrogenation (99.6%, Table 3)) and 95%
279 energy efficiency-retaining APR (Huber et al., 2004; Metzger, 2006), much higher than those
280 through microbial fermentations: ethanol, methane, palmitate acid (Table 3), and other biofuels
281 (Huang and Zhang, 2011; Li et al., 2010; Schirmer et al., 2010; Steen et al., 2010). The overall
282 reaction (Eq. 5) is composed of three reaction modules – Equation 3 ($\Delta_r G^{0'}$ = -3.3 kJ/mol and
283 $\Delta H^{o'}$ = -10.0 kJ/mol), Equation 1 ($\Delta_r G^{0'}$ = -207.5 kJ/mol and $\Delta H^{o'}$ = +268.9 kJ/mol), and

Equation 4 ($\Delta_r G^{0'}$ = -20.8 kJ/mol and $\Delta H^{o'}$ = -26.1 kJ/mol) at pH 7.0 (Table 3). Because the overall SyPaB reaction integrates the endothermic reaction (Eq. 1) and two exothermic reactions (Eqs. 3 and 4) in one pot, the resulting reaction has a very high energy-retaining efficiency (99.6%). This biocatalysis can utilize the sugars from the microorganism-toxic biomass hydrolysate and APR can decrease the product separation costs because alkanes are not water soluble. Therefore, the combination of biohydrogenation and ARP may be an important pathway for the production of sulfur-free jet fuel in the future.

One of the major advantages of cell-free SyPaB is very high product yields even including sugar use for the enzyme production. SyPaB does not rely on living microorganisms that insist on consuming a significant fraction of carbon and energy sources for cell growth and duplication as well as producing undesired by-products and has much longer running time (Zhang, 2010a). In microbial fermentations, glucose serves as both a growth substrate and a source of reducing equivalents (NADPH) mainly through the PPP, NADPH-dependent isocitrate dehydrogenase in the TCA cycle, and transhydrogenase (Cirino et al., 2006; Sauer et al., 2004). It is really challenging to isolate microbial basic metabolisms for its growth and duplication from the formation of desired products. As a result, only a small fraction of glucose is allocated to NADPH generation. For example, biotransformation mediated by growing cells exhibits a typical yield of ~2.3 NADPH/glucose (Cirino et al., 2006; Walton and Stewart, 2004). Much higher yields (up to 4.7 NADPH/glucose) are obtained by resting cells whose basic metabolisms are stopped (Cirino et al., 2006). In this study, the isolation of the high-yield product formation by SyPaB and the efficient enzyme production by microbial fermentation would bring several

benefits: increasing sugar utilization efficiency, simplifying the product separation (CO_2 as the final product), decreasing product inhibition, and avoiding complicated cellular regulation.

SIGNIFICANCE

Cell-free SyPaB is an emerging direction of synthetic biology for low-cost and high-yield biomanufacturing (Zhang, 2010a; Zhang et al., 2010). This work demonstrated another new application of SyPaB – the production of NAD(P)H. Nearly complete oxidation of glucose unit by using water as an oxidant through the synthetic enzymatic pathways can generate ~12 NADPH per glucose unit. In addition to the biohydrogenation of low-value sugars to polyols as already described, regenerated NADPH could also be used for the synthesis of high-value chiral compounds in the pharmaceutical industry. The highest-yield and projected low-cost biohydrogenation was achieved from a biomass sugar – cellobiose by using an enzyme cocktail consisting of 12 purified enzymes and coenzymes. This work also demonstrated that the enzyme cocktail systems can work in the presence of microorganism-toxic compounds from dilute-acid pretreated biomass hydrolysate, suggesting that enzyme systems do not require high-purity substrates for biotransformation. A hybrid of biohydrogenation mediated by SyPaB and catalysis APR may be used to produce sulfur-free high energy density liquid hydrocarbons as jet fuel from dilute acid-pretreated biomass hydrolysate at low costs and with high energy-retaining efficiencies. The main hurdles to SyPaB are a lack of Lego-like stable enzymes and high cost of the labile coenzymes but these are being addressed (Zhang, 2010a; Zhang et al., 2010).

EXPERIMENTAL PROCEDURES

328 **Chemicals.** All chemicals were reagent grade or higher, purchased from Sigma (St. Louis, MO)
329 or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Regenerated amorphous cellulose
330 (RAC) was prepared from Avicel as previously described (Zhang et al., 2006a). The
331 oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). Corn stover
332 was pretreated by dilute acid, conducted in the National Renewable Energy Laboratory (Boulder,
333 CO) (Zhu et al., 2009a; Zhu et al., 2009b). After centrifugation, the supernatant was neutralized
334 by either calcium carbonate powder or a 2.0 M sodium hydroxide solution for the enzymatic
335 reactions.
336 **Strains and media.** *Escherichia coli* DH5α was used as a host cell for DNA manipulation, and
337 *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) were used as the host for recombinant
338 protein expression. The Luria-Bertani (LB) medium plus 100 μg/mL ampicillin was used for *E.*
339 *coli* cell growth and recombinant protein expression.
340 **Gene cloning and plasmid construction.** Five new protein expression plasmids were
341 constructed for expressing *Clostridium thermocellum* cellobiose phophorylase (CBP), *Moorella*
342 *thermoacetica* 6-phosphogluconate dehydrogenase (6PGDH), *C. thermocellum* ribose 5-
343 phosphate isomerase (R5PI), *Thermus thermophilus* ribulose-5-phosphate 3-epimerase (Ru5PE),
344 and *T. thermophilus* triose phosphate isomerase (TIM) (Table S1). The genes were amplified
345 based on their genomic DNA templates, digested by the restriction enzymes, and ligated for
346 constructing the expression vectors (Table S1). *T. thermophilus* transketolase (TK) was
347 synthesized with N-terminal His-tag by DNA2.0 Inc. (Menlo Park, CA, USA) based on the *E.*
348 *coli* optimum codons.
349 **Recombinant protein production and purification.** The *E. coli* BL21 strain harboring the
350 protein expression plasmid was incubated in 200 mL of the LB culture in 1-L Erlenmeyer flasks

