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Modules for *in vitro* metabolic engineering: Pathway assembly for bio-based production of value-added chemicals

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ABSTRACT

Bio-based chemical production has drawn attention regarding the realization of a sustainable society. *In vitro* metabolic engineering is one of the methods used for the bio-based production of value-added chemicals. This method involves the reconstitution of natural or artificial metabolic pathways by assembling purified/semi-purified enzymes *in vitro*. Enzymes from distinct sources can be combined to construct desired reaction cascades with fewer biological constraints in one vessel, enabling easier pathway design with high modularity. Multiple modules have been designed, built, tested, and improved by different groups for different purpose. In this review, we focus on these *in vitro* metabolic engineering modules, especially focusing on the carbon metabolism, and present an overview of input modules, output modules, and other modules related to cofactor management.

Contents

1. Introduction

Enzymes catalyze numerous types of chemical reactions. More

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than ten thousand biochemical reactions have been described in the KEGG database (Kyoto Encyclopedia of Genes and Genomes) [68]. Thousands of enzymes are present inside cells, composing a complicated metabolic network [1,2]. Living organisms, including microorganisms, produce a wide array of chemical compounds through this metabolic network. Metabolic engineering has successfully utilized these microorganisms as platforms for the commercial production of useful metabolites by increasing the expression of desired genes and/or depleting that of undesired genes [3,4]. Precise control of natural metabolism is, however, still challenging due to the complicated regulatory architecture at the levels of transcription, translation, and post-translation [5,6].

One approach to circumvent this problem is to use simpler systems consisting of fewer components. The *in vitro* reconstitution of metabolic pathways with isolated enzymes allows for the optimization of metabolic flux in the absence of transcriptional and translational regulation, thus providing the pathway design without interference from the complex background of the host organism (Table 1) [7–9]. Different platforms have been invented for designing metabolic pathways with multiple enzymatic reactions *in vitro*, including synthetic pathway biotransformations (SyPaB) [10], minimized reaction cascades [11], the synthetic biochemistry system [12], synthetic metabolic engineering [13], and cell-free metabolic engineering (CFME) [14]. Depending on the platform, purified/semi-purified enzymes or crude cell lysates are used, and multiple modules for specific reaction cascades have been designed. In this review, the platforms using purified/semi-purified enzymes are collectively referred to as *in vitro* metabolic engineering [7,15–18].

In vitro metabolic engineering has high modularity. It allows for the free-hand design of an artificial metabolic pathway using a flexible combination of biocatalytic modules. These modules are sets of enzyme that catalyze a reaction cascade for a specific purpose, such as the conversion of starting materials (input) to intermediary metabolites (e.g., pyruvate, acetyl CoA) and biosynthesis of target products (output) from the intermediates. The functionality of different modules has been confirmed experimentally for the utilization and production of various compounds. The purpose of this review is to present an overview of the modules used for *in vitro* metabolic engineering, especially for carbon metabolisms. The modules are discussed in three sections: input modules, output modules, and other modules related to cofactor management. The first section describes how inputs are processed for product formation and energy production. The second section describes modules used for the production of output compounds, with particular focus on those involved in the derivatization of pyruvate and acetyl CoA. The third section focuses on other modules that solve general problems of cofactor balance and phosphorylation. We conclude with a discussion about the future of *in vitro* metabolic engineering.

2. Modules

2.1. Input modules

2.1.1. Carbon sources as input

For *in vitro* metabolic engineering, glucose is often used as an input owing to its relatively low price on both laboratory and industrial scales. With growing attention on the utilization of alternative carbon sources, many studies demonstrate the use of other carbon sources as inputs, including xylose [19,20], sucrose [17], maltodextrin [20], starch [21], cellulose [22], xylan [22], glycerol [23], and chitin [24]. Within *in vitro* metabolic engineering platforms, enzymes can access carbon sources without transporters, owing to the absence of cell membrane barrier. This feature enables the use of a variety of substrates as inputs, even a glycolysis intermediate [12].

2.1.2. Conversion of input for product formation

During normal glycolytic conversion of glucose to pyruvate, 2 molecules of ATP are produced per glucose, leading to the accumulation of ATP and depletion of ADP (Fig. 1A). In *in vitro* metabolic engineering platforms, an imbalance in cofactor availability can shut the system down, especially in platforms using purified/semi-purified enzymes. Therefore, different modules have been constructed to balance the cofactor availability or bypass cofactor usage to enable a continuous reaction. Ye et al. (2012) designed the Chimeric Embden-Meyerhof (EM) pathway for the conversion of glucose to lactate with balanced consumption and regeneration of ATP and ADP (Fig. 1A) [13]. This module employs the archaeal non-phosphorylating glyceraldehyde-3-phosphate (GAP) dehydrogenase (GAPN) instead of the GAP dehydrogenase and phosphoglycerate kinase of the classical EM pathway. Consequently, the net ATP generation/consumption in the module during the reaction is maintained at zero. Alternatively, Guterl et al. (2012) designed the Minimized Reaction Cascades module, in which no ADP/ATP is required (Fig. 1A) [11]. In this module, the use of two dehydrogenases, one dehydratase, and one aldolase enables the conversion of glucose to pyruvate with only six reactions catalyzed by four enzymes in a phosphorylation-independent manner. This

Table 1
Advantages and disadvantages of *in vitro* metabolic engineering over conventional metabolic engineering/fermentation.

