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Biohydrogenation from Biomass Sugar Mediated by In Vitro Synthetic Enzymatic Pathways

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SUMMARY

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.

INTRODUCTION

Hydrogenation by using dihydrogen has been widely applied in the food, pharmaceutical, and energy industries. Because most dihydrogen is produced from nonrenewable 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 nonrenewable hydrogen donor resources for NAD(P)H regeneration enable their applications to the synthesis of high-value chiral products while prevent from 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 (DA) pretreatment, 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., 2009a). Biomass hydrolysate, a liquid supernatant, contains xylose (mainly) and small amounts of glucose, cellobiose (CB), furfural, acetic acid, and phenolic compounds (Lau et al., 2009). Because biomass hydrolysate is highly toxic to most microorganisms, it must be detoxified before microbial fermentation (Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000). One of the common predetoxification approaches is neutralization by $CaCO_3$ so that the precipitate $CaSO_4$ can selectively absorb some inhibitors (Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000). Because thermodynamics decides economics for commodity production in the long term (Smil, 2008), high-energy efficiency production of liquid biofuels or their precursors (e.g., polyols) from the

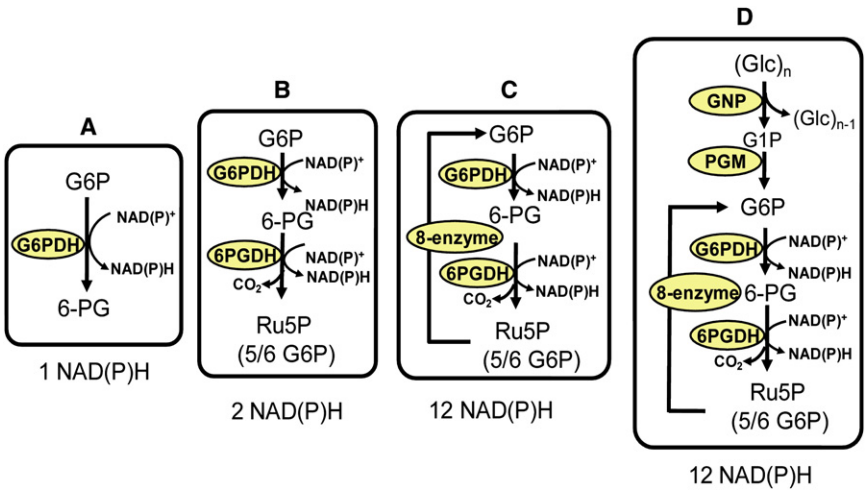

Figure 1. Enzymatic NAD(P)H Regeneration Comparison

Scheme of NAD(P)H regeneration from G6P mediated by: one enzyme, G6P dehydrogenase (G6PDH) (A); two enzymes, G6PDH and 6PGDH (B); G6PDH, 6PGDH, four enzymes in the nonoxidative PPP (#5–#8, in Figure 2), and four enzyme in the glycolysis and gluconeogenesis pathways (enzymes #9–#12 in Figure 2) (C); and a nonnatural synthetic pathway (D) with polysaccharides catalyzed by glucan phosphorylase (GNP), PGM, six enzymes in the PPP, and four enzymes in the glycolysis and gluconeogenesis pathways.

sugars in the biomass hydrolysate would 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, in 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 nonnatural 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 mol of NAD(P)H per one glucose unit of oligosaccharides by the in vitro synthetic enzymatic pathways. To avoid using any costly starch or its derived oligosaccharides, a biomass sugar—CB—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., 2006a) and a ready component of the dilute-acid pretreated biomass hydrolysate (Zhu et al., 2009b). Also, the enzyme mixture was shown to work in the presence of microorganism-toxic biomass hydrolysate, where the *Escherichia coli* strain cannot grow at all.

RESULTS

NAD(P)H Generation Pathway Design

One-enzyme reactions starting from glucose or G6P mediated by respective glucose dehydrogenase or G6P dehydrogenase usually generate only one reduced NAD(P)H factor (Figure 1A) (Wichmann and Vasic-Racki, 2005). A two-enzyme reaction from G6P mediated by G6P dehydrogenase and 6-phosphogluconate dehydrogenase (6PGDH) can produce two NADPH and one ribulose-5-phosphate (Ru5P) as well as release CO_2 (Figure 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 nonoxidative pentose phosphate pathway (PPP) (i.e., ribose 5-phosphate 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 phosphoglucose isomerase [PGI]) (Figure 1C). Therefore, one molecule of G6P can generate 12 NADPH, six CO_2, and one phosphate (Equation 1).