with a rotary shaking rate of 250 rpm at 37 ^{o}C for the cell growth. The recombinant protein expression was induced by adding IPTG when the absorbance (A_{600}) reached *ca.* 0.6. For the production of most of the recombinant proteins, the cultures were incubated at 37 ^{o}C for 4 h, unless otherwise noted. For the production of the difficult-to-express recombinant enzymes (i.e., R5PI, Ru5PE and PGM), the cultures were incubated at 18 ^{o}C for 23 h. The cells were harvested by centrifugation at 4 ^{o}C, washed once with 50 mM of Tris-HCl buffer (pH 7.5), and re-suspended with a 10 mL of 30 mM Tris-HCl buffer (pH 8.5) containing 0.5 M of NaCl and 1 mM of EDTA. The cell pellets were lysed by ultra-sonication by Fisher Scientific Sonic Dismembrator Model 500 (5-s pulse on and off, total 180 s, at 20% amplitude). After centrifugation, the supernatant of the cell lysate containing the His-tagged recombinant proteins (Table 1) was purified by using Ni-column. The recombinant FBPase was purified by using the GE Healthcare ion exchange Q column (Zhang et al., 2007). The supernatant of the cell lysate containing the fusion protein CBM-intein-PGM or –R5PI was purified through affinity adsorption on a large surface area RAC (Hong et al., 2007), followed by intein cleavage (Hong et al., 2008).

Enzyme activity assays. *C. thermocellum* cellobiose phosphorylase (CBP) activity was assayed as described elsewhere (Zhang and Lynd, 2004). *C. thermocellum* phosphoglucomutase activity was assayed as described previously (Wang and Zhang, 2010). *M. thermoacetica* 6-phosphogluconate dehydrogenase (6PGDH) activity was measured in a 50 mM Hepes buffer (pH 7.2) containing 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 2.5 mM 6-phosphogluconate, and 2.5 mM NAD^+ or $NADP^+$. It displayed similar NAD^+ (17 U/mg) specific activity over $NADP^+$ (13 U/mg) at 20^{o}C. *T. thermophilus* HB27 ribose 5-phosphate isomerase (R5PI) activity was determined on a substrate D-ribulose 5-phosphate. The reactions were carried out in a 50 mM

Tris/HCl (pH7.5) containing 2 mM D-ribulose 5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/mL BSA at 30^{o}C in a 5 minute reaction period. The product D-ribose 5-phosphate was enzymatically quantified by consumption of NADH in 50 mM Tris/HCl (pH7.5) containing 2 mM xylulose 5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/mL BSA, 0.2 mM thiamine pyrophosphate, 5 U/mL transketolase, 60 U/mL triose phosphate isomerase, 20 U/mL D-glyceraldehyde 3-phosphate dehydrogenase, and 0.25 mM NADH at 30^{o}C for 5 minutes. The specific activity of TmR5PI was 15 U/mg at 30^{o}C. *T. thermophilus* HB27 ribulose 5-phosphate epimerase (Ru5PE) activity was determined on a substrate D-ribulose 5-phosphate. The reactions were carried out in a 50 mM Tris/HCl (pH7.5) containing 2 mM D-ribulose 5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/mL BSA at 30^{o}C for 5 minutes. The product D-xylulose 5-phosphate was enzymatically quantified by consumption of NADH in 50 mM Tris/HCl, pH7.5, 2 mM ribose-5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/mL BSA, 0.2 mM thiamine pyrophosphate, 5 U/mL transketolase, 60 U/mL triose phosphate isomerase, 20 U/mL D-glyceraldehyde 3-phosphate dehydrogenase, and 0.25 mM NADH at 30^{o}C for 5 minutes. The specific activity of Ru5PE was 30 U/mg at 30^{o}C. *T. thermophilus* HB27 transketolase (TK) activity was measured on the substrates of D-xylulose 5-phosphate and D-ribose 5-phosphate. The reactions were carried out in a 50 mM Tris/HCl (pH7.5) containing 2 mM D-xylulose 5-phosphate, 2 mM D-ribose 5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/mL BSA at 30^{o}C for 5 minutes. The product D-glyceraldehyde 3-phosphate was quantified through the consumption of NADH in 50 mM Tris/HCl (pH7.5) containing 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/mL BSA, 0.2 mM thiamine pyrophosphate, 5 U/mL transketolase, 60 U/mL triose phosphate isomerase, 20 U/mL glycerol 3-phosphate dehydrogenase, and 0.25 mM NADH at 30^{o}C for 5 minutes. The activity of TK was 0.2 U/mg at 30^{o}C. *Thermus thermophilus* triose

phosphate isomerase (TIM) activity was measured in 50 mM Tris/HCl (pH7.5) containing 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/mL BSA, 20 U/mL glycerol 3-phosphate dehydrogenase, and 0.25 mM NADH at 30°C. Fructose 1,6-biphosphatase (FBP) activity was determined as described with modifications (Myung et al., 2010). The assay was carried out in a 50 mM Hepes buffer (pH 7.2) containing 1.5 mM fructose 1,6-bisphosphate, 0.5 mM $MnCl_2$, and 50 mM NaCl at 30°C.