	Metabolic Engineering/Fermentation	*In vitro* metabolic engineering
Controllability	× Expression levels have to be tuned by genetic modification × Complex regulation for cell growth is necessary by controlling oxygen/nutrition availability	○ Enzyme concentration can be tuned precisely on demand ○ Simple regulation is sufficient for enzyme reaction
Pathway design	× Metabolic product is limited × Side effects caused by pathway modification is difficult to be predicted ○ Optimization of pathways can be achieved by directed evolution or selection	○ Pathway can be designed with high flexibility ○ Artificial pathway design is possible ○ Production of cytotoxic compounds is possible
Production cost	○ Microorganism self-reproduces, producing enzymes and cofactors △ Condensation/purification can be costly when product concentration is low.	× Enzymes are necessary to be prepared × Cofactor addition is necessary ○ Separation/purification of products is easy
Product titer	○ High yield can be achieved by feeding sufficient amount of substrate	△ Cofactor regeneration is required for full conversion of substrate
Product yield	△ Substrate is also utilized for cell growth	○ Substrate is converted to product without byproduct formation
Membrane permeation	× Required	○ Not required
Scale up	× Difficult	○ Easy
Commercial implementation	○ Various examples	× At the study phase

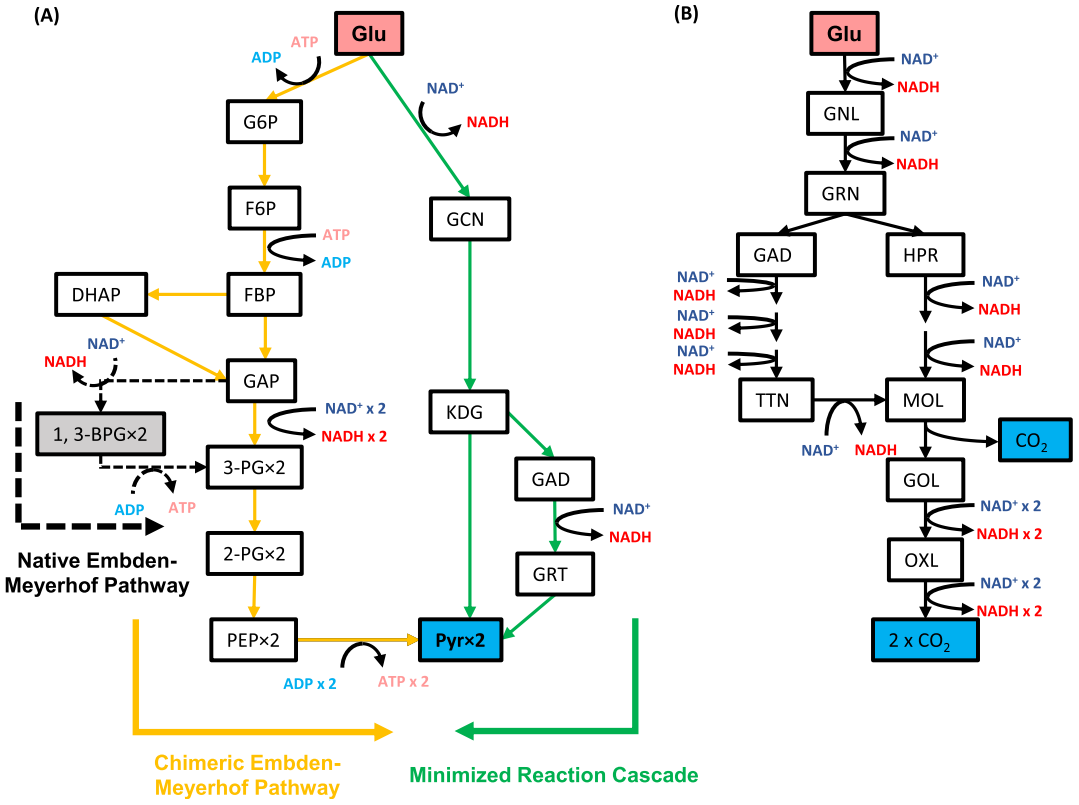

Fig. 1. Linear modules for conversion of glucose. Linear modules for glucose conversion to pyruvate (A) and complete oxidation of glucose (B). Input and output are represented by red and blue, respectively. Chimeric Embden Meyerhof Pathway and Minimized Reaction Cascades are indicated by yellow and green arrows, respectively. The native segment of Embden-Meyerhof pathway is shown in black dotted lines. Glu, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 3-PG, 3-phosphoglycerate; 1,3-PG, 1,3-bisphosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; GCN, gluconate; KDG, 2-keto-3-desoxy-gluconate; GAD, glyceraldehyde; GRT, glycerate; GNL, gluconolactone; GRN, glucuronic acid; TTN, tartronate; HPR, hydroxypyruvate; MOL, mesoxalate; GOL, glyoxylate; OXL, oxalate.

concept was applied to the conversion of glucuronate to α-ketoglutarate using two dehydrogenases and two dehydratases [15]. In nature, glucose is also assimilated through the pentose phosphate pathway (Fig. 2A). Bogorad et al. (2013) designed the Non-Oxidative Glycolysis (NOG) module, in which fructose 6-phosphate is converted to acetyl phosphate by bifid shunt, the non-oxidative pentose phosphate pathway, and gluconeogenesis (Fig. 2B) [25]. This cyclic module is ATP- and redox-cofactor-independent. Furthermore, no CO_2 release takes place during the assimilation; therefore, three molecules of acetyl phosphate can be produced per fructose 6-phosphate. Similarly, the Pentose–Bifido–Glycolysis (PBG) cycle module was designed using an efficient ATP-recycling phosphofructokinase that catalyzes the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate, with ATP regeneration [26]. This module can use non-phosphorylated glucose as a substrate and produce two molecules of acetyl CoA and four NAD(P)H per glucose, with balanced ATP formation/consumption (Fig. 2C). Apart from glucose conversion, glycerol was successfully converted to lactate in an ATP-balanced manner [23].

