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An *in vitro* synthetic biology platform for emerging industrial biomanufacturing: Bottom-up pathway design

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ABSTRACT

Although most *in vitro* (cell-free) synthetic biology projects are usually used for the purposes of fundamental research or the formation of high-value products, *in vitro* synthetic biology platform, which can implement complicated biochemical reactions by the *in vitro* assembly of numerous enzymes and coenzymes, has been proposed for low-cost biomanufacturing of bioenergy, food, biochemicals, and nutraceuticals. In addition to the most important advantage-high product yield, *in vitro* synthetic biology platform features 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 article, we present the basic bottom-up design principles of *in vitro* synthetic pathway from basic building blocks-BioBricks (thermoenzymes and/or immobilized enzymes) to building modules (e.g., enzyme complexes or multiple enzymes as a module) with specific functions. With development in thermostable building blocks-BioBricks and modules, the *in vitro* synthetic biology platform would open a new biomanufacturing age for the cost-competitive production of biocommodities.

Contents

1. Introduction

Synthetic biology is an interdisciplinary branch of biology, chemistry and engineering that combines the investigative nature of biology with engineering design principles [1]. Most efforts in synthetic biology have largely concentrated on the design and

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construction of artificial biological pathways *in vivo*, or on the redesign of existing natural biological systems for biological research [2–5]. The ultimate engineering goal of synthetic biology is the cost-competitive production of new drugs, biochemical, nutraceuticals, and bioenergy via engineered bioentities to replace current manufacturing methods [6–8].

Synthetic biology can be roughly divided into two areas, *in vivo* and *in vitro* synthetic biology. *In vivo* synthetic biology focuses on living bioentities, which can duplicate themselves. There are numerous breakthroughs, especially in terms of fundamental researches and publications [2,9,10], However, some inherent constraints of living organisms (e.g., net ATP generation for cell growth and maintenance, intact cellular membrane for maintaining basic metabolism and achieving selective mass transfer and exchange) prevent them from implementing some important reactions. Whereas, *in vitro* synthetic biology focuses on the construction of synthetic enzymatic pathways outside cells to convert substrates to desired products. For example, $12H_2$ can be obtained from one glucose and water via ATP-free *in vitro* synthetic pathways [11,12]. This pathways cannot be applied to living organisms due to no bioenergetic benefits. It is for the reason that living microorganisms have their H_2 yield limit (i.e., $4H_2$ per glucose), called the Thauer limit [13,14]. Another example is making starch from cellulose, whereas cellulose and starch, large-size polymers, cannot be transported across cellular membrane [15].

Although *in vitro* synthetic biology is largely ignored compared to *in vivo* synthetic biology, it has made great and rapid progress [7,16–19]. These *in vitro* synthetic biology systems can be based on cell extracts [20] or purified enzymes [21,22] or their combinations. Their potential applications include cell-free protein synthesis (CFPS) [23,24], vaccines [25–27], and potentially low-cost production of bioenergy [12,28–31], nutraceuticals [32] and biochemicals [33,34]. The *in vitro* synthetic biology platform has some distinctive advantages, such as high product yield, high volumetric productivity, high product titer, high tolerance in toxic environments, substrates, and/or products, easy product separation and easy process control and optimization [7], and so on. These features make it feasible to become a disruptive biomanufacturing platform [17].

The history of *in vitro* (cell-free) fundamental research and *in vitro* biomanufacturing accompanied with milestones is presented in Table 1. The development of *in vitro* (synthetic) biology originated from Eduard Buchner's paradigm-shifting discovery of "cell-free ethanol fermentation by non-living yeast lysate" (Nobel Chemistry Prize 1907). Later, whole-cell lysates were important scientific targets for understanding of biochemistry of natural organisms. Numerous scientists isolated and characterized individual enzymes, reconstituted metabolic pathways *in vitro* and *in vivo*, and understood natural organisms. For instance, Harden et al. discovered key enzymes in glycolysis (Nobel Chemistry Prize 1929), Krebs analyzed the citric acid cycle (Nobel Chemistry Prize 1952), and Calvin elucidated the CO_2 assimilation in plants (Nobel Chemistry Prize 1961). Subsequently, Jacob et al. discovered concerning genetic control of enzyme and virus synthesis, and Nirenberg and Matthaei interpreted the genetic code and its function in protein synthesis (Nobel Physiology or Medicine Prize 1968). The next major technique breakthroughs were the invention of the PCR method and the establishment of site-directed mutagenesis in the 1990s. Fundamental studies and tools development of *in vitro* biology offer a versatile workforce for understanding the operation principle of nature and for enabling redesigned biosynthetic pathways for the biosynthesis of novel chemicals, sustainable fuel, and new tunable materials. For example, CFPS, used for decades as a fundamental research tool for understanding transcription and translation, has been suggested to be the fastest way to make recombinant proteins, especially for membrane or complicated proteins [23,24]. CFPS has been expanded to a 100-L scale recently, showing great potential in industrial biomanufacturing [35].

The development of *in vitro* synthetic biology platform for biomanufacturing lags far behind fundamental research of *in vitro* biology-biochemistry [36]. Although Eduard Buchner discovered the phenomenon of cell-free ethanol fermentation in the 1890s, the use of one enzyme for industrial biomanufacturing came into being in the 1960s-1970s, for instance, high fructose corn syrup (i.e., more than 20 million tonnes yearly) and semi-synthetic antibiotics (e.g., cephalosporin) [37]. Such *in vitro* biosystems evolved to more complicated system containing two-three enzymes in one pot for enhancing volumetric productivity, decreasing product inhibition, shifting reaction equilibrium, and facilitating product/substrate separation [38,39]. For example, the pharmaceutical and fine

Table 1
The history of cell-free fundamental research and *in vitro* biomanufacturing accompanied with milestones.

