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Review

Metabolic engineering strategies for microbial utilization of methanol

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ABSTRACT

The increasing shortage of fossil resources and environmental pollution has renewed interest in the synthesis of value-added biochemicals from methanol. However, most of native or synthetic methylotrophs are unable to assimilate methanol at a sufficient rate to produce biochemicals. Thus, the performance of methylotrophs still needs to be optimized to meet the demands of industrial applications. In this review, we provide an in-depth discussion on the properties of natural and synthetic methylotrophs, and summarize the natural and synthetic methanol assimilation pathways. Further, we discuss metabolic engineering strategies for enabling microbial utilization of methanol for the bioproduction of value-added chemicals. Finally, we highlight the potential of microbial engineering for methanol assimilation and offer guidance for achieving a low-carbon footprint for the biosynthesis of chemicals.

1. Introduction

Large-scale industrial manufacturing has led to the shortage of fossil resources (such as coal and fossil oil) and environmental pollution (such as greenhouse effect and air pollution), limiting the sustainable development and breaking the ecological balance of the earth. Thus, it is urgent to find alternative raw materials that can replace fossil resources and promote a major change in traditional industrial manufacturing. As one of renewable resources, the production of chemicals from methanol has many advantages. (i) Methanol is easy to obtain. Methanol can be prepared from methane, carbon dioxide (CO_2) or other industrial waste gases. (ii) Methanol is easy to store. Methanol exists in liquid form under room temperature that is convenient for transportation and storage. (iii) The price of methanol is cheap. The simple production process of methanol results in a lower market price than other sugars (or carbon sources) for fermentation. (iv) The purity of methanol is high. Compared with other raw materials (such as straw and molasses), methanol is a single substrate that can be completely converted to chemicals in fermentation. (v) Methanol is one of highly reductive compounds. Methanol contains 50% or more electrons per carbon than carbohydrates [1–3]. However, methanol industrial manufacturing is not environmentally friendly. To deal with this issue, microbial biosynthesis based on methylotrophic strains provides an alternative strategy for methanol conversion.

Recently, many high-value biochemicals have been obtained by natural methylotrophic microorganisms, such as 3-hydroxybutyrate produced by *Methylobacterium rhodesianum* [4], L-glutamate produced by *Bacillus methanolicus* MGA3 [5], and acetone produced by *Acetobacterium woodii* [6]. However, there still remain many critical issues in using native methylotrophs, such as unclear genetic background, few reliable tools for genetic manipulation, low efficiency of methanol utilization, and narrow product spectrum [7,8]. As an alternative approach to solve these issues, industrial chassis such as *Escherichia coli, Corynebacterium glutamicum*, and *Saccharomyces cerevisiae* can be designed and engineered to achieve a complete conversion from non-methylotroph to methylotroph. After fine-turning or reconstructing the metabolic network of industrial chassis, these synthetic methylotrophic microorganisms can grow on methanol and produce high value-added biochemicals [3,9,10]. For example, *S. cerevisiae* was engineered to produce fatty acids from methanol by integrating the key enzymes of xylulose monophosphate cycle, such as alcohol oxidase, dihydroxyacetone synthase and dihydroxy-acetone kinase [11]. However, when natural methanol assimilation pathways are introduced into non-native methylotrophs, microbial metabolic burden usually limits the efficiency of microbial biosynthesis. Thus, there is considerable interest in developing synthetic methanol assimilation pathways and adaptive laboratory evolution for rewiring methanol assimilation pathways to enhance methanol assimilation efficiency [2,12]. For example, when *C. glutamicum* MX-11 was evolved under the increased methanol, the obtained mutants showed a large improvement in methanol tolerance [13].