Building C–C bonds from C1 compounds is challenging. Using *in vitro* metabolic engineering, the assimilation of methanol or CO_2 has been demonstrated. The Methanol Condensation Cycle (MCC) was designed by combining the non-oxidative glycolysis and ribulose monophosphate (RuMP) pathways (Fig. 2D) [27]. In this module, methanol is assimilated together with ribulose-5-phosphate and converted into acetyl phosphate using a bifid shunt. Even though a certain amount of ribose-5-phosphate or relevant phosphorylated pentose has to be supplied to initiate the cyclic reaction, two molecules of methanol can be converted to one molecule of acetyl phosphate. Schwander et al. (2016) designed the Crotonyl-CoA/Ethylmalonyl-CoA/Hydroxybutyryl-CoA (CETCH) Cycle module, which is an optimized synthetic CO_2 fixation pathway [28]. This module consists of 13 core reactions with 17 enzymes and includes cofactor regeneration. One CETCH cycle converts two molecules of CO_2 to one molecule of glyoxylate with the initial addition of propionyl-CoA or relevant intermediates.

2.1.3. *Conversion of input for the efficient use of electrons*

Sugars, including glucose, are good sources of electrons. For example, the oxidation of all carbon atoms in glucose to CO_2 yields 24 electrons per glucose molecule. Several types of modules have been designed to completely oxidize carbohydrates to CO_2 and to transfer the released electrons to biological redox mediators (e.g., nicotinamide and flavin cofactors). Arechederra and Minteer (2009) constructed a module in which the electrons in glycerol are efficiently extracted and utilized for electricity production in an ADP/ATP-independent manner [29]. In this module, glycerol is completely oxidized by three enzymes with broad substrate specificity: alcohol dehydrogenase, aldehyde dehydrogenase, and oxalate oxidase. This concept was further developed for the complete oxidation of glucose using three additional enzymes: glucose dehydrogenase, gluconate 2-dehydrogenase, and aldolase [30] (Fig. 1B). Another module was established by hybridizing the pentose phosphate pathway and gluconeogenesis (Fig. 2E). Woodward et al. (2000) demonstrated the full oxidation of glucose 6-phosphate along with the formation of 12 NADPH, corresponding to 24 electrons [31]. This module was further improved for the

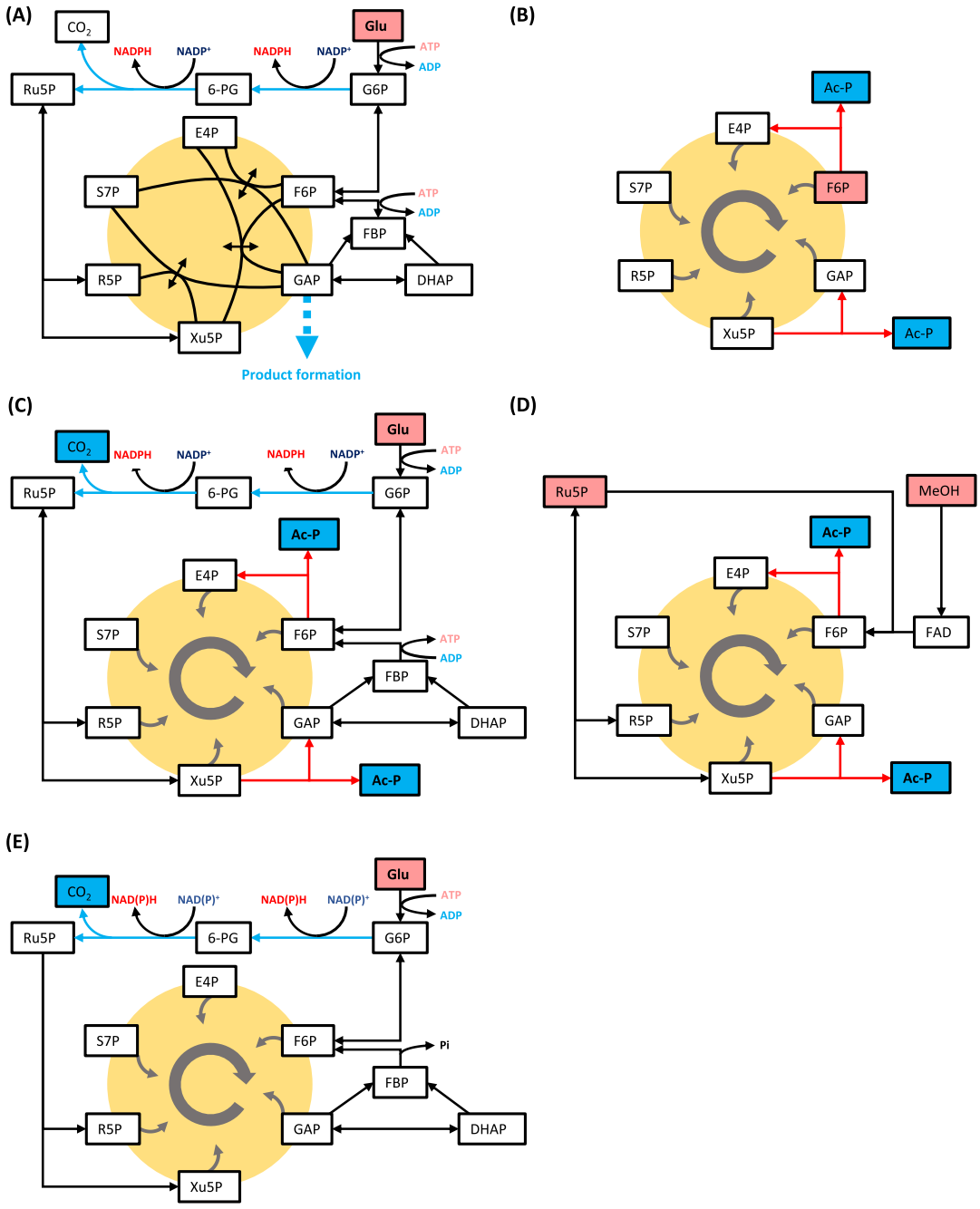

Fig. 2. Cyclic modules for conversion of glucose and other carbon source. Cyclic modules for the pentose phosphate pathway (A), Non-Oxidative Glycolysis (NOG) (B), Pentose–Bifido–Glycolysis (PBG) cycle (C), Methanol Condensation cycle (MCC) (D), and the complete oxidization of glucose by the pentose phosphate pathway (E). Blue and red arrows indicate the oxidative pentose phosphate pathway and bifid shunt for acetyl phosphate formation, respectively. Yellow circle indicates the sequential reactions of transketolase/transaldolase in the non-oxidative pentose phosphate pathway. Glu, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 6-PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; Xu5P, xylulose 5-phosphate; Ac-P, acetyl phosphate; MeOH, methanol; FAD, formaldehyde.

utilization of a variety of carbon sources, including starch [21], xylose [19], and mixed sugars [22].