Year	Leaders	Milestone (Award)	References
Milestones for cell-free fundamental research			
1907	Arthur Harden, Hans Karl August Simon von Euler-Chelpin	Fermentation of sugar and fermentative enzymes (Nobel Chemistry 1929)	[109]
1930s	James Batcheller Sumner, John Howard Northrop, Wendell Meredith Stanley	Preparation of enzymes and virus proteins in a pure form (Nobel Chemistry 1946)	[110–112]
1940s	Hans Krebs	Discovery of the citric acid cycle (Nobel Physiology 1953)	[113]
1940s	Melvin Calvin	Carbon dioxide assimilation in plants (Nobel Chemistry 1961)	[114]
1960s	Francois Jacob, Jacques Monod, Andre Lwoff	Discoveries concerning genetic control of enzyme and virus synthesis (Nobel Physiology or Medicine 1965)	[115]
1960s	Robert William Holley, Har Gobind Khorana, Marshall Warren Nirenbrg	Interpretation of the genetic code and its function in protein synthesis (Nobel Physiology or Medicine 1968)	[116,117]
1970s	Werner Arber, Daniel Nathans, Hamilton Othanel Smith	Discovery of restriction enzymes and their application to problems of molecular genetics (Nobel Physiology or Medicine 1978)	[118–120]
1990s	Kary Banks Mullis, Michael Smith	Invention of the polymerase chain reaction (PCR) method; Establishment of site-directed mutagenesis with application to protein studies (Nobel Chemistry 1993)	[121,122]
2000s-		Natural/non-natural product synthesis used as pharmaceuticals, biochemicals and biofules (e.g., CFPS)	[24,35]
Milestones for *in vitro* biomanufacturing			
1897	Eduard Buchner	Cell-free ethanol fermentation by nonliving yeast lysate (Nobel Chemistry 1907)	[123]
1960s-1970s		One-enzyme biotransformation for high fructose corn syrup production (i.e., more than 20 million tonnes yearly) and semi-synthetic antibiotics (e.g., cephalosporin)	[37]
1990s		Multi-enzyme biotransformation for fine chemicals and pharmaceuticals production	[36,40,41]
1990s		Cell-free protein synthesis	[35]
2000s		Hydrogen, artificial starch, and inositol production	[15,32,92]

chemistry industries adopted this platform to produce high-value chiral alcohols, α-hydroxy acids, and α-amino acids, such as, (S)-2-butanol, L-tert-leucine, (S)-ethyl-4-chloro-3-hydroxybutyrate, atorvastatin, and so on [36,40,41]. In the organic chemistry field, the synthesis of monosaccharides, activated monosaccharides, oligosaccharides, and glycopeptides by using two-three enzymes in one pot had been intensively investigated, such as, L-fructose, 5-deoxy-5-ethyl-D-xylulose, amylose, and so on [42–47]. In this century, some researchers propose to put more than four biocatalytic components or even tens of ones in one vessel to implement very complicated reactions that is comparable to microbial cell factories [17,28,29,31,33,48,49]. This represents the emerging area-the *in vitro* synthetic biology platform, distinct from *in vitro* biocatalysis based on one or multiple enzymes. The first industrial biomanufacturing example is the production of *myo*-inositol (called inositol later) from starch, which has been demonstrated in China [32].

In this review, we are focused on the bottom-up design principles of *in vitro* enzymatic pathways including pathway design and reconstruction, enzyme selection, and coenzyme management, and we highlight three examples for industrial biomanufacturing.

2. Basic design principles for *in vitro* synthetic pathways

The basic bottom-up design principles of *in vitro* synthetic pathways include (i) pathway design and reconstruction, (ii) enzyme selection, and (iii) coenzyme management. For pathway design and reconstruction, several points, such as coenzyme balance and involvement, enzyme selection, thermodynamics, reaction equilibrium, product separation et al., need to be carefully considered [17,22]. For enzyme selection, the discovery and utilization of thermostable enzymes can greatly simplify numerous biotechnological processes and decrease potential biomanufacturing costs [7,17]. Furthermore, coenzyme regeneration and balancing in *in vitro* synthetic pathways is another important issue. Depletion or imbalance of specific coenzymes slows down the reaction and finally stops the entire cascade. To overcome this problem, modules for regenerating and balancing coenzymes have been proposed and developed [16].

2.1. Pathway design and reconstruction: coenzyme-free or coenzyme balancing

The design and reconstruction of an enzymatic pathway is the central point of the *in vitro* synthetic biology platform, which starts from basic building blocks-BioBricks (i.e., thermoenzymes, immobilized enzymes) to building modules, that is, enzyme complexes or several enzymes with defined functions (e.g., ATP regeneration, NAD(P)H regeneration) to a complicated synthetic pathway or system for the purpose of biomanufacturing [17]. The pathway design usually starts from natural metabolic pathways with necessary modifications. Because the same biochemical reactions can be conducted by several different pathways sometimes, the pathways need to be designed carefully by considering ATP and NAD(P)H balance, thermodynamics, reaction equilibrium, product separation, and so on [17,22,50]. Owing to the expensive and unstable characteristics of coenzymes, it is best to design an *in vitro* synthetic coenzyme-free enzymatic pathway. For example, an *in vitro* non-fermentative enzymatic pathway has been constructed to convert starch to inositol in one vessel, which is composed of four enzymes without ATP or NAD^+ supplementation. Besides inositol, artificial starch [15,51] and fructose 1,6-diphosphate [33] have been produced by using similar *in vitro* synthetic enzymatic pathways without coenzyme involved.

However, most *in vitro* biocatalysts for biomanufacturing are restricted to coenzyme-independent enzymes such as hydrolases and isomerases. In comparison, coenzyme-dependent enzymes, such as oxidoreductases and transferases, are capable of performing more complex chemistry. As a result, by considering the increasing range of products, it is vital to maintain both ATP and reducing power carriers (NAD(P)H) recycling and balance for *in vitro* synthetic biosystems. As these coenzymes are too costly to be used as stoichiometric agents for preparative applications, the regeneration of coenzymes *in situ* are needed for low-cost production [52]. Abundant *in vitro* coenzyme regeneration methods have been developed to regenerate the required coenzymes, while simultaneously driving the reaction equilibrium toward desired products [52–54]. Besides, coenzyme regeneration can simplify product isolation and avoid the accumulation of inhibitory coenzymes [55]. Recently, a variety of *in vitro* synthetic pathways with coenzyme regeneration have been designed and implemented for the production of chiral alcohols [31,48,56], biopolymers [57], organic acids [49,58,59], hydrogen [12,60,61], and bioelectricity [28,62].

