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.

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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, biochemicals, nutraceuticals, and bioenergy via engineered biointerfaces 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 biointerfaces, which can duplicate themselves. There are numerous breakthroughs, especially in terms of fundamental research and publications [23,24,10]. 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 products, and maintenance, intact cellular membrane for maintaining basic 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.

Although in vitro synthetic biology is largely ignored compared to in vivo synthetic biology, it has made great and rapid progress in the past decades [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 bio-chemicals [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 to 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 lyses” (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 CO2 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 technical 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 next-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

| Year  | Leaders | Milestone (Award) | References |
|-------|---------|-------------------|------------|
| 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 Nirenberg | 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 biofuels (e.g., CFPS) | [24,35] |

| Year  | Milestones for in vitro biomanufacturing | References |
|-------|-----------------------------------------|------------|
| 1897  | Eduard Buchner | Cell-free ethanol fermentation by nonliving yeast lyses (Nobel Chemistry 1907) | [123] |
| 1960s– | | 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] |
| 1970s | | 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] |
been proposed and developed [16]. 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, 1-fructose, 5-deoxy-5-ethyl-o-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 biomanning 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 biomanning.

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 biomanning 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., theremoenzymes, immobilized enzymes) to building modules, that is, enzyme complexes or several enzymes with de-bilized enzymes) to building modules, that is, enzyme complexes or from basic building blocks-BioBricks (i.e., thermoenzymes, immo-

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 theremoenzymes from (hyper-) thermophilic hosts [64]. Several novel enzymes have been discovered from hyper-thermophilic microorganisms like Thermotoga maritima, Thermus thermophilus, Pyrococcus furiosus, Thermococcus kodakarenisi, Sulfolobus tokodaii, and so on [17]. In vitro biosynthetic biosystems have been constructed by using numerous recombinant theremoenzymes 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 theremoenzymes 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

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, 1-fructose, 5-deoxy-5-ethyl-o-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 biomanning example is the production of myo-inositol (called inositol later) from starch, which has been demonstrated in China [32].

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

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However, most in vitro biocatalysts for biomanning 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 [35]. 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 [37], 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].
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 endo- 


glucanase 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 thermozymes and protein engineering [71,72]. 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—TiO₂ 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 synthesise 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 *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 thermophilic 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
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/hydride [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 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 over-
potential must be solved before this technique becomes industri-
ally 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, ex-
tra 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 re-
move 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 with catalase (Fig. 3b) 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 conver-
sion of glucuronate to x-ketoglutarate [34] and the cell-free pro-
duction of monoterpenes from glucose [29]. Furthermore, extra
NADPH can also be removed by hydrogenase to produce H₂ 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) [91] or BV-conjugated
diaphorase system (Fig. 3d) [92]. Lastly, another way to remove extra
NADH occurs in enzymatic fuel cells through an electron mediat-
ator (Fig. 3e–f) [28,83].

NAD(P)⁺ and NAD(P)H are known to have relatively low thermal
Stability. Thermal instability of NAD(P)H is problematic, especi-
al 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 re-
mains 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 synergistic 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-hydroxybutyrate [96],
amyllose [15], n-butanol [31], isobutanol [56,63], terpenoids [29],
and so on. Here three representative examples are highlighted for the
ingeniousness of pathway design.

3.1. Pathway design for inositol production

Inositol is important in the cosmetics, pharmaceutical and func-
tional food industries, which is predominantly obtained by acid
hydrolysis of inositol hexakisphosphate (IP6). However, this pro-
duction 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
synthesize the 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 sub-
strate with starch; (ii) decreased phosphorous pollution and COD
emission; (iii) easy product separation; (iv) scalable low-cost pro-
duction of all theromoenzymes; and (v) nearly no odds for microbial
contamination.

3.2. High-yield production of hydrogen

Hydrogen (H₂) 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 H₂ beyond the Thauer
limit (4H₂/glucose) [101–103]. Moreover, in vitro hydrogen pro-
duction from low-cost biomass and water is an excellent solution
for producing low-cost H₂ 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% H₂ mass.
Zhang and his coworkers have carried out a proof-of-concept
experiment for H₂ production from glycogen (animal starch) using an *in vitro* enzymatic pathway [105] with the maximum H₂ production yield (43% of the theoretical yield of 12H₂ per glucose) exceeding the Thauer limit. Later they have redesigned and demonstrated several *in vitro* pathways for H₂ 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 H₂ 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 (H₂ and CO₂) 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 CO₂ 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...
Glucose with a molar yield of 82% at a rate of 8.2 and regulation, are motivating the predominant fermentation. Here, comparable with the best product yield in ABE fermentation [108].

![Scheme of the in vitro synthetic enzymatic pathway for the production of n-butanol from glucose. The enzymes are HK, hexokinase; PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; PDC, pyruvate decarboxylase; HBD, hydroxybutyryl-CoA dehydrogenase; HPD, 3-hydroxypropionyl-CoA dehydratase; NFO, NADH-dependent flavinoxidoreductase; and NADP, 3-hydroxyacyl-CoA dehydratase. Metabolites are g6p, glucose 6-phosphate; f6p, fructose 6-phosphate; fdp, fructose 1,6-diphosphate; gap, glyceraldehyde 3-phosphate; dhap, dihydroxyacetone phosphate; 3pg, 3-phosphoglycerate; 2pg, 2-phosphoglycerate; and pep, phosphoenolpyruvate.](image)

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 $-265.9 \text{ kJ/mol}$ 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 mmol·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 isoinol 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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