2.2. Output modules

2.2.1. Modules extendable from pyruvate

Pyruvate is one of the main precursors for the biosynthesis of various metabolites (Fig. 3). Conversion of pyruvate to ethanol has been mediated by pyruvate decarboxylase and alcohol dehydrogenase, with concomitant oxidation of NADH to NAD^+, such that the intra-module balance of the redox cofactor is maintained when glucose is used as the starting substrate [11]. Pyruvate conversion has also performed using lactate dehydrogenases to yield lactate, maintaining the redox balance [13]. Pyruvate has been converted to isobutanol using five reactions in a CoA-independent manner [11]. In addition, pyruvate has been converted to malate along with CO_2 assimilation using the reverse reaction of the NAD(P)H-dependent malic enzyme [32].

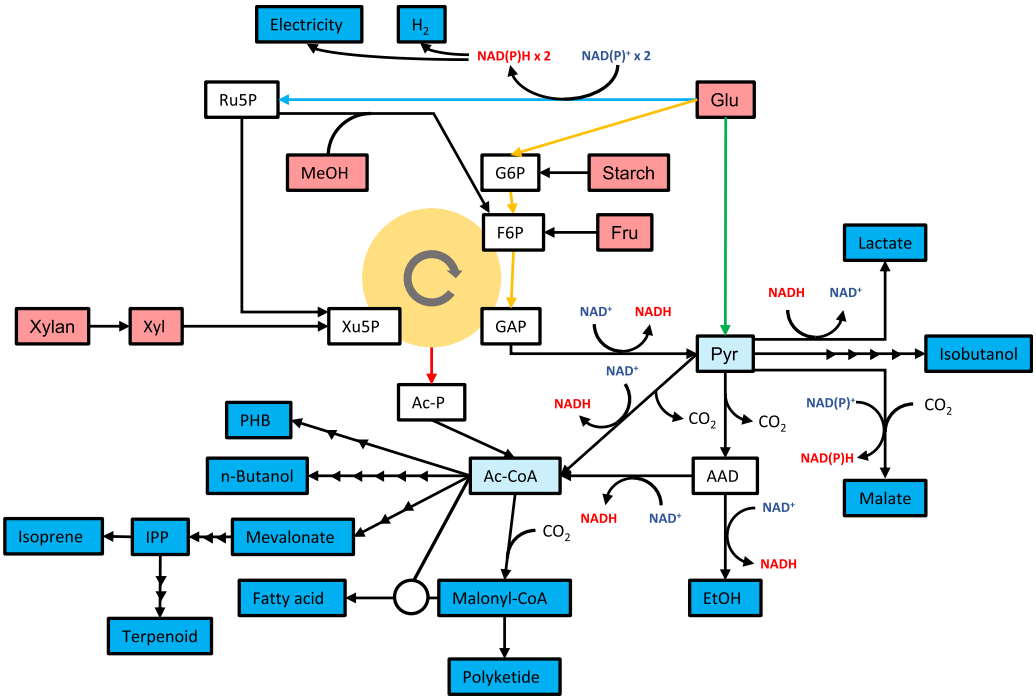

Fig. 3. Modules for chemical production from pyruvate and acetyl CoA. Overview of *in vitro* metabolic engineering modules. Red and blue boxes indicate the possible inputs and outputs, respectively. Pale blue boxes indicate pyruvate and acetyl CoA. Colored arrows indicate reactions of the oxidative pentose phosphate pathway (blue), Chimeric Embden Meyerhof Pathway (Yellow), Minimized Reaction Cascades (green) and bifid shunt (red). Yellow circle indicates the carbon rearrangement in the non-oxidative pentose phosphate pathway. Glu, glucose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; Fru, fructose; GAP, glyceraldehyde 3-phosphate; Ru5P, ribulose-5-phosphate; MeOH, methanol; Xu5P, xylulose 5-phosphate; Xyl, xylose; Pyr, pyruvate; AAD, acetaldehyde; EtOH, ethanol; Ac-P, acetyl phosphate; Ac-CoA, acetyl CoA; PHB, polyhydroxybutyrate; IPP, isopentenyl pyrophosphate.

2.2.2. *Modules extendable from acetyl CoA*

Acetyl CoA is an important precursor for the biosynthesis of bioplastics, higher alcohols, terpenoids, fatty acids, and polyketides. Acetyl CoA can be produced from pyruvate with production of NAD(P)H and CO_2 or from glucose by the non-oxidative pentose phosphate pathway and bifid shunt without carbon loss (Fig. 3).

n-Butanol production from acetyl CoA has been achieved using six reactions by two slightly different pathways, one through crotonaldehyde [33] and the other through butyryl CoA [27]. Isoprene production was demonstrated using a module starting from phosphoenolpyruvate via acetyl CoA [12]. Three molecules of acetyl CoA were converted to mevalonate via three reactions, and mevalonate was further converted to isoprene via five reactions. Polyhydroxybutyrate (PHB), known as bioplastic, has been produced from acetyl-CoA using three reactions *in vitro* by extending the PBG cycle [26].

2.2.3. *Modules extendable from other molecules*

Several studies have investigated the synthesis of complicated compounds from other key intermediates by reconstituting native biosynthesis pathways [34]. The understanding of reaction cascades in nature can be used in *in vitro* metabolic engineering for the design of modules.