Moreover, phenomena of ATP hydrolysis or spontaneous NAD(P)H oxidation or undesired side-reactions may take place when the cell lysates containing undesired enzyme components were used or the system is exposed to the air [30,57,63]. Thus, the system would wind down when the levels of ATP or NAD(P)H dissipate over time. Coenzyme balancing must be maintained owing to the economic viability of *in vitro* synthetic biosystems, which run the systems in a self-sustaining manner for a long time. Several strategies can be implemented, such as, a molecular purge valve module for NADPH balance [29,48,57], a molecular ATP rheostat [63], and integration of an additional enzyme set of thermophilic adenylate kinase and polyphosphate kinase for the deceleration of ATP degradation [30].

2.2. Enzyme selection: stable enzymes as standardized building blocks

Stable enzymes used as BioBricks for *in vitro* synthetic biosystems are essentially significant to decrease production costs and increase the carbohydrate allocation to the desired products [7,17]. In general, three major strategies can be conducted for the selection of stable enzymes: enzyme mining and discovery from (hyper-) thermophilic hosts, protein engineering, and enzyme immobilization. The best and simplest starting point is mining and discovery of thermoenzymes from (hyper-) thermophilic hosts [64]. Several novel enzymes have been discovered from hyper-thermophilic microorganisms like *Thermotoga maritima*, *Thermus thermophilus*, *Pyrococcus furiosus*, *Thermococcus kodakarensis*, *Sulfolobus tokodaii,* and so on [17]. *In vitro* biosynthetic biosystems have been constructed by using numerous recombinant thermoenzymes produced in *E. coli* BL21(DE3), such as, artificial starch production [15], hydrogen generation [12,60], bioelectricity generation [28,62], fructose 1,6-diphosphate production [33], and inositol production [32]. Now several websites have provided valuable collections for putative enzyme sources, such as, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) and the comprehensive information of characterized enzymes-BRENDA (http://www.brenda-enzymes.org/).

Poor thermostability of enzymes is one of the main limiting factors preventing the industrial application of enzymes [65]. When thermoenzymes are not available in the database and literature, an enzyme from a mesophilic source needs to be modified to enhance the stability by enzyme engineering, which involves rational design and directed evolution or their combination. Rational design usually requires both the availability of the structure of the enzyme and knowledge about the relationships among sequence, structure, and mechanism/function. For example, the

thermostability of *Pseudoalteromonas carrageenovora* arylsulfatase has been improved by using rational design [66]. Among the mutants, K253H/H260L is the best one with improved thermal stability, and structure modeling demonstrates that the additional hydrogen bonds, optimization of surface charge-charge interactions, and increasing of hydrophobic interaction could account for the improved thermostability imparted by K253H/H260L. On the other side, directed evolution is another potent protein engineering tool for improving enzyme performance without in-depth understanding of protein structure and enzyme-substrate interactions. By using error-prone PCR or other mutation strategies accompanied with thermal stress for screening, mutants of endoglucanase and cellobiohydrolases have been identified and characterized with improved thermostability [67,68]. Recently, new strategies have been developed for the improvement of enzyme thermostability. For example, Cornvik and his coworkers developed a new screening method for protein thermostability engineering, so called HotCoFi method [69]. Unlike the traditional screening methods based on activity, this method relies on the unfolding and aggregation quality of the protein above a critical temperature. Rather than playing off one approach against the others, future efforts should focus on how to combine these alternative approaches in order to improve the thermostability of the desired enzyme. A successful first study of this type has been reported by Cherry et al. [70]. In their endeavor to improve the stability of a haem peroxidase for laundry applications, four mutations have been rationally designed: one to increase the enzyme's thermostability and three to increase resistance to oxidative damage. The combination of these mutations with favorable amino acid exchanges identified in directed evolution experiments yields a final mutant with 174 times the thermal stability and 100 times the oxidative stability of the wild-type haem peroxidase.

Enzyme immobilization is a classic method to increase enzyme stability even before mining & discovery of thermoenzymes and protein engineering [7,17]. The underlying benefits for immobilization are improved stability, easy recyclability of immobilized enzymes, and easy separation of biocatalysts and products. Besides, low risk of production contamination and low allergenicity are further advantages of enzyme immobilization. Methods for enzyme immobilization can be classified into three principal types: adsorption, encapsulation and cross-linking [71]. Furthermore, combinations of two or more immobilization methods are designed to improve the performance of immobilized enzymes. For instance, Antrim and his coworkers immobilized glucose isomerase (GI) from *Streptomyces rubiginosus* to DEAE-cellulose−polystyrene−TiO_2 resin using electrostatic binding, resulting in immobilized glucose isomerase (IGI) with catalytic densities of up to 1500 U g^{-1}. IGI is very stable, with a half-life of over 1800 h under recommended operating conditions at a pH range of 7.2–8.2 with a preferred range of 7.6–7.8, and a temperature range of 54–62 °C with the temperature of optimum productivity being about 57 °C [72,73]. Novozyme 435 (a lipase) is utilized to synthesize specialty esters industrially. Lipase B from *Candida antarctica* (CaLB) is adsorbed on Lewatit VP OC 1600 (Lanxess, Germany), whose protein loading can be up to 1–10% and the thermal stability can be up to 110 °C in solvent-free systems [74–76]. As we known, the ideal immobilization should have no/little influence on enzyme activity. However, several essential trade-offs occur when considering the immobilization method, as immobilization procedures often inactivate a percentage of the enzymes prepared and mass transfer can become a limitation, slowing the reaction rate.

2.3. In vitro ATP regeneration or balancing

Adenosine triphosphate (ATP), the most influential energy currency for all living organisms, is essentially important for biosynthesis, mobility, signaling, and cell division [52]. Different from *in vivo* synthetic pathways where whole cells can obtain or depose extra ATP from or to cellular metabolism, *in vitro* synthetic enzymatic pathways must have a balance in ATP production and consumption although ATP may be needed for some enzymes. If net ATP is generated for the case of *in vitro* ethanol fermentation via the glycolytic pathway, the accumulation of ATP stops the cell-free system from running for a long time [77]. The best solution is cautious design of pathways without ATP involvement or with ATP balance. By contrast, unwanted ATP hydrolysis should be taken into account as a form of metabolite proofreading for maintaining high-energy coenzyme balance. A simple molecular ATP rheostat has been developed to regulate ATP levels by controlling the flow down either an ATP-generating or non-ATP-generating pathway in a function of free-phosphate concentration (Fig. 1a) [63]. This rheostat maintains adequate ATP concentrations even in the presence of ATPase contamination. Meanwhile, it is critical to use a low-cost sacrificial substrate for the regeneration of ATP due to high cost of ATP.