Xylitol production. Synthesis of xylitol from xylose was conducted in 300 μL of the reaction mixture at 25°C. The reaction buffer contained 100 mM HEPES buffer (pH 7.2) supplemented with 5 mM Mg^{2+}, 0.5 mM Mn^{2+}, 1 mM thiamine pyrophosphate, and 2 mM NA(D)P^+, unless otherwise noted. The substrates were 3 mM G6P or cellobiose and 100 mM xylose. The enzyme loadings were shown in Table 1. Ten μL of the samples were withdrawn and quenched by 90 μL of 5 mM H_2SO_4 at different time intervals. The concentrations of xylose, cellobiose, and xylitol were measured by a HPLC equipped with the Bio-Rad Aminex HPX-87H column (Zhang and Lynd, 2005). The NAD(P)H yield ($Y_{NAD(P)H}$) in terms of mole of NAD(P)H generated per mole of H_2 donor (G6P or CB) consumed was calculated as below

$$Y_{NAD(P)H} = \frac{\Delta[xylitol] + \Delta[NAD(P)H]}{\Delta[H_2\ donor]}$$

The concentration of residual G6P was determined by using an enzymatic assay by adding 1 U/mL of G6PDH in a 50 mM Tris-HCl buffer (pH7.5) containing 1.5 mM NAD^+, with G6P as the standard. The NAD(P)H concentrations were quantified based on the net increase in the absorbance at 340 nm. Total turnover number (TTN) of NADPH was calculated by dividing the moles of xylitol formed by the number of moles of $NADP^+$ added.

ACKNOWLEDGEMENTS

This work was supported mainly by the Air Force Office of Scientific Research and MURI (FA9550-08-1-0145), and partially by the DOE Bioenergy Science Center (BESC), the USDA Biodesign and Bioprocess Center, and ICTAS. We are grateful for the data of metabolites measured by Prof. An-Ping Zeng and Dr. Wei Wang of Hamburg University of Technology (Germany).

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581

582 **FIGURE LEGENDS**

583 **Figure 1**. Scheme of NAD(P)H regeneration from glucose-6-phosphate (G6P) mediated by one
584 enzyme G6P dehydrogenase (G6PDH) (a); by two enzymes G6PDH and 6-phosphogluconate
585 dehydrogenase (6PGDH) (b); by G6PDH, 6PGDH, four enzymes in the non-oxidative pentose
586 phosphate pathway (#5-8, in Fig. 2), and four enzyme in the glycolysis and gluconeogenesis
587 pathways (enzymes #9-12 in Fig. 2) (c); and a non-natural synthetic pathway (d) with
588 polysaccharides catalyzed by glucan phosphorylase (GNP), phosphoglucomutase (PGM), six
589 enzymes in the PPP, and four enzyme in the glycolysis and gluconeogenesis pathways. Ru5P =
590 ribulose-5-phosphate.

591 **Figure 2**. Scheme of NADPH regeneration from cellobiose coupled with xylitol synthesis from
592 xylose. The enzymes are: #1 CBP, cellobiose phosphorylase (1); #2 PGM, phosphoglucomutase
593 (1); #3 G6PDH, G-6-P dehydrogenase (6); #4 6PGDH, 6-phosphogluconate dehydrogenase (6);
594 #5 Ru5PE, ribulose 5-phosphate epimerase (4); #6 R5PI, phosphoribose isomerase (2); #7 TKL,
595 transketolase (4); #8 TAL, transaldolase (2); #9 TIM, triose phosphate isomerase (2); #10 ALD,
596 aldolase (1); #11 FBP, fructose-1, 6-bisphosphatase (1); #12 PGI, phosphoglucose isomerase (5);
597 and #13 XR, xylose reductase, where the number in parentheses represents the stoichiometric
598 coefficient for each enzyme for generation of 12 NAD(P)H from one glucose unit and water.
599 The metabolites and chemicals are: g1p, glucose-1-phosphate; g6p, glucose-6-phosphate; 6pg, 6-
600 phosphogluconate; ru5p, ribulose-5-phosphate; x5p, xylulose-5-phosphate; r5p, ribose-5-
601 phosphate; s7p, sedoheptulose-7-phosphate; g3p, glyceraldehyde-3-phosphate; e4p, erythrose-4-
602 phosphate; dhap, dihydroxacetone phosphate; fdp, fructose-1,6-diphosphate; f6p, fructose-6-
603 phosphate; and P_i, inorganic phosphate.

Figure 3. Profile of xylitol formation from xylose mediated by the enzyme cocktails. G6P and CB denote substrate glucose-6-phophate and cellobiose, respectively. 1-E denotes 1 enzyme (Fig. 1a), 2-E denotes 2 enzymes (Fig. 1b), 10-E denotes 10 enzymes (Fig. 1c), and 12-E denotes 12 enzymes (Fig. 2).

Figure 4. Cost analysis of biohydrogenation based on different hydrogen sources (a) and the TTN values and production costs of the enzymes in the SyPaB system (b). Costs of bulk chemicals: hydrogen ($2.50/kg), ethanol ($2.50/gallon), propanol ($1.27/kg), formic acid ($1.00/kg), and glucose or carbohydrates ($0.18/kg). The enzyme costs are calculate based on the assumptions that all of the enzymes in the 12-enzyme SyPaB systems have the same TTN values from 10^3 to 10^9 and their production costs ranges from $40 to $4000 per kg of the purified enzyme, and the average molecular weight of the enzymes in the SyPaB is 50,000 (i.e., one kg of enzyme equals 0.02 mole of enzyme).

Table 1. Complete list of enzymes and their loadings used for NAD(P)H regeneration.