For example, *in vitro* synthesis of the polyketides enterocin and wailupemycin has been performed from benzoic acid and malonyl CoA by reconstituting a complete polyketide synthase [35]. For nucleotide synthesis, the *de novo* purine nucleotide synthesis pathway was reconstituted using 28 enzymes and requiring glucose, glutamine, serine, ammonia, and CO_2 as inputs [36]. The *de novo* pyrimidine nucleotide synthesis pathway has been reconstituted using 18 enzymes and requiring glucose, aspartate, ammonia, and CO_2 as inputs [37]. The initiation and elongation reactions of fatty acid production were reconstituted starting from malonyl CoA and acetyl CoA [38]. For terpenoid synthesis, the production of amorpha-4,11-diene, a key precursor to artemisinin, was demonstrated using mevalonate as an input [39].

2.2.4. *Electron-based production*

Carbohydrates, including glucose, serve not only as starting materials for chemical production but also as good electron sources when completely oxidized. Different types of modules have been developed to efficiently utilize carbohydrate-derived electrons. For example, electrons from glucose have been utilized for electricity production by several pyrroloquinoline-quinone-dependent enzymes immobilized at the anode [30]. Zhu et al. (2014) established the system to harvest electrons as electricity by completely oxidizing maltodextrin in an enzymatic fuel cell [20]. In this module, electrons were harvested in the form of NADH and transferred to the anode by diaphorase and the electron mediator vitamin K3. This module was further refined using enzymes from different species and a different electron mediator (anthraquinone-2,7-disulfonic acid [AQDS]) [40]. As a result, the new module achieved a faraday efficiency as high as 98.8% during the production of electrons from glucose.

These electrons were further utilized for hydrogen production by coupling to NADPH and hydrogenase. Hydrogenase purified from the hyperthermophilic archaeon *Pyrococcus furiosus* was used to produce hydrogen, with NADPH obtained by the complete oxidization of glucose through the oxidative pentose phosphate cycle [31]. This concept was further developed and refined for use with other inputs and improving the efficiency [19,21,22].

2.3. Other modules related to cofactor management

One important issue in *in vitro* metabolic engineering is cofactor regeneration and balancing. The depletion or imbalance of specific cofactors slows down the reaction and finally stops the entire cascade. To overcome this problem, modules for regenerating and balancing cofactors such as ADP/ATP and $NAD(P)^+$/NAD(P)H have been developed. Furthermore, the direct phosphorylation of substrates without requiring ATP has been established.

2.3.1. ATP-dependent and -independent phosphorylation

Substrate phosphorylation is necessary for modules that use reaction cascades such as glycolysis and the pentose phosphate pathway. In general, such phosphorylation is performed using ATP, which is made in sufficient amounts in living organisms through oxidative phosphorylation. For *in vitro* metabolic engineering, two different approaches to substrate phosphorylation have been explored: ATP-dependent and ATP-independent phosphorylation (Fig. 4A).

For ATP-dependent phosphorylation, regeneration of ATP from ADP using a low-cost sacrificial substrate is critical due to the high cost of ATP. The regeneration can be performed through glycolysis or by using different phosphate donors. Although regeneration through glycolysis requires only a cheap sacrificial substrate (i.e., glucose), ATP consumption by glucokinase and phosphofructokinase has to be carefully balanced [13,33]. For phosphorylation with phosphate donors, several combinations of enzymes and sacrificial substrates are known, including acetyl phosphate/acetate kinase, phosphoenol pyruvate/pyruvate kinase, creatine phosphate/creatine kinase, and carbamoyl phosphate/carbamate kinase [41]; however, the use of these phosphate donors is not economically feasible. Alternatively, polyphosphate can be used as a phosphate donor. Polyphosphate is a linear polymer of orthophosphate with high-energy phosphoanhydride bonds and serves as a phosphate