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

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 (Equation 2) (Figure 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 nonnatural synthetic pathway (Figure 2) was designed to contain cellobiose phosphorylase (CBP) and PGM for producing G6P from CB (Equation 3), followed by the 6-enzyme PPP and 4-enzymes in the glycolysis and gluconeogenesis pathways for generating 12 NADPH per G6P (Equation 1).

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

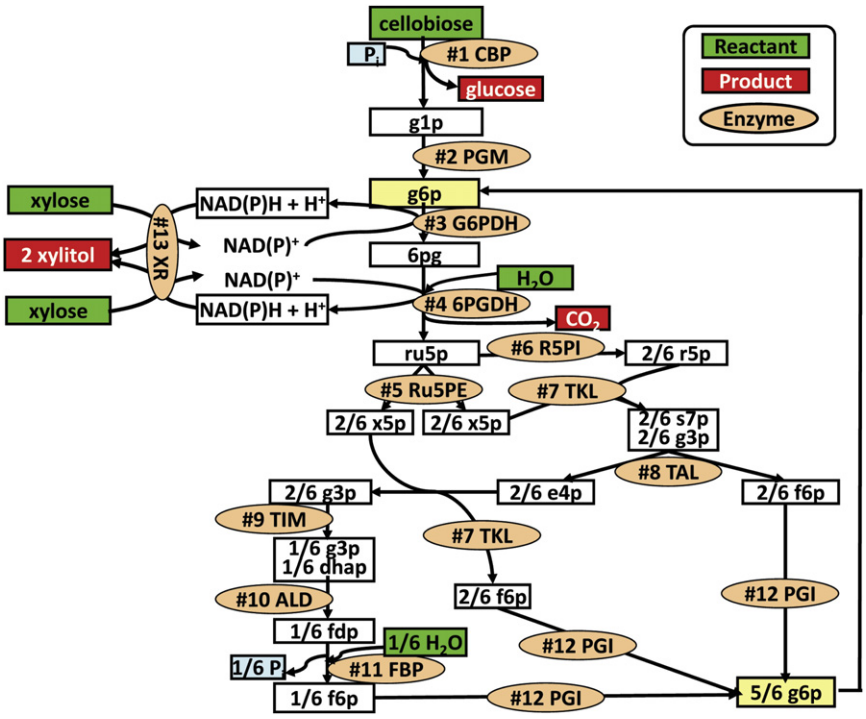

Figure 2. Scheme of NADPH Regeneration from CB Coupled with Xylitol Synthesis from Xylose

The enzymes are: #1 CBP (1); #2 PGM (1); #3 G6PDH (G-6-P dehydrogenase) (6); #4 6PGDH (6); #5 Ru5PE (4); #6 R5PI (2); #7 TKL (4); #8 TAL (2); #9 TIM (2); #10 ALD (1); #11 FBP (1); #12 PGI (5); and #13 XR, where the number in parentheses represents the stoichiometric coefficient for each enzyme for generation of 12 NAD(P)H from one glucose unit and water. The metabolites and chemicals are: g1p, glucose-1-phosphate; 6pg, 6-phosphogluconate; x5p, xylulose-5-phosphate; r5p, ribose-5-phosphate; s7p, sedoheptulose-7-phosphate; g3p, glyceraldehyde-3-phosphate; e4p, erythrose-4-phosphate; dhap, dihydroxyacetone phosphate; fdp, fructose-1,6-diphosphate; f6p, fructose-6-phosphate; and P_i, inorganic phosphate.