No.	Enzyme	E.C.	Source/ORF*	purification	Enzyme loading (U/mL)				
					G6P/1-enzyme	G6P/2-enzyme	G6P/10-enzyme	CB/12-enzyme	G6P/10-enzyme (NAD)
#1	Cellobiose phosphorylase (CBP)	2.4.1.1	REE, Cthe0357	His/NTA				10	
#2	Phosphoglucomutase (PGM)	5.4.2.2	REE, Cthe1265	CBM/intein (Wang and Zhang, 2010)				10	
#3	Glucose-6-phosphate dehydrogenase (G6PDH, $NADP^+$)	1.1.1.49	Sigma, *S. cerevisiae*	NA	10	10	10	10	
	Glucose-6-phosphate dehydrogenase (G6PDH, NAD^+)	1.1.1.49	Sigma, *Leuconostoc mesenteroides*	NA					10
#4	6-Phosphogluconate dehydrogenase (6PGDH, $NADP^+$)	1.1.1.44	Sigma, *S. cerevisiae*	NA		10	10	10	
	6-Phosphogluconate dehydrogenase (Moth6PGDH, NAD^+)	1.1.1.44	REE, Moth1283	His/NTA					10
#5	Ribose 5-phosphate Isomerase (R5PI)	5.3.1.6	REE, Cthe2597	CBM/intein			1	1	1
#6	Ribulose-5-phosphate 3-epimerase (Ru5PE)	5.1.3.1	REE, Ttc1898	His/NTA			1	1	1
#7	Transketolase (TK)	2.2.1.1	REE, Ttc1896	His/NTA			1	1	1
#8	Transaldolase (TAL)	2.2.1.2	Sigma, *S. cerevisiae*	NA			1	1	1
#9	Triosephosphate isomerase (TIM)	5.3.1.1	REE, Ttc0581	His/NTA			1	1	1
#10	F1,6-biphosphate aldolase (ALD)	4.1.2.13	Sigma, Rabbit muscle	NA			1	1	1
#11	Fructose 1,6-bisphosphatase (FBPase)	3.1.3.11	REE, *E. coli glp*X	Ion exchange Q (Zhang et al., 2007)			1	1	1
#12	Phosphoglucose isomerase (PGI)	5.3.1.9	Sigma, *S. cerevisiae*	NA			1	1	1
#13	Xylose reductase (XR)	1.1.1.21	REE, NCU08384	His/NTA (Woodyer et al., 2005)	10	10	10	10	10

* REE, recombinant expression in *E. coli*; His/NTA, purified by His-tag of recombinant protein binding with nickel resin; Sigma, purchased from Sigma; *S. cerevisiae*, *Saccharomyces cerevisiae;* Cthe, *Clostridium thermocellum*; Ttc, *Thermus thermophilus* HB27; Moth, *Moorella thermoacetica*; NCU, *Neurospora crassa*.

Table 2. Summary of NAD(P) yields and reaction conditions.

H_2 donor	Substrate	Enzyme No.	Cofactor	NAD(P)H Yield (mol/mol)
G6P	xylose	1	$NADP^+$	0.98
G6P	xylose	2	$NADP^+$	1.97
G6P	xylose	10	$NADP^+$	8.74
G6P	xylose	10	NAD^+	5.40
G6P	xylose	10 [a]	$NADP^+$	11.5
G6P	xylose	10 [a]	$NADP^+$ [b]	8.30
cellobiose	xylose	12	$NADP^+$	6.66
cellobiose	xylose	12 [a]	$NADP^+$	11.4
cellobiose	xylose	12 [a]	$NADP^+$ [b]	7.20
cellobiose	xylose [c]	12	$NADP^+$	5.60
cellobiose	xylose [d]	12	$NADP^+$	6.20
cellobiose	xylose [d]	12 [a]	$NADP^+$	10.9

[a] enzyme optimization (10 U/mL of fructose 1,6-bisphosphate aldolase).
[b] $NADP^+$ concentration is 0.02 mM.
[c] NaOH-neutralized hydrolysate from dilute acid-pretreated corn stover.
[d] $CaCO_3$-neutralized hydrolysate from dilute acid-pretreated corn stover.

Table 3. Standard Gibbs free energies and enthalpies of reaction, and energy conversion efficiency (at pH = 7.0, 298.15 K, 1 bar, and zero ionic strength in dilute aqueous solutions)

Reaction	$\Delta_r G^{0'}$/(kJ/mol)	$\Delta_r H^{0'}$/(kJ/mol)	Efficiency (%)*
$C_{12}H_{22}O_{11} + P_i \rightarrow G6P + C_6H_{12}O_6$ [a]	-3.3	-10.0	
$G6P + 12\ NADP^+ + 7\ H_2O \rightarrow 12\ NADPH + 12\ H^+ + P_i + 6\ CO_2$ [a]	-207.5	+268.9	
$NADPH + H^+ + C_5H_{10}O_5 \rightarrow C_5H_{12}O_5 + NADP^+$ [a]	-20.8	-26.1	
$C_{12}H_{22}O_{11} + 12\ C_5H_{10}O_5 + 7\ H_2O \rightarrow C_6H_{12}O_6 + 12\ C_5H_{12}O_5 + 6\ CO_2$ [a]	-460.1	-53.9	99.6
$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$ [b]	-347.2	-96.9	96.6
$C_6H_{12}O_6 \rightarrow 3\ CH_4 + 3\ CO_2$ [b]	-419.4	-138.4	95.1
$4\ C_6H_{12}O_6 + O_2 \rightarrow C_{15}H_{31}COOH + 8\ CO_2 + 8\ H_2O$ [c]	-1704.8	-1206.3	89.3
$4\ C_6H_{12}O_6 \rightarrow C_{15}H_{31}COOH + 8\ CO_2 + 2\ H_2 + 6\ H_2O$ [b][d]	-1230.5	-634.6	94.3

* The energy conversion efficiency = $1 - \Delta_r H^{0'} / \sum (\Delta_f H^0 \text{ of reactants})$.