To better understand the recent developments in engineering non-native methylotrophic microorganisms, we describe natural and

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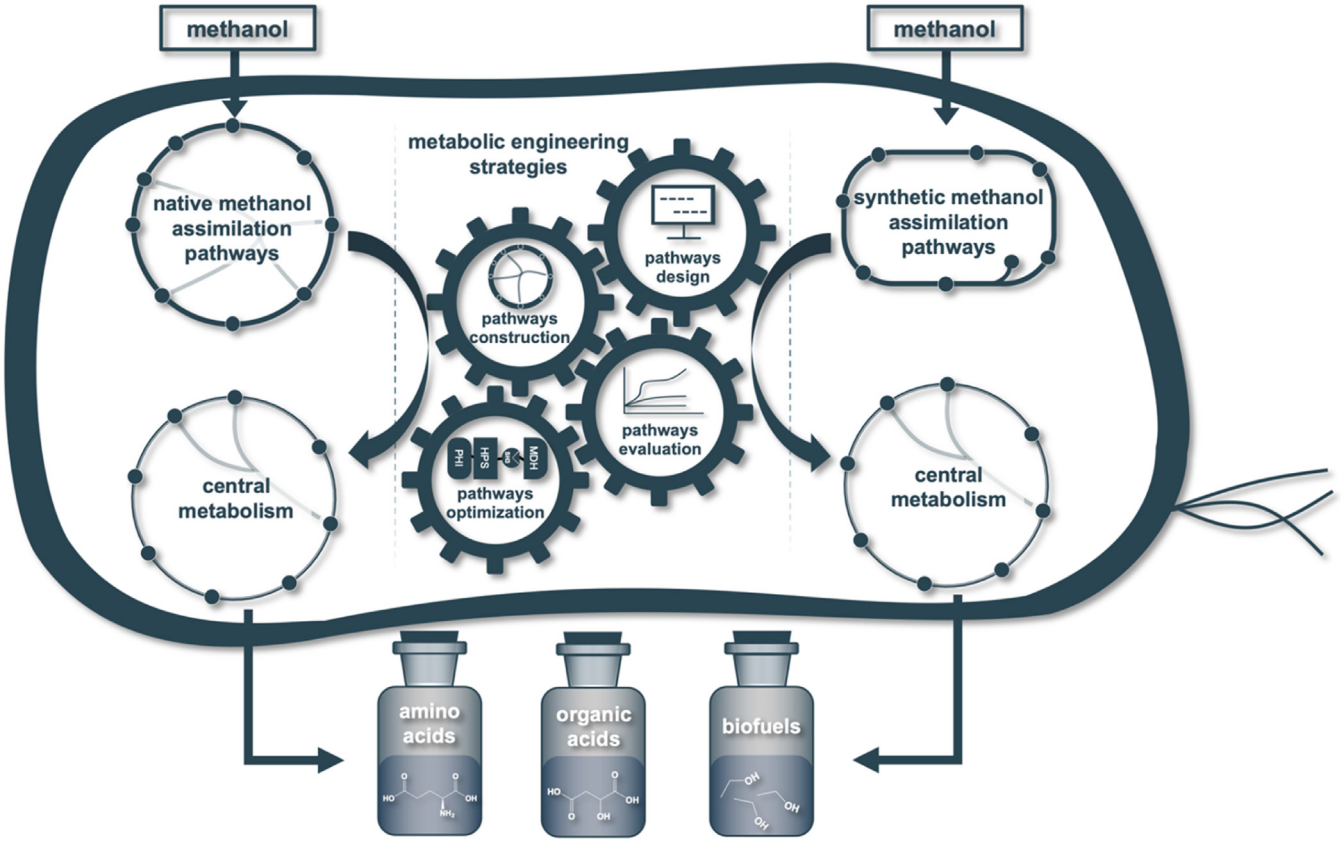

Fig. 1. Methanol-based biomanufacturing. Methanol can be converted into high value-added biochemicals such as amino acids, organic acids, and biofuels by engineering native or synthetic methanol assimilation pathways in industrial hosts.

synthetic microbial hosts and metabolic pathways for methanol utilization, and then advances in metabolic engineering strategies for converting methanol to high-value chemicals are discussed in this review (Fig. 1). Finally, we also highlight future perspectives of methanol-based biomanufacturing for achieving a low-carbon footprint for the biosynthesis of chemicals.