By coupling NADPH generation from CB with a NADPH-consumption reaction mediated by xylose reductase (XR) that can convert xylose to xylitol (Woodyer et al., 2005) in Equation 4, the proof-of-principle reaction contained 13 enzymes in one pot for implementing Equation 5. In it, nine enzymes of them were produced heterologously in *E. coli*, including CBP, PGM, 6PGDH, R5PI, Ru5PE, TKL, TIM, FBP, and XR, and were purified with near homogeneity (see Figure S1 available online); the others were 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 (Figure 3). Our previous study showed that the reactions mediated by two dehydrogenases (G6PDH and 6PGDH) were a 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 dehydrogenase loadings were increased to 10 U 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 CB (2.8 mmol/hr/l, 12-E) was slower than those based on G6P (11.0 mmol/hr/l, G6P/10-E; and 3.2 mmol/hr/l, G6P/2-E), consistent 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 G6P and 6-phosphogluconate that strongly inhibited the activities of G6PDH and 6PGDH. The highest xylitol level obtained was 26.2 mM at hr 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 mol/mol 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, Ru5P, 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, ~5.40 per glucose unit (Table 2) because XR 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 CB 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.

Table 1. Complete List of Enzymes and Their loadings Used for NAD(P)H Regeneration

Number	Enzyme	Enzyme Catalog	Source/ORF[a]	Purification	Enzyme Loading (U/ml) G6P/1-Enzyme	G6P/2-Enzyme	G6P/10-Enzyme	CB/12-Enzyme	G6P/10-Enzyme (NAD)
1	CBP	2.4.1.1	REE, Cthe0357	His/NTA				10	
2	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	10	10		10
4	6PGDH ($NADP^+$);	1.1.1.44;	Sigma, *S. cerevisiae*;	NA	10	10	10	10	
	6PGDH (Moth6PGDH, NAD^+)	1.1.1.44	REE, Moth1283	His/NTA	10	10	10		10
5	R5PI	5.3.1.6	REE, Cthe2597	CBM/intein			1	1	1
6	Ru5PE	5.1.3.1	REE, Ttc1898	His/NTA			1	1	1
7	TKL	2.2.1.1	REE, Ttc1896	His/NTA			1	1	1
8	TAL	2.2.1.2	Sigma, *S. cerevisiae*	NA			1	1	1
9	TIM	5.3.1.1	REE, Ttc0581	His/NTA			1	1	1
10	F1,6-biphosphate ALD	4.1.2.13	Sigma, Rabbit muscle	NA			1	1/10	1
11	Fructose 1,6-bisphosphatase (FBP)	3.1.3.11	REE, *E. coli glpX*	Ion exchange Q (Zhang et al., 2007)			1	1	1
12	PGI	5.3.1.9	Sigma, *S. cerevisiae*	NA			1	1	1
13	XR	1.1.1.21	REE, NCU08384	His/NTA (Woodyer et al., 2005)	10	10	10	10	10

[a] 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, *C. thermocellum*; Ttc, *T. thermophilus* HB27; Moth, *M. thermoacetica*; NCU, *Neurospora crassa*.

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) was rate limiting when each enzyme has 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 the overall reaction rates (Table 1). The updated model in metabolite pathway flux with the new settings (e.g., new enzyme parameters and loadings) suggested that the reaction linking glyceraldehyde-3-phosphate and dihydroxyacetone phosphate for forming fructose 1,6-bisphosphate might be enzyme limited. When we increased fructose 1,6-bisphosphate ALD from 1 to 10 U/ml (Table 1), the NADPH yields were enhanced to 11.5 and 11.4 for the G6P/10-E and the CB/12-E, respectively (Table 2). The levels of G6P, other metabolites, and NADPH by the end of the reactions (data not shown) were not detectable (e.g., below 0.1 mM). For $CaCO_3$-neutralized biomass hydrolysate, the NADPH yield was 10.9. In the future, enzymatic conversion of toxic biomass hydrolysate to polyols would be very important for direct utilization of the microorganism-toxic biomass hydrolysate without significant detoxification.

The decreases in an initial $NADP^+$ concentration from 2 to 0.02 mM resulted in nearly two orders of magnitude increases in coenzyme total turnover number (TTN), from 23 and 23 to 1440 and 1665 based on G6P and CB, respectively, but these attempts drastically decreased NADPH yields to 8.30 and 7.20, respectively (Table 2).