[a], enzymatic reaction.

[b], microbial anaerobic fermentation with a balanced NAD(P)H.

[c], microbial aerobic fermentation, where extra reducing cofactors generated through fatty acid synthesis pathway are consumed through oxidative phosphorylation.

[d], anaerobic fermentation with a hypothetic mechanism that can convert extra reducing cofactors to hydrogen. Such microorganisms are not available.

Figure 1

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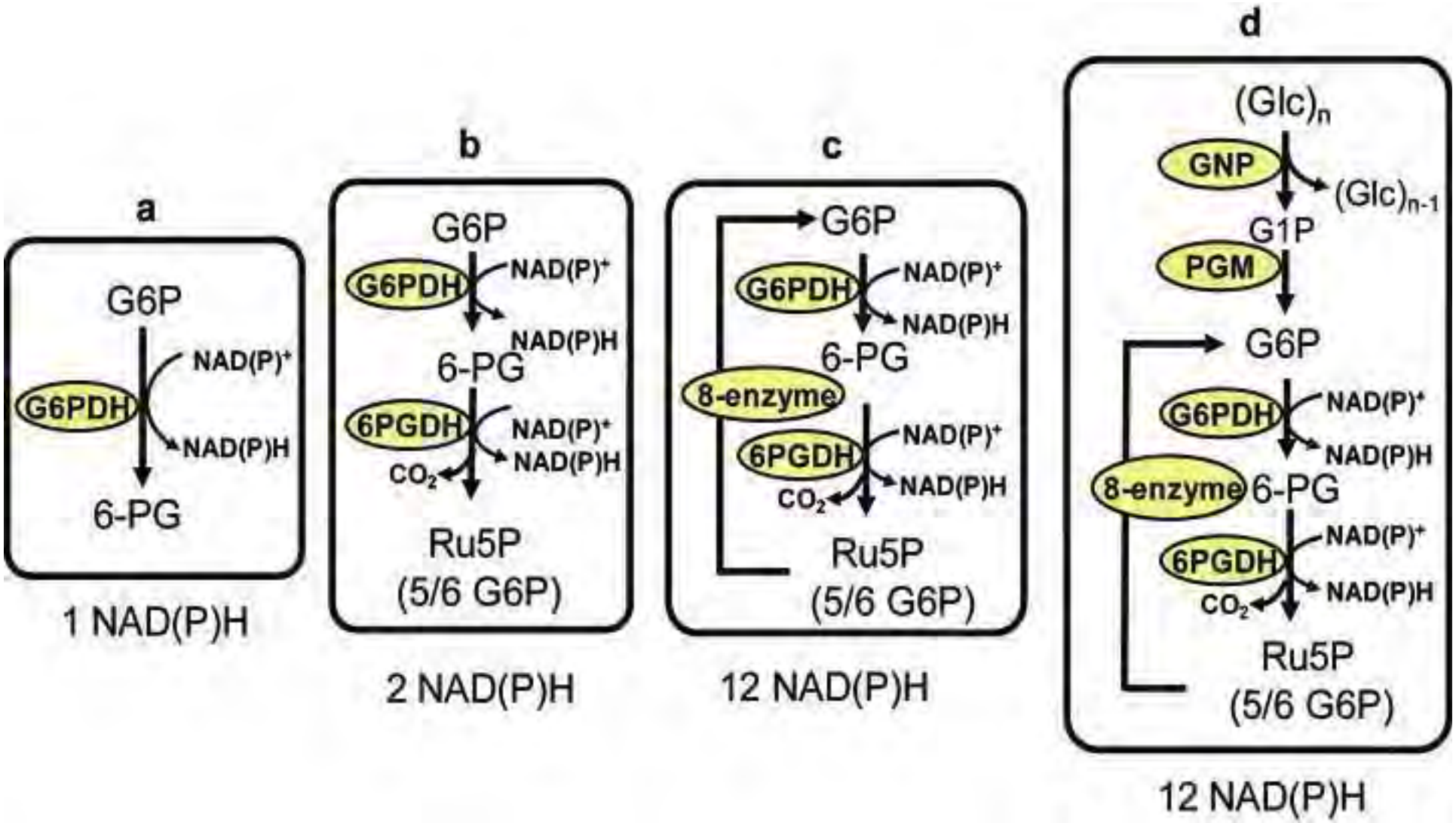