2. Microbial hosts for methanol utilization

It is necessary to develop next-generation platforms for biotechnological application using native methylotrophs (e.g., acetogens and methylotrophs) that can grow naturally on one-carbon substrates such as methanol. Compared with industrial chemical manufacturing of methanol, bio-catalysis is more environmentally friendly. However, the engineered native methylotrophs have produced value-added products at low rates and titers so far (Fig. 2(A) and (B)), because genetic tools and specific metabolic network are still limited. Thus, the introduction of methanol assimilation pathway into industrial hosts such as *E. coli* and *C. glutamicum* has received an increasing attention (Fig. 2(D) and (E)).

2.1. Acetogens

Acetogens, anaerobic bacteria, can use the acetyl-CoA pathway for the reductive synthesis of acetyl-CoA from CO_2, namely, reductive acetyl-coenzyme A (rAcCoA) pathway (Fig. 2(A)). This rAcCoA pathway is a terminal electron-accepting and energy-conserving process, which is also used for biomass synthesis from CO_2 fixation [14]. Many biofuels and biochemicals such as hexanol and butyrate can be generated by acetogens using syngas (H_2, CO_2, and CO) as carbon and energy sources through the rAcCoA pathway. According to carbon and energy sources, most of acetogenic bacteria (also utilizing methanol) fall into three categories: (i) acetogens grow with H_2 and CO_2 as energy and carbon sources, such as *Acetoanaerobium carbinolicum, Clostridium methoxybenzovorans*, and *Sporomusa paucivorans*; (ii) acetogens grow with H_2, CO_2, and CO as energy and carbon sources, including *Acetoanaerobium bakii, Moorella thermoautotrophica*, and *Sporomusa termitida*; and (iii) acetogens grow with CO as carbon and energy sources, including *Clostridium formicaceticum* [15]. Different types of acetogens obtain energy in different ways. When acetogens grow autotrophically on CO or syngas, the oxidation of CO to CO_2 produces the reduced ferredoxin. In addition, when acetogens grow on H_2 and CO_2, electron-bifurcating [Fe-Fe]- or [Ni-Fe]-hydrogenases can convert two H_2 to reducing power, with the low-potential electron pairs from H_2 for NADH (exergonic reaction) to facilitate the high-potential electron pairs for the reduced ferredoxin (endergonic reaction) [16]. This reduced ferredoxin can be utilized to drive a transmembrane ion gradient for ATP generation by the energy-conserving respiratory enzymes, Rnf (ferredoxin: NAD^+ reductase complex) and Ech (ferredoxin-dependent hydrogenase complex) to reduce NAD^+ and protons, respectively [17]. Acetogens display high energetic efficiency for the conversion of substrates to products, characteristically 70–90% [18]. Most acetogens, such as *Acetobacterium*, can produce acetate as the sole end product through the rAcCoA pathway which is an ATP-yielding reaction for the conversion of acetyl-CoA to acetate by phosphotransacetylase and acetate kinase [15], and is the only metabolic route to provide energy to the cell for assimilating and reducing CO_2 and/or other one-carbon substrates [18]. Apart from to acetate, acetogens can also produce other high-value biochemicals using one-carbon substrates. For example, *Clostridium carboxidivorans* can produce hexanoate and hexanol using CO as carbon source [19]; *Eubacterium limosum* is known for their ability to produce hexanoate using methanol as carbon source [20]. Despite several advantages, the acetogens-based