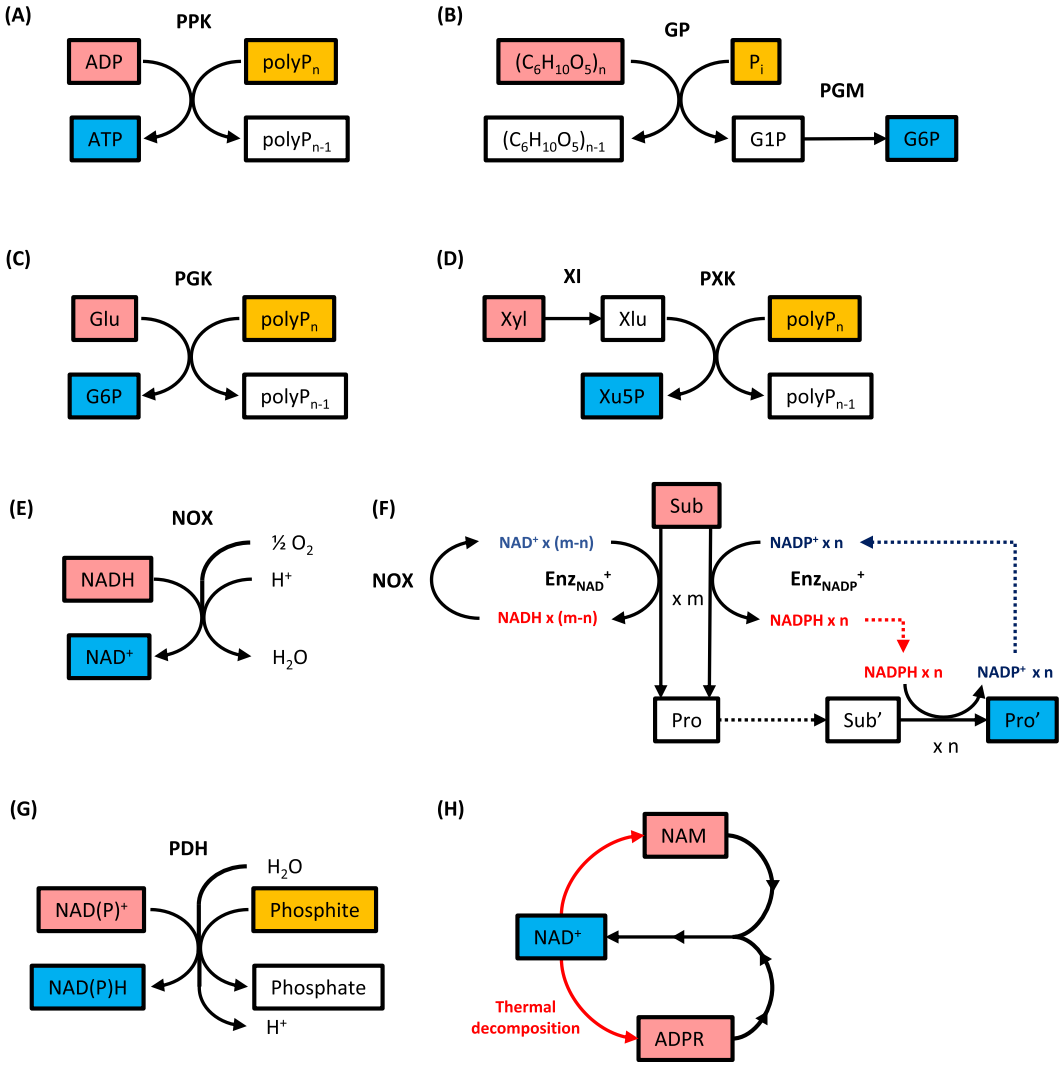

Fig. 4. Modules for cofactor balancing and phosphorylation. Modules of (A) ATP regeneration by polyphosphate, (B) direct phosphorylation of starch by inorganic phosphate (C) direct phosphorylation of glucose by polyphosphate, (D) direct phosphorylation of xylulose by polyphosphate, (E) NAD^+ regeneration by water-forming NADH oxidase, (F) the purge valve for $NADP^+$/NADPH, (G) NAD(P)H regeneration by phosphite, and (H) NAD^+ salvage synthesis. Red, blue, and orange boxes indicate the input, output, and the substrate required for the module. PPK, polyphosphate kinase; $polyP_n$, polyphosphate; GP, glucan phosphorylase; PGM, phosphoglucomutase; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; PGK, polyphosphate-dependent glucokinase; XI, xylose isomerase; PXK, polyphosphate-dependent xylulokinase; Xyl, xylose; Xlu, xylulose; Xu5P, xylulose 5-phosphate; NOX, NADH oxidase; PDH, phosphite dehydrogenase; NAM, nicotinamide; ADPR, adenosine diphosphate ribose. ENZ^*_{NAD} and ENZ^*_{NAPD} indicates enzymes that use NAD^+ or $NADP^+$ as a cofactor; m indicates the time of NADH and NADPH regeneration; n indicates the time of NADPH consumption downstream of the module.

donor for reversible conversion of ADP to ATP by polyphosphate kinases [42-44]. Because the price of polyphosphate is relatively low, the combination of polyphosphate/polyphosphate kinase is a good candidate for ATP regeneration (Fig. 4A).

Alternatively, the direct phosphorylation of substrates in an ATP-independent manner has been explored. In nature, several enzymes are known to accept inorganic phosphate as a phosphate donor for substrate phosphorylation. Using glucan phosphorylase and phosphoglucomutase, the direct phosphorylation of glucose with inorganic phosphate has been performed using starch as a starting material [21] (Fig. 4B). A new polyphosphate-dependent glucokinase, which catalyzes the direct phosphorylation of glucose with polyphosphate, has been characterized in *Thermobifida fusca* YX [45]; this enzyme was successfully integrated into hydrogen production [22] (Fig. 4C). Similarly, a new polyphosphate-dependent xylulokinase was isolated from *Thermotoga maritima* and used with xylose isomerase to convert xylose to xylulose 5-phosphate using polyphosphate [19] (Fig. 4D). GAP dehydrogenase in the classical Embden-Meyerhof pathway also incorporates inorganic phosphate into its substrate, which is subsequently utilized for ATP formation. Pyrophosphate dependent kinase such as pyrophosphate:D-fructose 6-phosphate 1-phosphotransferase exists in nature [46]. Those enzymes also have the potential to be integrated into *in vitro* metabolic pathway as one of the module for substrate phosphorylation.

2.3.2. NAD(P)$^+$/NAD(P)H regeneration and balancing

NAD$^+$/NADH and NADP$^+$/NADPH are involved in more than 2000 redox reactions. In conventional glycolysis, two NADH are produced from two NAD$^+$ per glucose molecule (Fig. 1A). Two NADPH are produced from two NADP$^+$ by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the usual oxidative pentose phosphate pathway (Fig. 2A). Accumulation of the reduced form of the cofactor, NAD(P)H, leads to depletion of the corresponding oxidized form of the cofactor, NAD(P)$^+$, which is necessary for continuous conversion of substrate. Consequently, NAD(P)$^+$ depletion leads to the termination of the cascade reaction. By contrast, some modules that have multiple reduction steps require additional NAD(P)H. Therefore, the intra-module balance of NAD(P)$^+$/NAD(P)H has to be controlled, depending on the module in use. To address this problem, several modules to regenerate NAD(P)$^+$ from NAD(P)H or produce NAD(P)H from NAD(P)$^+$ have been developed.

NADH can be converted to NAD$^+$ using a water-forming NADH oxidase and oxygen (Fig. 4E). For example, a water-forming NADH oxidase was characterized from *Lactobacillus pentosus* [47]. This enzyme was further used for the regeneration of NAD$^+$ from NADH during the conversion of glucuronate to α-ketoglutarate [15]. Several other water-forming NADH oxidases are also reported in different organisms [48,49].