DISCUSSION

This study shows that a biomass sugar—CB—can be used as a hydrogen donor for high-yield biohydrogenation. As compared to other enzyme-mediated biohydrogenation approaches, complete oxidation of biomass sugars for biohydrogenation has the lowest substrate cost ($1.35/kg H_2 added), much lower than the costs of hydrogen generated from natural gas (e.g., ~$2.00/kg H_2) or other substrates, such as alcohols, formic acid, and phosphite (Figure 4A). Although nearly 12 mol of hydrogen has been produced per mole of glucose unit by SyPaB

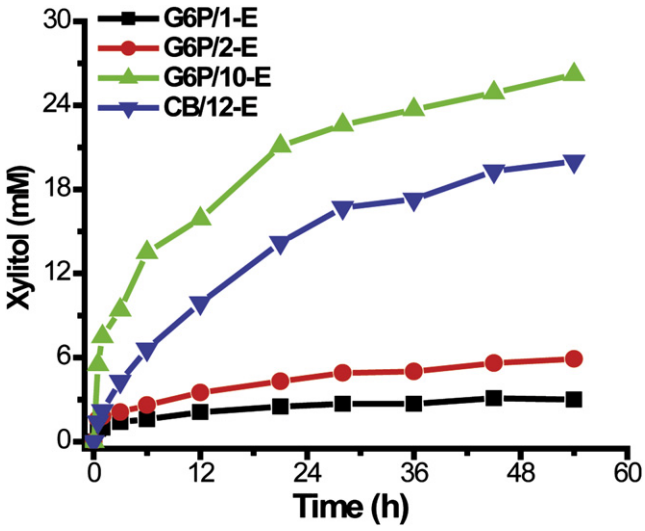

Figure 3. Profile of Xylitol Formation from Xylose Mediated by the Enzyme Cocktails

G6P and CB denote substrate glucose-6-phophate and CB, respectively. 1-E denotes one enzyme (Figure 1A), 2-E denotes two enzymes (Figure 1B), 10-E denotes ten enzymes (Figure 1C), and 12-E denotes 12 enzymes (Figure 2). See also Figure S1.

as described before (Ye et al., 2009; Zhang et al., 2007), direct biohydrogenation through NAD(P)H, but not through hydrogen, is more advantageous because of: (i) consolidating hydrogen generation and hydrogen consumption in one reactor; (ii) avoiding use of costly hydrogenase (Mertens and Liese, 2004; Wong et al., 1981); (iii) lowering safety risk for hydrogen-containing bioreactors; and (iv) no strict anaerobic conditions needed for enzymatic biohydrogen reaction.

In addition to substrate costs, there are two cost factors influencing biohydrogenation—coenzyme and enzymes. When TTN values of the coenzyme are the same for all enzymatic biohydrogenation approaches, coenzyme expenditures are the same regardless of enzyme number in the enzymatic reactions. Both increasing TTN of enzymes (i.e., enzyme stability) and decreasing their production costs can drastically decrease expenditures in enzymes (Figure 4B). When enzyme costs are \$400 per kg, TTN values must be higher than 3×10^7 mol reaction catalyzed by enzyme per mol of enzyme to decrease the enzyme costs to a critical low level (e.g., \$1.00/per kg of hydrogen added). When the enzyme production costs can be reduced to \$40/kg or as low levels as the bulk (recombinant non-membrane) industrial enzymes at \$5–\$20 per kg, the expenditure of the enzyme mixture would be lower than \$1.00/kg of hydrogen added at TTN values of each enzyme $\geq 3 \times 10^6$ or \$0.10/kg of hydrogen added at TTN values of each enzyme $\geq 3 \times 10^7$ (Figure 4B). In practice it is relatively easy to discover the thermo enzymes with the TTN values of more than 10^7 from (hyper-)

Table 2. Summary of NAD(P) Yields and Reaction Conditions

H_2 Donor	Substrate	Enzyme Number	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
CB	Xylose	12	$NADP^+$	6.66
CB	Xylose	12[a]	$NADP^+$	11.4
CB	Xylose	12[a]	$NADP^+$[b]	7.20
CB	Xylose[c]	12	$NADP^+$	5.60
CB	Xylose[d]	12	$NADP^+$	6.20
CB	Xylose[d]	12[a]	$NADP^+$	10.9

[a] Enzyme optimization (10 U/ml of fructose 1,6-bisphosphate ALD).
[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.