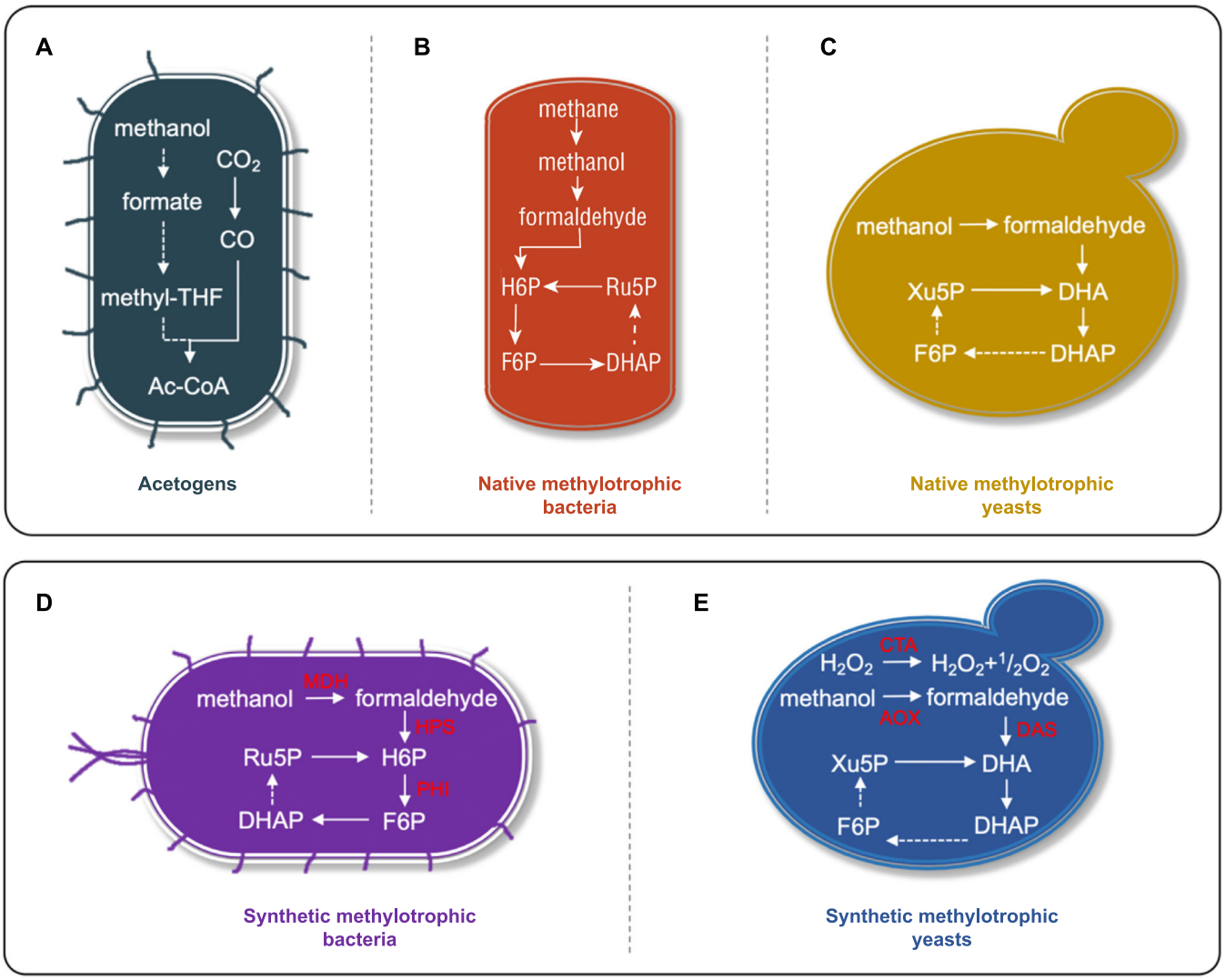

Fig. 2. Microbial hosts for methanol utilization. (A), (B), and (C) native methylotrophs. (D) and (E) synthetic methylotrophs. The enzymes in red are heterologously expressed in chassis cells. H6P, hexulose-6-phosphate; F6P, fructose-6-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate.; MDH, methanol dehydrogenase; HPS, 3-hexulose-6-phosphate synthase; PHI, 6-phospho-3-hexuloisomerase; CAT, catalase; AOX, alcohol oxidase; DAS, dihydroxyacetone synthase.

bioproduction has distinct drawbacks: (i) the product spectrum is still limited. For example, acetogenic production is constrained to producing chemicals such as acetate, butyrate, ethanol, butanol and 2,3-butanediol [21]; (ii) the low growth rate also limits the overall productivity of acetogens when compared with aerobic microbes, such as *Cupriavidus necator* [22] and *S. cerevisiae* [23], due to the low microbial resistance to ethanol or other products [24].