As a different approach, a synthetic biochemistry molecular purge valve module for balancing the availability of NADP$^+$/NADPH was designed [50] (Fig. 4F). This purge valve is useful for the reaction module where NADPH production upstream in the reaction is in excess over its consumption downstream. For proof of concept, two pyruvate dehydrogenase complexes (PDC) with different cofactor preferences were simultaneously used for a single reaction. NADP$^+$-dependent PDC produces the NADPH required for the formation of the target product, while NAD$^+$-dependent PDC enables the continuous supply of substrates for NADP$^+$ regeneration under the NADP$^+$ limiting condition; therefore, the availability of NADP$^+$/NADPH can be maintained. The NADH produced is recycled to NAD$^+$ by a water-forming NADH oxidase, as described previously. The versatility of this purge valve module was further demonstrated for two reactions in the oxidative pentose phosphate pathway, one catalyzed by glucose 6-phosphate dehydrogenase and the other catalyzed by 6-phosphogluconate dehydrogenase [26].

A supply of redox power in the form of NAD(P)H is necessary for product formation in some modules. Several substrate/enzyme combinations have been established to regenerate NAD(P)H from NAD(P)$^+$, including formate/formate dehydrogenase, glucose/glucose dehydrogenase, hydrogen/hydrogenase, and isopropanol/alcohol dehydrogenase [51]. Of these combinations, regeneration of NAD(P)H with phosphite/phosphite dehydrogenase is most promising because of the availability and low cost of phosphite (Fig. 4G). The native phosphite dehydrogenase from *Pseudomonas stutzeri* accepts only NAD$^+$ as a redox partner [52]; however, protein engineering was used to successfully alter its cofactor preference from NAD$^+$ to NADP$^+$ [53,54]. Other phosphite dehydrogenases have been reported from different organisms [55,56].

NAD(P)$^+$ and NAD(P)H are known to have relatively low thermal stability. For *in vitro* metabolic engineering, thermal instability is problematic, especially at high temperatures, causing a decrease in the cofactor pool size [13,33]. To overcome this obstacle, the NAD$^+$ salvage module was designed to re-synthesize NAD$^+$ from its thermal decomposition products of nicotinamide and ADP-ribose using eight thermophilic enzymes [57] (Fig. 4H). The authors report that the NAD$^+$ concentration was kept constant for 15 h at 60 °C with the NAD$^+$ salvage module, while the concentration decreased by half in 6 h without the module.

3. Discussion

Developments in organic chemistry have allowed for the design of different reaction modules and their use in combination. Computer technology has developed very quickly over the past few decades, owing to its high modularity at the levels of hardware as well as software. Similarly, bio-based chemical production has progressed with the accumulation of biological knowledge and various types of reaction modules. As a platform for bio-based production, *in vitro* metabolic engineering has great potential because of its simplicity, high modularity, and suitability for quick trial-and-error experimentation. This review presents the *in vitro* metabolic engineering modules that have been designed, constructed, and shown to work functionally.

The pathway without no/little byproduct formation can be designed using *in vitro* metabolic engineering. Therefore, the stoichiometric balances as well as Gibbs free energy changes of the whole pathway can be estimated from starting substrates and final products, leading to the precise prediction of conversion yields. For example, the stoichiometric balances and Gibbs energy changes of the modules introduced in this review are summarized in Table 2. In fact, the some of the modules have achieved conversion yields above 90% for input to output compounds [13,15,19,26,40,58]. These results indicate that *in vitro* metabolic engineering is an efficient approach for bio-based chemical production. On the other hand, the product titers are still low compared to those of whole-cell-based chemical production in most of the cases. Enzyme engineering and the optimization of enzyme concentrations will be helpful toward improving product titers. The performance of a module depends greatly on the efficiency of enzymes; therefore, increasing the stability, solubility, and catalytic efficiency of enzymes by engineering will markedly improve module performance. In fact, thermostable enzymes have been intensively used in modules because of their high operational stability [13,59,60]. In addition, the activity of several enzymes has been altered using enzyme engineering, allowing for the design of artificial pathways [11,28,61]. Rational optimization of enzyme concentrations in a module will increase the efficiency of the module by balancing

Table 2
Stoichiometric balances and Gibbs free energy changes for each modules.