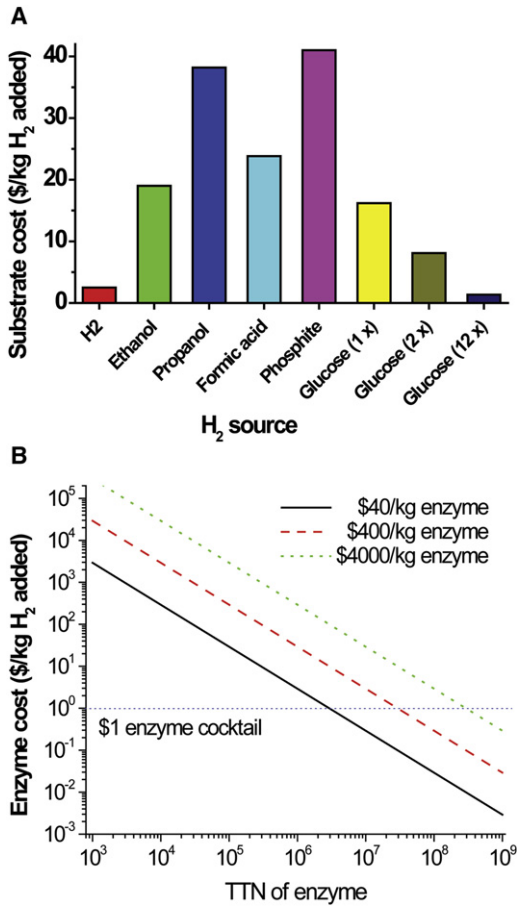

Figure 4. Cost Analysis of Biohydrogenation

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 calculated 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 \$4,000 per kg of the purified enzyme, and the average molecular weight of the enzymes in the SyPaB is 50,000 (i.e., 1 kg of enzyme equals 0.02 mol of enzyme).

Table 3. Standard Gibbs Free Energies and Enthalpies of Reaction, and Energy Conversion Efficiency

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

At pH = 7.0, 298.15 K, one bar, and zero ionic strength in dilute aqueous solutions.

[a] The energy conversion efficiency = $1 - \Delta_rH^{0'}/\sum(\Delta_fH^0$ of reactants).

[b] Enzymatic reaction.

[c] Microbial anaerobic fermentation with a balanced NAD(P)H.

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

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

thermophilic microorganisms by utilizing their available genomic DNA sequences and bioinformatics analysis (Blumer-Schuette et al., 2008; Nelson et al., 1999), e.g., *Clostridium thermocellum* PGM (Wang and Zhang, 2010), *Thermotoga maritima* 6PGDH (Wang and Zhang, 2009b), and so on. In the food industry, immobilized thermostable glucose isomerase has reached a TTN value of more than 250 million (Vasic-Racki, 2006). In addition the TTN value of the immobilized *C. thermocellum* PGI by simple adsorption on a low-cost cellulosic material is as high as 10^9 (unpublished data). Considering the cost contributions from the sugar feedstock ($1.35/kg H_2 added), the enzyme mixture ($0.10–$1.00/kg H_2 added), and future use of less costly and more stable biomimetic coenzyme (Ryan et al., 2008; Zhang, 2010a; Zhang et al., 2010), biohydrogenation would be very cost competitive with hydrogenation, where hydrogen is made from natural gas ($2.0–$2.7/kg H_2).