In vitro ATP regeneration technologies are performed through glycolysis or by using different phosphate donors based on substrate-level phosphorylation. Various metabolic pathway modules from glucose or anhydroglucose from starch to pyruvate can be implemented in a function of different numbers of ATP generated, from zero to four (Fig. 1b–g). The *in vitro* ATP-free pathway has been shown to produce two pyruvate from glucose (Fig. 1b) [56], but this pathway suffer from very slow reaction rates. Alternatively, another *in vitro* ATP-balanced pathway has been designed by modification of the glycolytic pathway, generating two pyruvate and two NADH from one glucose with zero net ATP produced (Fig. 1c) [31]. Furthermore, if a small amount of ATP is necessary for the synthesis of desired products, several pathways can be selected as below. The Entner-Doudoroff pathway (ED pathway) can produce a net yield of one ATP per glucose (Fig. 1d) and the Embden-Meyerhof-Parnas pathway can generate two net ATP per glucose (Fig. 1e). The use of alpha-glucan phosphorylase and phosphoglucomutase to phosphorylate starch to generate glucose 6-phosphate, following the glycolytic pathway can generate three net ATP per glucose (Fig. 1f). When the introduction of a pyrophosphate-dependent fructose 6-phosphate kinase to replace ATP-dependent fructose 6-phosphate kinase enables the generation of four net ATP for a glucose unit of starch (Fig. 1g).

For phosphorylation with phosphate donors, low-cost polyphosphate and pyrophosphate will be economically feasible for biocommodity production. Meanwhile, numerous enzymes are found to be able to accept polyphosphate as phosphate donor for ATP regeneration. For instance, a thermophilic polyphosphate-dependent glucokinase from *Thermobifida fusca* YX has been applied into hydrogen production from glucose [60,78]; A new polyphosphate-dependent xylulokinase from *T. maritima* has been used to convert xylose to xylulose 5-phosphate along with xylose isomerase by using polyphosphate instead of ATP [79]; Pyrophosphate-dependent phosphofructokinase from *T. maritima* has been used along with three thermopilic enzymes to produce a high-energy phosphate metabolite fructose 1,6-diphosphate from starch and pyrophosphate [33].

2.4. In vitro $NAD(P)^+$/NAD(P)H regeneration and balance

While living organisms can adjust $NAD(P)^+$/NAD(P)H balance through anabolism and catabolism, *in vitro* synthetic biosystem must have $NAD(P)^+$/NAD(P)H balanced in its pathway design at the beginning [16,17]. It means that the amount of reduced NAD(P)H generated from substrates should match that of NAD(P)H