Reaction	Stoichiometry	Gibbs free energy change[a] [kJ/mol]	Ref
Input module			
Classical EM pathway	Glucose (aq) + 2 NAD^+ (aq) + 2 ADP (aq) + 2 Phosphate (aq) ↔ 2 Pyruvate (aq) + 2 NADH (aq) + 2 ATP (aq) + 2H_2O	−63.7 ± 3.1	–
Chimeric Embden-Meyerhof (EM) Pathway/ Minimized Reaction Cascades module	Glucose (aq) + 2 NAD^+ (aq) ↔ 2 Pyruvate (aq) + 2 NADH (aq)	−150.8 ± 3.4	[10,12]
Complete oxidation of glucose for electricity generation	Glucose (aq) + 12 NAD^+ (aq) + 6H_2O ↔ 6 CO_2 (aq) + 12 NADH (aq)	−256.4 ± 37.0	[29,39]
Non-Oxidative Glycolysis (NOG) module	D-Fructose-6-phosphate (aq) + 2 Phosphate (aq) ↔ 3 Acetyl phosphate (aq) + 2H_2O	−203.2 ± 6.2	[24]
	Glucose (aq) + ATP (aq) + 2 Phosphate (aq) ↔ 3 Acetyl phosphate (aq) + ADP (aq) + 2H_2O	−217.9 ± 6.2	–
Pentose–Bifido–Glycolysis (PBG) cycle	Glucose (aq) + 4 $NADP^+$ (aq) + 2 Phosphate (aq) ↔ 2 Acetyl phosphate (aq) + 4 NADPH (aq) + 2 CO_2 (aq)	−197.8 ± 13.1	[25]
Methanol Condensation Cycle (MCC)	2 Methanol (aq) + 2 NAD^+ (aq) + Phosphate (aq) ↔ Acetyl phosphate (aq) + 2 NADH (aq) + H_2O	−18.4 ± 12.0	[26]
Output module			
Lactate production from pyruvate	Pyruvate (aq) + NADH (aq) ↔ Lactate (aq) + NAD^+ (aq)	−27.5 ± 0.9	[12]
Malate production from pyruvate	Pyruvate (aq) + HCO_3^- (aq) + NADPH (aq) ↔ Malate (aq) + $NADP^+$ (aq) + H_2O	6.3 ± 6.5	[31]
Isobutanol production from pyruvate	2 Pyruvate (aq) + 2 NADH (aq) ↔ Isobutanol (aq) + 2 CO_2 (aq) + 2 NAD^+ (aq) + H_2O	−141.2 ± 12.5	[10]
n-Butanol production from pyruvate	2 Pyruvate (aq) + 2 NADH (aq) ↔ n-Butanol (aq) + 2 CO_2 (aq) + 2 NAD^+ (aq) + H_2O	−149.4 ± 13.0	[32]
n-Butanol production from acetyl-CoA	2 Acetyl-CoA (aq) + 4 NADH (aq) ↔ n-Butanol (aq) + 2 CoA (aq) + 4 NAD^+ (aq) + H_2O	−78.7 ± 7.1	[26]
Isoprene production from phosphoenol pyruvate	3 Phosphoenolpyruvate (aq) + 3 NAD^+ (aq) + 2 NADPH (aq) + 2H_2O ↔ Isoprene (aq) + 4 CO_2 (aq) + 3 NADH (aq) + 2 $NADP^+$ (aq) + 3 Phosphate (aq)	−365.3 ± 24.5	[11]
Hydrogen production from glucose	Glucose (aq) + ATP (aq) + 7H_2O ↔ 6 CO_2 (aq) + 12H_2 (aq) + ADP (aq) + Phosphate (aq)	−90.5 ± 78.5	[20,21,30]

[a] Gibbs free energy changes were calculated using eQuilibrator (http://equilibrator.weizmann.ac.il/) [67] with 1 mM concentration for each compounds.

substrate and cofactor supplies and allow for reducing the required amount of enzyme, which accounts for a large portion of the manufacturing costs. In fact, the optimization of enzyme concentrations is reported to improve productivity enough to match that of organism-based production, indicating the further potential of *in vitro* metabolic engineering [15,40]. Since fewer enzymatic reactions take place in *in vitro* metabolic pathways, their optimization is not as complex as for conventional *in vivo* metabolic engineering. For each enzyme of the module, the kinetic parameter such as K_m and k_{cat} can be determined experimentally. This information is helpful to predict and optimize the production rate for the whole pathway. The modeling-based approach can be a powerful tool for addressing the issue of optimization. For example, Rollin et al. (2015) determined the kinetic parameter of enzymes in the pathway producing hydrogen from glucose, and used those parameters for the optimization of the production rate through the whole pathway [22]. As a result, they identified the bottle neck reactions, and the hydrogen productivity was improved three fold to 32 mmol $H_2 \cdot L^{-1} \cdot h^{-1}$ by optimizing enzyme amounts. Ensemble Modeling for Robustness Analysis (EMRA) was used to analyze the effects of enzyme concentrations in the present modules. EMRA demonstrated the potential for investigating the stability of modules without a priori knowledge of specific enzyme parameter values, drawing conclusions consistent with those attained from the actual experiments [62].

In vitro metabolic engineering enables us to design the pathway by connecting the input and output module without considering the inhibition at the level of transcription and translation; however, several constrains exists upon the pathway construction with different modules. For example, a certain reaction in a module can be allosterically inhibited by a compound/intermediate of other modules. Ye et al. (2012) reported that the lactate dehydrogenase from *T. thermophilus* is inhibited by NAD^+ and cannot be used with Chimeric EM pathway for the production of lactate from glucose (Fig. 1A) [13]. They solved this problem by employing an alternative enzyme, malate/lactate dehydrogenase (MLDH) from *T. thermophilus*, and demonstrated the lactate production through Chimeric EM pathway without inhibition by NAD^+. The natural substrate of MLDH is unknown and it may more preferably accept a metabolite other than pyruvate in physiological conditions; however, *in vitro* metabolic pathways involves only a limited number of metabolites, and thus an enzyme with a broad substrate specificity is also available. The cofactor balancing between the input module and output module is also necessary to be considered upon combining the input and output module. For example, the production of PHB from glucose connecting Pentose-Bifido-Glycolysis (PBG) cycle and the PHB output module produces two NADPH in PBG cycle, and consume one NADPH in the PHB module (Figs. 2C and 3), therefore one NADPH is necessary to be regenerated to $NADP^+$ by other module. This cofactor imbalance was solved by the synthetic biochemistry molecular purge valve module (Fig. 4F).

The discovery of enzymes with new activities increases the flexibility of pathway design [28,63]. The combination of bioinformatics and molecular modeling can be helpful to rationally identify an enzyme catalyzing the desired reaction [64]. In addition, large sequencing data from different environments enable us to use the genetic information of 'microbial dark matter' [65]. These data can be a great source of enzymes with newly-described activities. Conventional screening-based bioprospecting will, of course, continue to be an important approach to acquire novel biocatalysts. Higher organisms, including plants, insects, and animals, which have been less explored than microorganisms, will also be promising sources of novel enzymes in the future [66].

Many improvements are still possible for *in vitro* metabolic engineering. Owing to its high modularity and relative simplicity, *in vitro* metabolic engineering will be advanced by the combined use of technologies such as enzyme engineering, *in silico* modeling, and bioprospecting, thereby refining this practical approach to bio-based production.

Competing interests

The authors declare no competing financial interest.

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