2.2. Methylotrophs

2.2.1. Methylotrophic bacteria

Methylotrophic bacteria mainly contain two types, type I (methane-utilizing methanotrophs) and type II (non-methane-utilizing methylotrophs) (Fig. 2(B)). Type I strains have three features: (i) intracytoplasmic membranes throughout the cell; (ii) the ribulose monophosphate (RuMP) cycle for carbon assimilation; and (iii) the phospholipid fatty acids in the length of 14 and 16 carbons, such as the genera of *Methylobacter, Methylococcus, Methylomicrobium, Methylomonas* and *Methylosoma* [25–27]. Type II strains are characterized by: (i) intracytoplasmic membranes aligned along the periphery; (ii) the serine pathway for carbon assimilation; and (iii) the phospholipid fatty acids in the length of 18 carbons, including the genera of *Methylocystis, Methylosinus, Methylocella* and *Methylocapsa* [25,28,29]. Type I strains have the ability to grow with methane as sole carbon and energy source through the dissimilation and assimilation pathways. One of key steps in methane utilization is that methane is oxidated into methanol for driving biomass production by soluble methane monooxygenase with NADH as cofactor and oxygen as terminal electron acceptor [30,31]. Therefore, type I strains not only play a key role in the global carbon cycle, but also can help to sustain diverse microbial communities through the conversion of methane to complex organic compounds [25]. Type II strains, such as pink-pigmented *Methylobacterium* strains, are able to grow on methanol or other one-carbon substrates but not on methane [32–34]. No matter in type I or type II strains, methanol is always oxidized to formaldehyde by three enzymes, quinoprotein methanol dehydrogenases (qMDH), NAD^+-dependent methanol dehydrogenases (nMDH) [35,36], and N,N-dimethyl-4-nitrosoaniline oxidoreductase (MNO) [32]. Periplasmic qMDH consists of two different subunits forming $\alpha_2\beta_2$ structure and contains the cofactor pyrroloquinoline quinone (PQQ). The reduced PQQ prosthetic group can capture electrons from methanol oxidation, transport them to *c*-type cytochrome and then proceed through electron transport chain until reaching a

membrane-bound oxidase for energy regeneration [36,37]. nMDHs have low affinity towards methanol. Under standard conditions, microbial methanol oxidation is favored in utilizing qMDHs but not nMDHs, as indicated by a positive reaction Gibbs energy [36]. However, when intracellular formaldehyde is rapidly consumed and maintained at low levels, nMDHs are more active to oxidize methanol and generate NADH for metabolite production [35]. Thus, it is widely used in methylotrophic bacteria for biosynthesizing amino acids, 1-butanol and polyhydroxyalkanoate through the ribulose monophosphate cycle or the serine cycle [38].

2.2.2. Methylotrophic yeast

Methylotrophic yeasts are capable to grow solely on methanol (Fig. 2(C)). Methanol utilization follows a similar metabolic pathway in all methylotrophic yeasts, and several enzymes are localized in peroxisomes, which can proliferate during growth in methanol. *Ogataea philodendra* and *Pichia pastoris* are the common methylotrophic yeasts that grow on extract of woods and other pectic material [39]. This type of yeast is characterized by unique methanol metabolism with the expression of alcohol oxidase (AOX), dihydroxyacetone synthase (DAS), formate dehydrogenase (FDH), and dihydroxyacetone kinase (DAK) genes [39]. Thus, methylotrophic yeasts always use AOX for the oxidation of methanol, in which methanol is converted to formaldehyde with hydrogen peroxide as a by-product. Hydrogen peroxide is then decomposed into water and oxygen by catalase (CAT), while formaldehyde from methanol oxidation can be converted into xylulose 5-phosphate by DAS and then entering to the central metabolism [40]. In addition, the dissimilation of formaldehyde results in the generation of NADH, which leads to the production of ATP via mitochondrial oxidative phosphorylation [41]. Recently, methylotrophic yeasts have been developed as cell systems for heterologous protein expression [42]. For example, when bovine enterokinase was heterologously expressed in *P. pastoris*, the production of bovine enterokinase was up to 6.3 mg/L [43].