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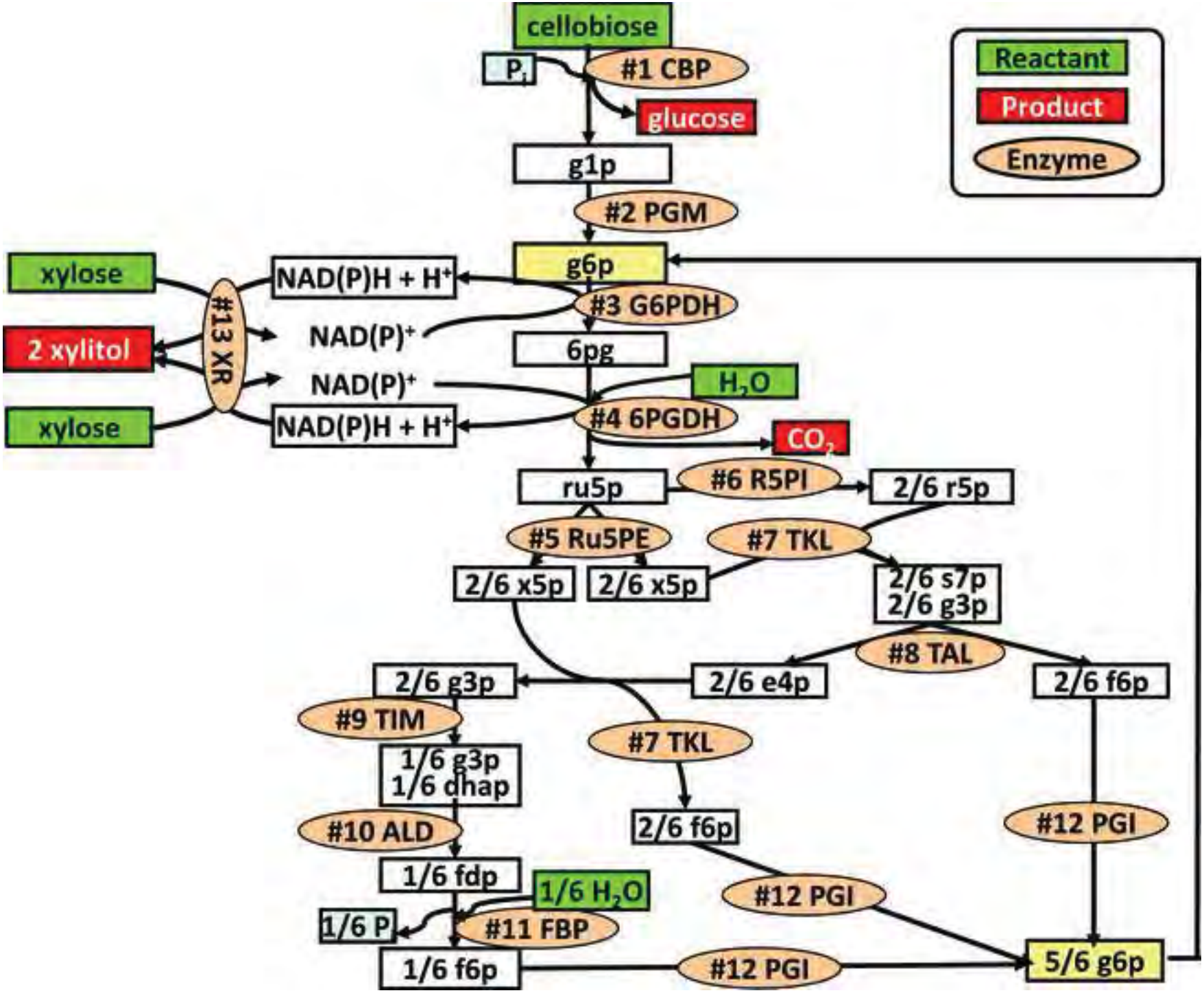

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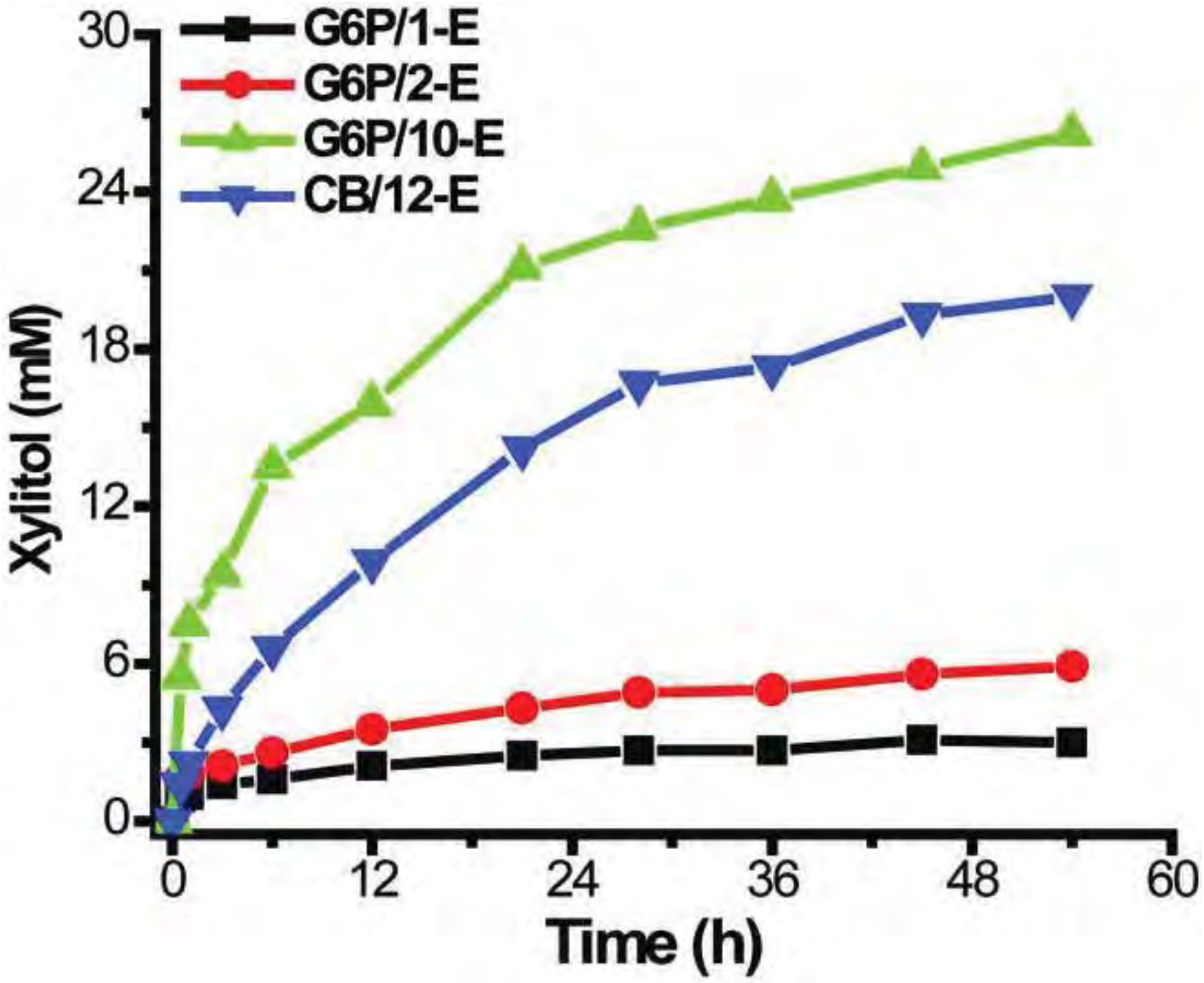