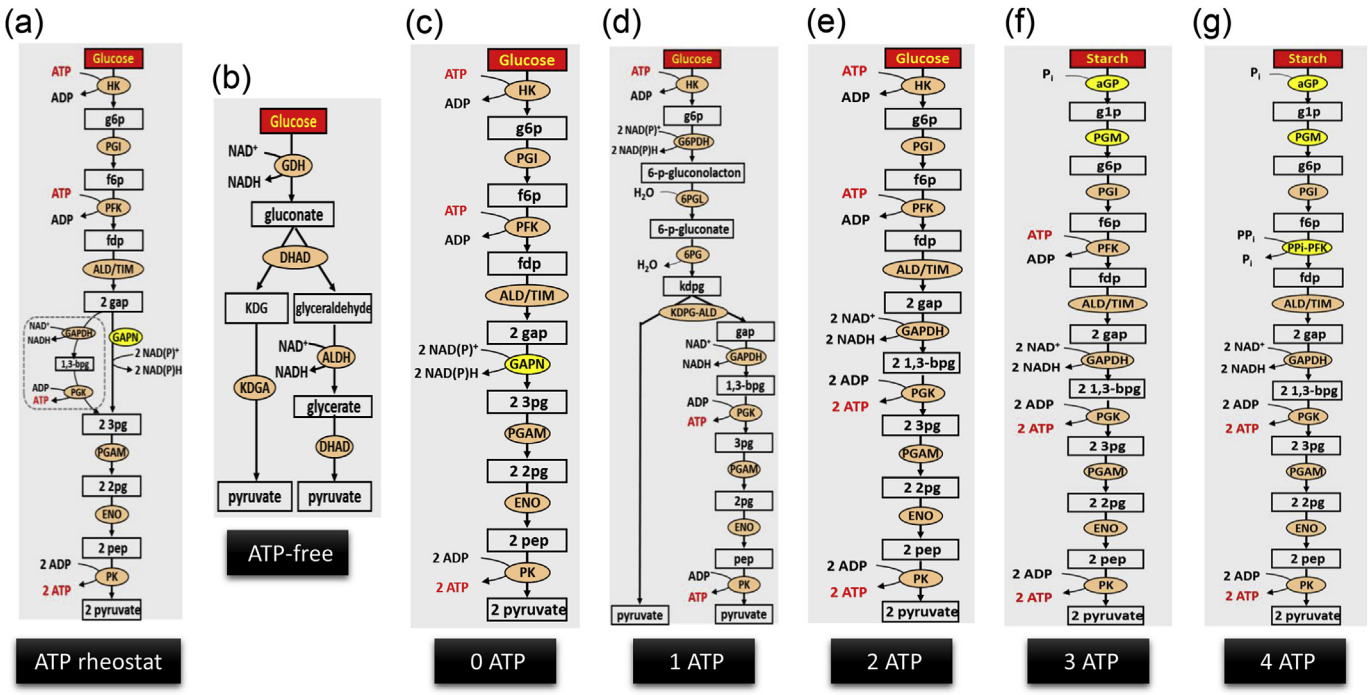

Fig. 1. Pathway design for ATP regeneration or balancing. The enzymes are GDH, glucose dehydrogenase; DHAD, dihydroxy acid dehydratase; KDGA, 2-keto-3-desoxygluconate aldolase; ALDH glyceraldehyde dehydrogenase; HK, hexokinase; PGI, phosphoglucose isomerase; PFK, 6-phosphofructokinase; ALD, fructosebisphosphate aldolase; TIM, triosephosphate isomerase; GAPN, non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase; PGAM, cofactor-independent phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; G6PDH, glucose 6-phosphate dehydrogenase; 6PGL, 6-phosphogluconolactonase; 6PG, 6-phosphogluconate dehydratase; KDPG-ALD, 2-keto-3-deoxy-phosphogluconate aldolase; GAPDH glyceraldehyde 3-phosphate dehydrogenase; PGK phosphoglycerate dehydrogenase; aGP, alpha-glucan phosphorylase; PGM, phosphoglucomutase; PP_i-PFK, pyrophosphate-dependent fructose-6-phosphate 1-phosphotransferase. Metabolites are KDG, 2-keto-3-deoxygluconate; g6p, glucose 6-phosphate; f6p, fructose 6-phosphate; fdp, fructose 1,6-diphosphate; gap, glyceraldehyde 3-phosphate; 3pg, 3-phosphoglycerate; 2pg, 2-phosphoglycerate; pep, phosphoenolpyruvate; kdpg, 2-keto-3-deoxy-6-phosphogluconate; 1,3-bpg, 1,3-diphosphoglycerate; dhap, dihydroxacetone phosphate.

consumption for the production of desired products. The accumulation of the reduced NAD(P)H leads to depletion of the corresponding oxidized NAD(P)$^+$, which is necessary for continuous utilization of substrate. For example, a molecular purge valve module for balancing the availability of NAD(P)$^+$/NADPH has been designed, which is useful for the reaction module where NADPH production upstream in the reaction is in excess over its consumption downstream [57].

Most *in vitro* NAD(P)H regeneration methods can be implemented by using another substrate and its respective enzymes (Fig. 2). NAD(P)H can be generated by using a hydrogen-donor substrate and one of the followings: a single enzyme, cascade enzymes, and bioelectrochemistry. Single-enzyme systems include alcohol/alcohol dehydrogenase [80], formate/formate dehydrogenase [81], glucose/glucose dehydrogenase [82], glucose 6-phosphate (G6P)/G6P dehydrogenase [83], dihydrogen/hydrogenase [84,85], and phosphite/phosphite dehydrogenase [86]. Single-enzyme NAD(P)H regeneration systems have been widely used in the synthesis of high-value chiral compounds in the pharmaceutical industry. Four representative single-enzyme substrates to regenerate NADH are the dehydrogenation of isopropanol, formate, glucose, and phosphite (Fig. 2a–d). As an example of cascade enzymes for NADH regeneration, three enzymes-formate dehydrogenase, formaldehyde dehydrogenase, and alcohol dehydrogenase-can completely oxidize methanol to carbon dioxide, generating three NADH (Fig. 2e) [87]. A 12-enzyme system is utilized to produce nearly 12 NADPH from one glucose unit of cellobiose (Fig. 2f) [88]. In addition, our group has designed an NAD$^+$- based electron transport chain (ETC) for *in vitro* NADH regeneration, where diaphorase as a transhydrogenase was used to convert NADPH and

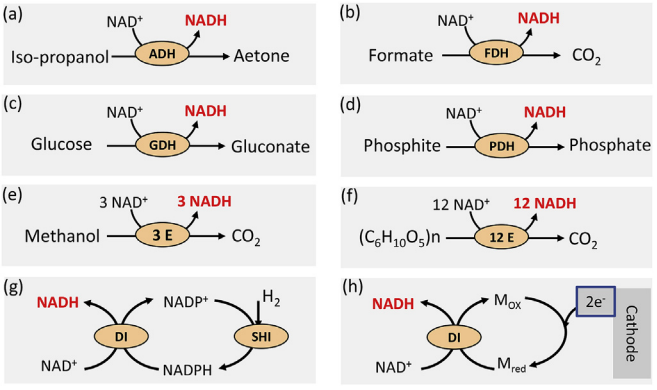

Fig. 2. *In vitro* NAD(P)H generation catalyzed by one enzyme or synthetic enzymatic pathways. NAD(P)H can be generated by using a hydrogen-donor substrate and one of the followings: a single enzyme (a–d), cascade enzymes (e–f), and bioelectrochemistry (g–h). The enzymes are ADH, alcohol dehydrogenase; FDH, formate dehydrogenase; GDH, glucose dehydrogenase; PDH, phosphite dehydrogenase; DI, diaphorase; SHI, soluble [NiFe] hydrogenase I. M_{ox} and M_{red} are oxidized and reduced mediator.

NAD$^+$ to NADH and NADP$^+$, matching with NADP$^+$-preferred hydrogenase (submitted for publication) (Fig. 2g). NADH can also be regenerated by electrochemistry based on the mediator-conjugated diaphorase system (Fig. 2h) [89]. Among all hydrogen-donor compounds, renewable sugars have the lowest substrate costs, but they require more enzymes and increase system complexity. Utilization of electrochemistry to generate reduced cofactors is

low-cost and clean, but the instability of NADH under high overpotential must be solved before this technique becomes industrially feasible.

Sometimes designed products have a lower degree of reduction than those of substrates, for example, the production of 1,3-butanediol or fatty acid ethyl esters from glucose [90], that is, extra NAD(P)H is generated in *in vitro* pathways. Unlike microbial fermentation that can consume NAD(P)H through oxidation or cell mass synthesis, it is vital to remove extra NAD(P)H from *in vitro* synthetic biosystems. Fig. 3 presents four different ways to remove extra NAD(P)H: enzymatic (Fig. 3a–d) and electrochemical (Fig. 3e–f). NADH can be converted to NAD^+ by using a water-forming NADH oxidase (Fig. 3a) or a hydrogen peroxide-forming NADH oxidase combined with catalase (Fig. 3b). For example, a water-forming NADH oxidase from *Lactobacillus pentosus* has been used for the regeneration of NAD^+ from NADH during the conversion of glucuronate to α-ketoglutarate [34] and the cell-free production of monoterpenes from glucose [29]. Furthermore, extra NADPH can also be removed by hydrogenase to produce H_2 via the biomimetic ETC, which has been designed by the introduction of an electron mediator benzyl viologen (BV) and an enzyme NADPH rubredoxin oxidoreductase (NROR) (Fig. 3c) [91] or BV-conjugated diaphorase system (Fig. 3d) [92]. Lastly, another way to remove extra NADH occurs in enzymatic fuel cells through an electron mediator (Fig. 3e–f) [28,93].