To solve problems such as low production rates and titers with acetogens and methylotrophs, future work can be carried out to expand the product spectrum and improve microbial resistance under extreme conditions: (i) developing novel genetic manipulation tools to rewire metabolic flux toward target products in acetogens based on their complex metabolic network; (ii) inhibiting the production of metabolic byproduct to reduce metabolic burden of acetobacter through deleting redundant genes via CRISPR/Cas9-based technologies; (iii) adaptive evolution makes acetobacter tolerant to extreme environments; (iv) construction of a compartmentalized methanol assimilation pathway helps to improve methanol utilization and reduce intermediate metabolites toxicity.

2.3. Synthetic methylotrophs

It is feasible to develop next-generation platforms for biotechnological applications based on native methylotrophic organisms that can naturally utilize methanol for cell growth. However, engineering native methylotrophs to produce chemicals has been limited so far, due to the fact that the difficulty of understanding and engineering the complex metabolic processes of all native methylotrophic microorganisms. Therefore, there is considerable interest in the application of synthetic biology approach to integrate methanol assimilation pathways into more tractable and industrially relevant hosts such as *E. coli, C. glutamicum*, and *S. cerevisiae* (Fig. 2(D) and (E)) [3]. This bioprocess technology using synthetic methylotrophs has many advantages: (i) methane and methanol can serve as the sole carbon and energy source for producing high-value chemicals avoiding the undesirable competition with the food-derived feedstocks, such as sugars; (ii) methane and methanol can be converted biologically into value-added and even novel products that is difficult to manufacture or even inaccessible to chemical conversion; (iii) synthetic methylotrophs can compensate the limitations of natural methylotrophic microorganisms. For example, *E. coli, C. glutamicum*, and *S. cerevisiae* are the tractable model hosts that provide the most flexible, biologically well-understood, and genetically tractable strategies for further engineering; (iv) synthetic methylotrophy has a major potential to assimilate and convert one-carbon substrates into high-value bio-commodities [44].

2.3.1. Escherichia coli

In theory, it is straightforward to introduce the missing enzymes in industrial hosts for the construction of synthetic methylotrophic microorganisms. The RuMP cycle is more appealing for engineering methylotrophy due to its relatively high energy efficiency and compatibility with *E. coli* metabolism. Thus, recent studies have been successfully introduced RuMP cycle into *E. coli*. For example, the heterologous expression of MDH, hexulose-6-phosphate synthase (HPS) and 6-phospho-3-hexuloisomerase (PHI) in *E. coli* to establish the RuMP cycle, eventually achieved up to 40% incorporation of methanol into central metabolites [45]. Moreover, fine-tuning carbon fluxes through methanol assimilation pathway and central metabolic pathways such as the pentose phosphate pathway, may pave the way for enhancing the methanol assimilation efficiency of synthetic methylotrophs [3]. For example, the engineered *E. coli* CFC526.0, in which the pentose phosphate pathway was disrupted by deleting *rpiAB* and *pfkA*, methanol utilization genes (*mdh, hps*, and *phi*) were installed, and *gapA* with *gapC* were replaced, was evolved to obtain a synthetic methylotroph with methanol as the sole carbon source for cell growth [46]. However, the carbon can be lost during the formation of acetyl-CoA in the RuMP cycle. The modified serine cycle constructed in *E. coli* might resolve carbon lost and enhance ethanol production by 62% [47].

2.3.2. Corynebacterium glutamicum

In addition to *E. coli*, methanol assimilation pathways have also been integrated into *C. glutamicum*, another industrial host with broad substrate profiles and complete genetic tools [48,49]. Through genetic operation and carbon flux regulation, the optimal methanol assimilation pathway, the RuMP cycle, was integrated in *C. glutamicum* to enable conversion of xylose into ribulose 5-phosphate (Ru5P) but deactivate the conversion of Ru5P to ribose 5-phosphate. Moreover, introducing nMDH from *Bacillus stearothermophilus*, HPS and PHI from *Bacillus methanolicus* could obtain a methanol-dependent *C. glutamicum* MX-10, and then methanol-dependent growth was improved by 20-fold via adaptive laboratory evolution. Finally, the ^{13}C-labeling experiments demonstrated that ^{13}C-labeling methanol up to 63% was assimilated into building blocks, high-energy carriers, cofactors, and biomass [50]. Notably, deleting some dehydrogenase genes such as *adhE, ald* and *FdhF* in *C. glutamicum* could avoid methanol oxidation and toxicity, thereby improving the efficiency of methanol assimilation [51].