It was vital to analyze the carbohydrate allocation based on glucose between the product and biocatalyst (e.g., microbial cell and purified enzyme) because bulk enzyme in the SyPaB system must be produced through microbial fermentation, which consumes carbohydrate. Typical values of recombinant enzyme yield based on glucose ($Y_{P/S}$) ranges from ~0.005 to 0.175 g of protein/g of glucose consumed, based on several well-known facts of: (1) cell mass yield based on glucose in aerobic fermentation ($Y_{X/S}$) equals ~0.5 g cell mass/g glucose (Huang and Zhang, 2011; Shuler and Kargi, 2001; Zhang et al., 2010); (2) typical intracellular cellular protein content equals ~0.5 g protein/g cell mass (Zhang and Lynd, 2003); and (3) typical recombinant enzyme percentage in total cellular protein equals 2%–70% (Myung et al., 2010; Wang and Zhang, 2010; Wang and Zhang, 2009b; Ye et al., 2010). When each enzyme in the SyPaB systems has TTN values of $\geq 3 \times 10^7$ and $Y_{P/S}$ values of ~0.03 (a very conservative value for recombinant protein production in *E. coli*), the carbohydrate allocation to the production of the enzyme mixtures was ~0.01, i.e., ~99% of carbohydrate would be used to produce the desired product. The calculation of carbohydrate allocation to the enzyme mixture is presented as below. When $Y_{P/S}$ is 0.03 g of protein/g of glucose, 1 kg of glucose can produce 0.03 kg of enzyme (i.e., 0.6×10^{-3} mol of enzyme, MW = 50,000). So, 35 kg of glucose is needed to produce $18 \times 10^{-3} = 35 \times 0.6 \times 10^{-3}$ mol of the enzyme mixture, where an elemental enzyme used in the 12-E system is 0.6×10^{-3} mol, and the lumped stoichiometric coefficient for the 12-E system is 35. In this system the exact use for each enzyme is 0.6×10^{-3} mol multiplied by its stoichiometric coefficient (Figure 2), e.g., 0.6×10^{-3} mol of PGM and 3.6×10^{-3} mol of G6PDH. When each enzyme has TTN values of more than 3×10^7, a 12-E cocktail made from 35 kg of glucose can produce 432 kg of H equivalent equaling 24 H equivalent (i.e., 12 NAD(P)H) per anhydroglucose ($C_6H_{10}O_5$) and seven water (or per glucose, $C_6H_{12}O_6$, and six water) $\times 3 \times 10^7$ mol reaction/mol enzyme $\times 0.6 \times 10^{-3}$ mol enzyme $\times 10^{-3}$ kg/mol H equivalent. So, 432 kg of H equivalent is made from 3240 kg of glucose and 1944 kg of water. Therefore, the allocation of carbohydrate to the enzyme cocktail is 35/(35 + 3240) × 100% = 1.07%. The higher $Y_{P/S}$ values, the lower allocation of carbohydrate to the synthesis of the enzyme cocktails, the more allocation to the desired product.

We suggest a high-energy efficiency way for producing liquid hydrocarbons from the biomass hydrolysate through this biohydrogenation followed by aqueous phase reforming (APR) because the overall energy conversion efficiency mainly decides the economics of biofuel production. This hybrid process has a very high energy-retaining efficiency (94.6%) involving nearly no loss in combustion energy through biohydrogenation (99.6%) (Table 3) and 95% energy efficiency-retaining APR (Huber et al., 2004; Metzger, 2006), higher than those through microbial fermentations: palmitate acid (Table 3), and other biofuels (Huang and Zhang, 2011; Li et al., 2010; Schirmer et al., 2010; Steen et al., 2010). The overall reaction (Equation 5) is composed of three reaction modules: Equation 1 ($\Delta_rG^{0'} = -207.5$ kJ/mol and $\Delta H^{o'} = +268.9$ kJ/mol); Equation 3 ($\Delta_rG^{0'} = -3.3$ kJ/mol and $\Delta H^{o'} = -10.0$ kJ/mol); and Equation 4 ($\Delta_rG^{0'} = -20.8$ 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 (Equation 1) and two exothermic reactions (Equations 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 is different from 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 it would have 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 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

Chemicals

All chemicals were reagent grade or higher, purchased from Sigma (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. Regenerated amorphous cellulose (RAC) was prepared from Avicel as previously described (Zhang et al., 2006b). The oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA, USA). Corn stover was pretreated by dilute acid, conducted in the National Renewable Energy Laboratory (Boulder, CO, USA) (Zhu et al., 2009a, 2009b). After centrifugation, the supernatant was neutralized by either calcium carbonate powder or a 2.0 M sodium hydroxide solution for the enzymatic reactions.

Strains and Media

E. coli DH5α was used as the host cell for DNA manipulation, and *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA, USA) was used as the host cell for recombinant protein expression. The Luria-Bertani (LB) medium plus 100 μg/ml ampicillin was used for *E. coli* cell growth and recombinant protein expression.