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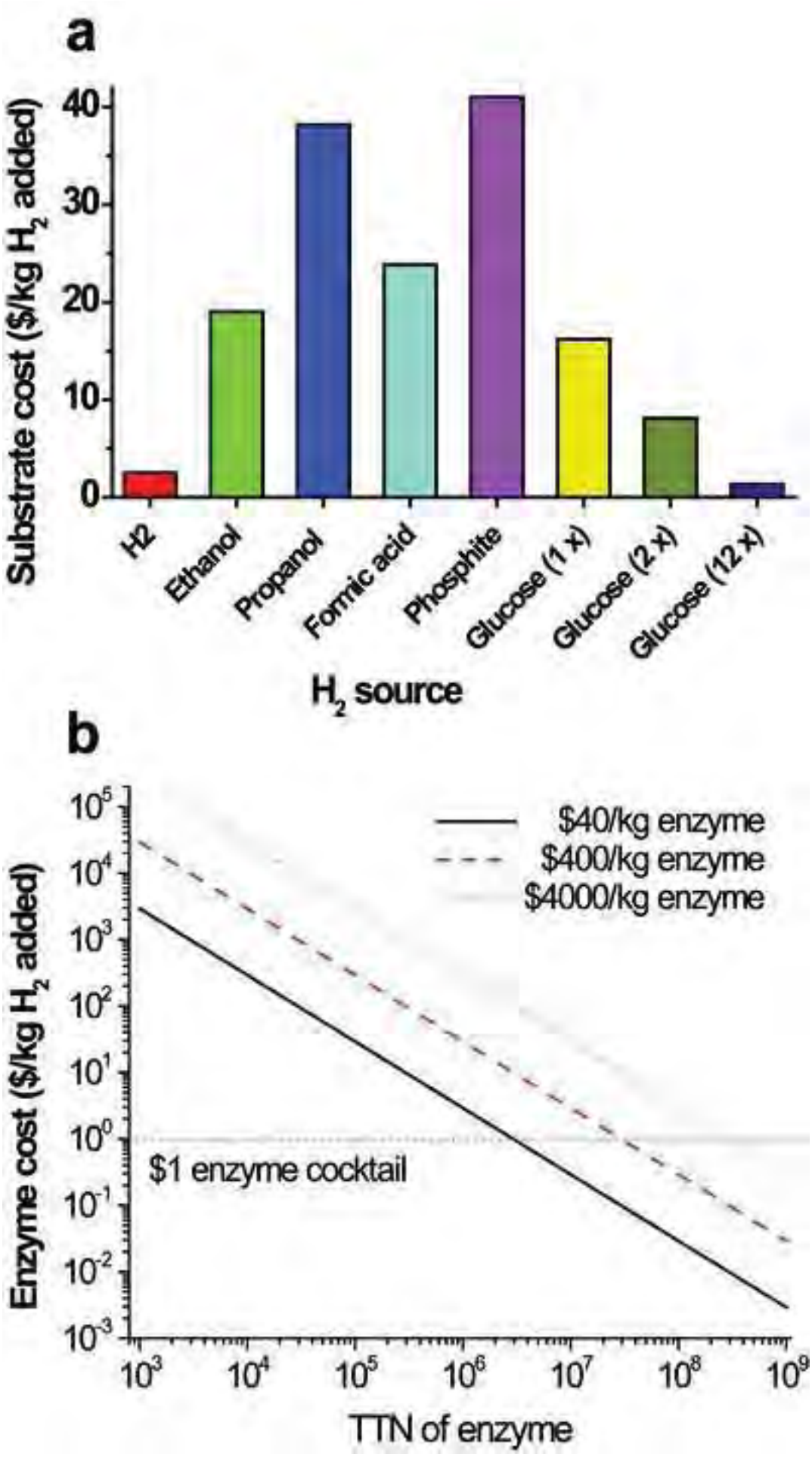

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Supplementary Materials

Biohydrogenation from Biomass Sugar Mediated by *in vitro* Synthetic Enzymatic Pathways