$NAD(P)^+$ and NAD(P)H are known to have relatively low thermal stability. Thermal instability of NAD(P)H is problematic, especially at high temperature [31,59]. To overcome this obstacle, the NAD^+ salvage module has been designed to re-synthesize NAD^+ from its thermal decomposition products of nicotinamide and ADP-ribose using eight thermophilic enzymes [94]. NAD^+ concentration remains nearly constant for 15 h at 60 °C with the NAD^+ salvage module, while the concentration decreased by a half in 6 h without the module [94].

3. Representative examples of biomanufacturing

It is highly likely that more biocommodities with huge-market sizes could be preferentially produced by the *in vitro* synthetic biology platform if synergetic efforts are taken for the design of enzymatic pathway, production of low-cost stable enzyme, enzyme immobilization and recycle, utilization of biomimetic coenzymes, coenzyme recycle, and product separation. Although the *in vitro* synthetic biology is just an emerging frontier, many high-value biochemicals and biofuels have been produced via this platform, such as 1,3-propanediol [95], poly-3-hydroxylbutyrate [96], amylose [15], n-butanol [31], isobutanol [56,63], terpenoids [29], and so on. Here three representative examples are highlighted for the ingeniousness of pathway design.

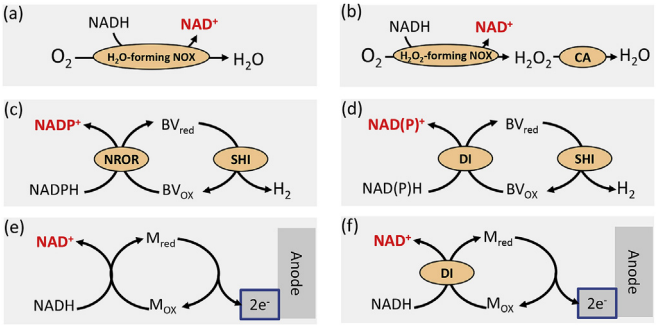

Fig. 3. In vitro NAD(P) oxidative regeneration. NAD(P) oxidative regeneration by a water-forming NADH oxidase (a), a hydrogen peroxide-forming NADH oxidase combined with catalase (b), cascade enzymes combined with an electron mediator (c–d), and electrochemistry through a mediator with or without enzyme (e–f). The enzymes are NOX, NADH oxidase; CA, catalase; NROR, NADPH rubredoxin oxidoreductase; DI, diaphorase; SHI, soluble [NiFe] hydrogenase I. BV_{ox} and BV_{red} are oxidized and reduced benzyl viologen; M_{ox} and M_{red} are oxidized and reduced electron mediator.

3.1. Pathway design for inositol production

Inositol is important in the cosmetics, pharmaceutical and functional food industries, which is predominately obtained by acid hydrolysis of inositol hexakisphosphate (IP6). However, this production method suffers from costly feedstock, serious phosphorous pollution, and complicated feedstock and product separation, resulting in relatively high price and limited supply.

You and his coworkers have constructed an *in vitro* synthetic enzymatic pathway that can convert starch to inositol without external coenzyme supplement [32]. This pathway is comprised of four steps (Fig. 4): (i) glucose 1-phosphate (G1P) generation from starch and phosphate; (ii) G6P generation from G1P; (iii) inositol 1-phosphate (I1P) generation from G6P; and (iv) inositol generation accompanied by phosphate generation from I1P. Phosphate generated from the fourth module is recycled in the first step. The consolidation of four step reactions has an overall Gibbs energy of −80.1 kJ/mol, that is, this pathway has a very high equilibrium constant to push the overall reaction toward completeness with very high product yield. Later, Atomi and his coworkers also demonstrate the synthesis of inositol from starch [97]; Tao and his coworkers demonstrate the synthesis of inositol from glucose with ATP regeneration from polyphosphate [98]; and Zhang and his coworkers demonstrate inositol production from sucrose [99].

This new synthesis of inositol from starch is a disruptive method for green production of inositol compared to the acid hydrolysis of IP6. It has many biomanufacturing advantages: (i) less costly substrate with starch; (ii) decreased phosphorous pollution and COD emission; (iii) easy product separation; (iv) scalable low-cost production of all thermoenzymes; and (v) nearly no odds for microbial contamination.

3.2. High-yield production of hydrogen

Hydrogen (H_2) as a future transportation fuel offers enhanced energy conversion efficiency and tremendous potential to reduce greenhouse gas emissions [100]. In spite of intensive efforts in metabolic engineering and synthetic biology, none of natural or engineered microorganisms can produce H_2 beyond the Thauer limit ($4H_2$/glucose) [101–103]. Moreover, *in vitro* hydrogen production from low-cost biomass and water is an excellent solution for producing low-cost H_2 without net carbon emissions [12,92,104].