Gene Cloning and Plasmid Construction

Five protein expression plasmids were constructed for expressing *C. thermocellum* CBP, *M. thermoacetica* 6PGDH, *C. thermocellum* R5PI, *Thermus thermophilus* Ru5PE, and *T. thermophilus* TIM (Table S1). The genes were amplified based on their genomic DNA templates, digested by the restriction enzymes, and ligated for constructing the expression vectors (Table S1). *T. thermophilus* TKL was synthesized with N-terminal His-tag by DNA2.0 Inc. (Menlo Park, CA, USA) based on the *E. coli* optimum codons.

Recombinant Protein Production and Purification

The *E. coli* BL21 strain harboring the 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°C for the cell growth. The recombinant protein expression was induced by adding IPTG when the absorbance (A_{600}) reached ~0.6. For the production of most of the recombinant proteins, the cultures were incubated at 37°C for 4 hr, 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°C for 23 hr. The cells were harvested by centrifugation at 4°C, washed once with 50 mM of Tris-HCl buffer (pH 7.5), and resuspended with a 10 ml of 30 mM Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl and 1 mM of EDTA. The cell pellets were lysed by ultrasonication 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 FBP 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 CBP activity was assayed as described elsewhere (Zhang and Lynd, 2004). *C. thermocellum* PGM activity was assayed as described previously (Wang and Zhang, 2010). *M. thermoacetica* 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°C. *T. thermophilus* HB27 R5PI activity was determined on a substrate D-ribulose 5-phosphate. The reactions were carried out in a 50 mM Tris/HCl (pH 7.5) containing 2 mM D-ribulose 5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, and 0.5 mg/ml BSA at 30°C in a 5-min reaction period. The product D-ribose 5-phosphate was enzymatically quantified by consumption of NADH in 50 mM Tris/HCl (pH 7.5) 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 TKL, 60 U/ml TIM, 20 U/ml D-glyceraldehyde 3-phosphate dehydrogenase, and 0.25 mM NADH at 30°C for 5 min. The specific activity of TmR5PI was 15 U/mg at 30°C. *T. thermophilus* HB27 Ru5PE activity was determined on a substrate D-ribulose 5-phosphate. The reactions were carried out in a 50 mM Tris/HCl (pH 7.5) containing 2 mM D-ribulose 5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, and 0.5 mg/ml BSA at 30°C for 5 min. The product D-xylulose 5-phosphate was enzymatically quantified by consumption of NADH in 50 mM Tris/HCl (pH 7.5), 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 TKL, 60 U/ml TIM, 20 U/ml D-glyceraldehyde 3-phosphate dehydrogenase, and 0.25 mM NADH at 30°C for 5 min. The specific activity of Ru5PE was 30 U/mg at 30°C. *T. thermophilus* HB27 TKL 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 (pH 7.5) containing 2 mM D-xylulose 5-phosphate, 2 mM D-ribose 5-phosphate, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, and 0.5 mg/ml BSA at 30°C for 5 min. The product D-glyceraldehyde 3-phosphate was quantified through the consumption of NADH in 50 mM Tris/HCl (pH 7.5) containing 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mg/ml BSA, 0.2 mM thiamine pyrophosphate, 5 U/ml TKL, 60 U/ml TIM, 20 U/ml glycerol 3-phosphate dehydrogenase, and 0.25 mM NADH at 30°C for 5 min. The activity of TKL was 0.2 U/mg at 30°C. *T. thermophilus* TIM activity was measured in 50 mM Tris/HCl (pH 7.5) 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. 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 FBP, 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 $NAD(P)H^+$, unless otherwise noted. The substrates were 3 mM G6P or CB and 100 mM xylose. The enzyme loadings are shown in Table 1. Ten microliters of the samples were withdrawn and quenched by 90 μl of 5 mM H_2SO_4 at different time intervals. The concentrations of xylose, CB, 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:

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

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 (pH 7.5) 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. The TTN of NADPH was calculated by dividing the moles of xylitol formed by the number of moles of $NADP^+$ added.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and one table and can be found with this article online at doi:10.1016/j.chembiol.2010.12.019.

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