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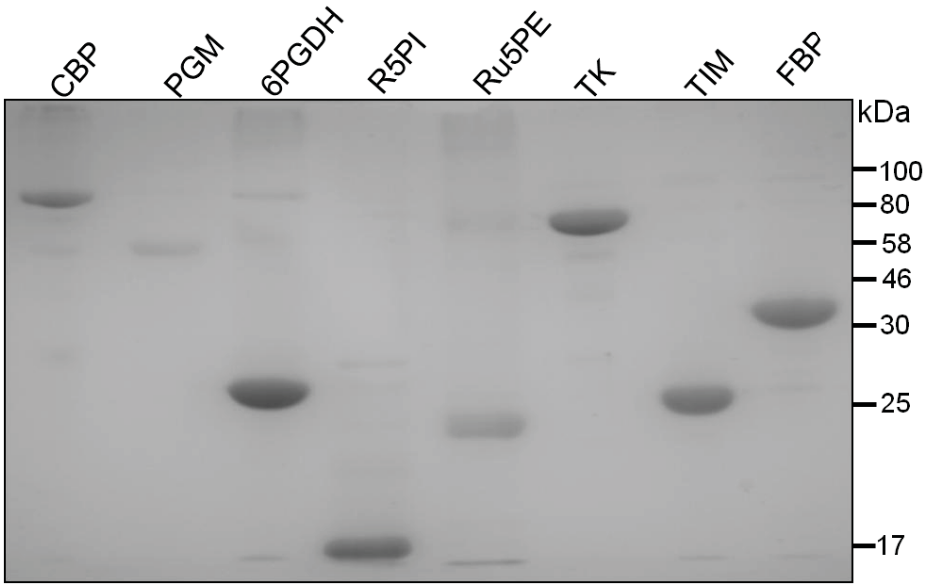

Figure S1. SDS-PAGE analysis for the purified recombinant enzymes.

Table S1. The strains, genomic DNA, plasmids, and oligonucleotides used in this study.

Description	Contents	Reference/sources
Strain		
E. coli Bl21^{star}(DE3)	B F$^-$ *ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm rne131* (DE3)	Invitrogen
Genomic DNA		
C. thermocellum	*Clostridium thermocellum* genomic DNA	(Zhang and Lynd, 2005a; Zhang and Lynd, 2005b)
M. thermoacetica	*Moorella thermoacetica* genomic DNA	ATCC
T. thermophilus HB27	*Thermus thermophilus* HB27 genomic DNA	ATCC
Plasmid		
pET21a-cbp	*C. thermocellum* cellobiose phosphorylase (CBP) gene subcloned into pET21a	This study
pCIP	Amp^r, T7 promoter, lacO, ColE1 ori, parental DNA, replacing *pgm* gene with *wt6pgdh*, *co6pgdh* or *g6pdh*	(Hong et al., 2008)
pET33b-6pgdh	Ka^r, T7 promoter, lacO, ColE1 ori, moth-6pgdh	This study
pET-ci-r5pi	*C. thermocellum* ribose 5-phosphate isomerase (R5PI) gene subcloned into pCIP	This study
pET21a-ru5pe	*T. thermophilus* Ribulose-5-phosphate 3-Epimerase (Ru5PE) gene subcloned into pET33b	This study
pJ404-tk	*E. coli* codon optimized *T. thermophilus* transketolase (TK) gene	DNA2.0
pET33b-tim	*T. thermophilus* triose phosphate isomerase (TIM) gene subcloned into pET33b	This study
pJB300B	Expression of *E. coli* fructose 1,6-bisphosphatase (*glp*X)	(Donahue et al., 2000)
pET26b-xr	Expression of *Neurospora crassa* xylose reductase (XR)	(Woodyer et al., 2005)
Primers*		Final plasmid
cbp-F	5'-gactgactgctagcatgaagttcggttttttgatg-3'	pET21a-cbp
cbp-R	5'-gactgactctcgagtcccataattacttcaactttgtg-3'	
6pg-F	5'- gactgactccatgggcttgcatatcggcttagtcggtc-3'	pET33b-6pgdh
6pg-R	5'- gactgactctcgagtttccgggccacgtcgtg -3'	
r5pi-F	5'- ccagtctactcgaggtgaaatctcatattggtctcatcggtc-3'	pET-ci-r5pi
r5pi-R	5'- ccagtctagtcgaccctatctctccttcctcccag-3'	
ru5pe-F	5'- ccagtctacatatgttgattaaaattgctccatcc -3'	pET21a-ru5pe
ru5pe-R	5'- ccagtctactcgagctagtggtggtggtggtggtgtttcctgtacgagttcattct -3'	
tim-F	5'- gactgactccatgggccaccaccaccaccaccaccggcgcgtcttggtgg -3'	pET33b-tim
tim-R	5'- gactgactggatccctaacccgcgatcctgaggag -3'	

* Underlined nucleotide sequences indicate restriction endonuclease sites.

Supplemental References

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Hong, J., Wang, Y., Ye X, and Zhang, Y.-H.P. (2008). Simple protein purification through affinity adsorption on regenerated amorphous cellulose followed by intein self-cleavage. J. Chromatogr. A 1194, 150-154.

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

Zhang, Y.-H.P., and Lynd, L.R. (2005a). Cellulose utilization by *Clostridium thermocellum*: Bioenergetics and hydrolysis product assimilation. Proc. Natl. Acad. Sci. USA 102, 7321-7325.

Zhang, Y.-H.P., and Lynd, L.R. (2005b). Regulation of cellulase synthesis in batch and continuous cultures of *Clostridium thermocellum*. J. Bacteriol. 187, 99-106.

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Short paragraph (E-TOC)

We put 12 enzymes in one pot so that these enzymes self-assemble a synthetic pathway for low-cost biohydrogenation; that is, desired biological reactions work without the other complex interactions that take place within a cell. In microbial fermentations, only a small fraction of glucose is allocated to NAD(P)H generation; while the cell-free synthetic pathway process both increases product yield and reaction rate. A novel hybrid of biocatalysis and catalysis would produce sulfur-free jet fuel with an unprecedented high energy conversion efficiency, much higher than those of fatty acid esters and bioalkanes by aerobic fermentations.