Starch has been proposed as a new high-density hydrogen storage carrier with its gravimetric density of up to 14% H_2 mass. Zhang and his coworkers have carried out a proof of-concept

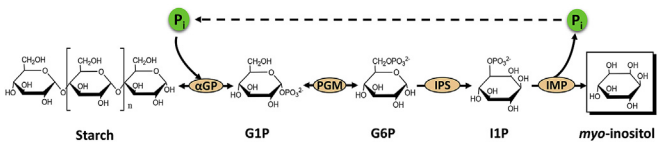

Fig. 4. Scheme of the *in vitro* synthetic enzymatic pathway for the production of inositol from starch. The enzymes are aGP, alpha-glucan phosphorylase; PGM, phosphoglucomutase; IPS, inositol 1-phosphate synthase; IMP inositol monophosphatase. Metabolites are G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; I1P, inositol 1-phosphate; P_i, inorganic phosphate.

experiment for H_2 production from glycogen (animal starch) using an *in vitro* enzymatic pathway [105] with the maximum H_2 production yield (43% of the theoretical yield of $12H_2$ per glucose) exceeding the Thauer limit. Later they have redesigned and demonstrated several *in vitro* pathways for H_2 production from various carbohydrates, including cellulosic materials [106], xylose [79], sucrose [61], a mixture of biomass monosaccharides [60], and xylooligosaccharides [104]. Recently, Kim and his coworkers have constructed an *in vitro* synthetic pathway for generating H_2 at theoretical yield from starch with the maximum volumetric productivity of 90.2 mmol/L/h [12]. This reconstituted ATP-free and cofactor-balanced enzymatic pathway composed of 17 enzymes and it can be grouped into four modules (Fig. 5): (i) ATP-free phosphorylation of starch generating G6P; (ii) NADPH generation via the oxidative pentose phosphate pathway (PPP); (iii) hydrogen generation catalyzed by soluble [NiFe]-hydrogenase I from a hyperthermophilic archaeon *P. furiosus* (SHI) from NADPH via a biomimetic ETC comprised of NROR and BV as an abiotic electron mediator [91]; and (iv) G6P regeneration via the non-oxidative PPP (iv-a) and partial gluconeogenesis pathway (iv-b). Phosphate generated from the fourth module is recycled by αGP for starch phosphorolysis in the first module.

Thermodynamic analysis indicates that the overall reaction is spontaneous with an overall Gibbs free energy change of −48.9 kJ/mol. Meanwhile, due to the gaseous products (H_2 and CO_2) are simultaneously removed from the liquid reaction solution, the real Gibbs free energy change is much less than −48.9 kJ/mol to drive the overall reaction toward completeness.

3.3. N-butanol production

N-butanol, a primary 4-carbon alcohol, is regarded as the advanced liquid biofuel with an energy density (27 MJ/L) comparable to gasoline (32 MJ/L). It is traditionally produced by acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum* [7,107]. However, its fermentation involves a complicated transition from acidogenesis to solvogenesis and suffers from low product yields and severe product inhibition, resulting in low product titers and yields [108].

Honda and his coworkers have constructed a non-natural, cofactor-balanced, and oxygen-insensitive pathway for the direct conversion of glucose to n-butanol using 16 thermostable enzymes [31]. This pathway comprises three modules (Fig. 6): (i) generation of two pyruvate and two NADH from one glucose without ATP accumulation, (ii) generation of acetyl-CoA from pyruvate; and (iii) production of one n-butanol from acetyl-CoA consuming two NADH. As a consequence, one glucose can produce one n-butanol, two CO_2 and one water. This synthetic pathway has three key features pertaining to the regenerations of ATP and redox cofactors (i.e., NADH and CoA): (i) ATP balance, where the ATP consumption during the conversion of glucose to fructose-1,6-diphosphate matches the ATP regeneration from phosphoenolpyruvate to pyruvate mediated by pyruvate kinase; (ii) NADH balance, where NADH regeneration by non-phosphorylating GAP dehydrogenase (GAPN) and CoA-acylating aldehyde dehydrogenase (ADDH) matches its consumption by hydroxybutyryl-CoA dehydrogenase (HBD), ADDH, NADH dependent flavinoxidoreductase (NFO), and 3-hydroxyacyl-CoA dehydrogenase (HAD); (iii) CoA balance, where

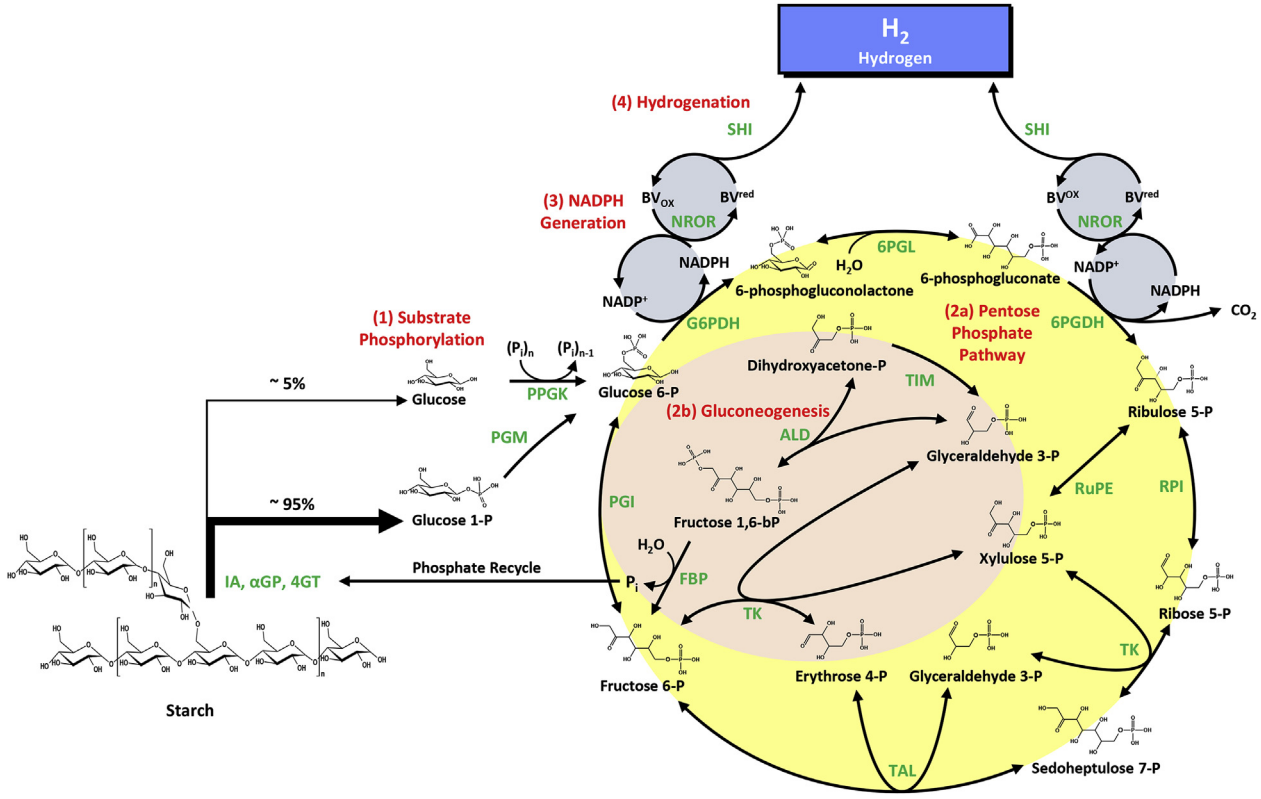

Fig. 5. Scheme of the *in vitro* synthetic pathway for the production of hydrogen through complete utilization of starch. The enzymes are IA, isoamylase; 4GT, 4-alpha-glucanotransferase; αGP, alpha-glucan phosphorylase; PPGK, polyphosphate glucokinase; PGM, phosphoglucomutase; G6PDH, glucose 6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; NROR, NADPH rubredoxin oxidoreductase; SHI, soluble [NiFe] hydrogenase I; 6PGL, 6-phosphogluconolactonase; RPI, ribose 5-phosphate isomerase; RuPE, ribulose 5-phosphate 3-epimerase; TK, transketolase; TAL, transaldolase; TIM, triose phosphate isomerase; ALD, aldolase; FBP, fructose 1,6-biphosphatase; PGI, phosphoglucose isomerase. P_i and $(P_i)_n$ are inorganic phosphate and polyphosphate with a degree of polymerization of n. BV_{ox} and BV_{red} are oxidized and reduced benzyl viologen.

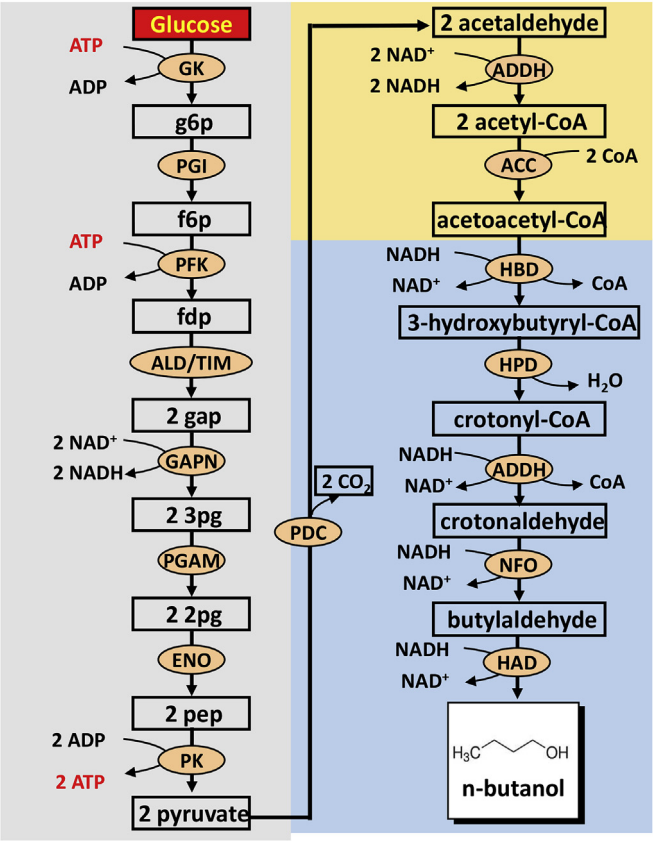

Fig. 6. Scheme of the in vitro synthetic enzymatic pathway for the production of n-butanol from glucose. The enzymes are HK, hexokinase; PGI, phosphoglucose isomerase; PFK, 6-phosphofructokinase; FBA, fructosebisphosphate aldolase; TIM, triosephosphate isomerase; GAPN, non-phosphorylating GAP dehydrogenase; PGAM, cofactor-independent phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; PDC, pyruvate decarboxylase; ADDH, CoA-acylating aldehyde dehydrogenase; HBD, hydroxybutyryl-CoA dehydrogenase; HPD, 3-hydroxypropionyl-CoA dehydratase; NFO, NADH-dependent flavinoxidoreductase; and HAD, 3-hydroxyacyl-CoA dehydrogenase. Metabolites are g6p, glucose 6-phosphate; f6p, fructose 6-phosphate; fdp, fructose 1,6-diphosphate; gap, glyceraldehyde 3-phosphate; dhap, dihydroxacetone phosphate; 3pg, 3-phosphoglycerate; 2pg, 2-phosphoglycerate; and pep, phosphoenolpyruvate.

CoA is needed by ADDH and is released by acetyl-CoA acetyltransferase (ACC) and ADDH. The overall reaction is an enthalpy-driven reaction with an overall Gibbs free energy change of −265.9 kJ/mol and a slight loss of chemical energy exists in the reaction. By optimizing enzyme loading and replenishing of redox cofactors NAD^+ and NADH, n-butanol could be produced from glucose with a molar yield of 82% at a rate of 8.2 $\mu mol\, l^{-1}\, min^{-1}$, comparable with the best product yield in ABE fermentation [108].

4. Conclusions

The appealing advantages, such as high product yield, fast reaction rate, broad reaction condition, as well as easy process control and regulation, are motivating the *in vitro* synthetic biology platform to be a novel biomanufacturing platform compared to the predominant fermentation. Here, *in vitro* synthetic pathways are comprised of stable BioBricks (e.g., thermoenzymes, immobilized enzymes) and modules (e.g., enzyme complexes or multiple enzymes as a module) with specific functions (e.g., coenzyme regeneration). Mining and discovery of thermoenzymes, protein engineering, and enzyme immobilization would result in ultra-stable enzymes as basic BioBricks. Many efficient coenzyme regeneration systems, including ATP and NAD(P)H, have been developed as building modules. The assembly of BioBricks and modules would make cost-competitive production of bio-commodities. The value-added inositol from starch is the first manufactured on an industrial scale [32]. In a word, the *in vitro* synthetic biology platform would open a new biomanufacturing age for the cost-competitive manufacturing of bioenergy, food, biochemicals, and nutraceuticals [7].

Declaration of interest

None.

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