2.3.3. Pseudomonas putida

P. putida has the ability of formaldehyde tolerance [52]. Thus, it is expected to transform this microbe into a non-native methylotrophic strain. For example, a solvent-tolerant *P. putida* was engineered to utilize methanol and formaldehyde as auxiliary substrates by introducing the HPS and PHI from *Bacillus brevis* to construct a pathway for assimilating methanol oxidation intermediate formaldehyde. The biomass yield of this engineered strain was increased from 35% without formaldehyde to 91% with mixture formaldehyde (60%) and glucose as co-substrate. Moreover, the endogenous enzymes for methanol oxidization allowed the engineered *P. putida* S12 to growth when replacing formaldehyde with methanol, resulting in an 84% biomass yield [53]. *P. putida* has the ability to efficiently utilize a variety of carbon sources without the formation of byproducts, even when exposed to stress conditions [54]. Thus, the engineered *P. putida* will be an efficient platform for methanol conversion.

2.3.4. Bacillus subtilis

B. subtilis is generally recognized as safe, and thus always used as a modal strain in the large-scale industrial chemical manufacturing [55].

B. subtilis possesses endogenous HPS and PHI which can enable these species to assimilate formaldehyde [56]. Thus, it is suitable for reconstructing metabolic network to obtain synthetic methylotrophs. Recently, a series of strategies have been adopted to construct a synthetic methylotrophic *B. subtilis* M, including the heterologous expression of MDH, enhancement of the endogenous HPS, PHI, and NADH dehydrogenase expression, deletion of glucose-6-phosphate isomerase, expression of key enzymes at both the translational and transcriptional levels, stabilization of the key enzyme expression through a dual-system, and improvement of the catalytic activity of MDH with a recycling strategy for NAD^+. After cultivating in M9 medium with 10 g/L methanol, 4.02 g/L methanol was utilized by the synthetic *B. subtilis* [57]. The construction of this synthetic methylotrophic *B. subtilis* will be helpful to reduce the use of traditional sugar as carbon sources, such as xylose and glucose.

2.3.5. Saccharomyces cerevisiae

S. cerevisiae is now used widely to produce a large number of value-added bioproducts, such as organic acids, fatty acids, and biofuels [58]. *S. cerevisiae* as chassis host provides significant advantages for methanol conversion due to its robust tolerance to high methanol concentration and other extreme conditions [11,59,60]. According to these features, alcohol oxidase (AOX), catalase (CAT), dihydroxyacetone synthase (DAS) and dihydroxyacetone kinase (DAK) were introduced into the chromosome of *S. cerevisiae* to construct a complete xylulose monophosphate (XuMP) cycle for methanol assimilation. This engineered strain showed 1.04 g/L consumption of methanol, and 0.26 g/L production of pyruvate [61]. However, the XuMP cycle may not be the optimal methanol assimilation pathway constructed in *S. cerevisiae*. Recently, by introducing the enzymes MDH, HPS and PHI into *S. cerevisiae*, a synthetic RuMP cycle was formed, leading to a better growth than that of the XuMP cycle [62]. To further improve the methanol-utilizing efficiency, the engineered *S. cerevisiae*, in which the glyoxylate-serine pathway is essential to relieve cytoplasmic formaldehyde toxicity, achieved an improvement in methanol-utilizing efficiency after adaptive laboratory evolution [63]. Nevertheless, the low growth rate, high cost of cultivation, and less flexible metabolism remain the disadvantage of engineered yeast compared with synthetic methylotrophic bacteria [60].

The synthetic methylotrophs can be used as production platform to obtain diverse biofuels and biochemicals, such as acetone, ethanol, butanol, succinate, glutamate and cadaverine, which paves the way for the bio-catalysis of renewable one-carbon resources such as methanol [12]. However, a lot of laboratory-synthesis methylotrophs can grow normally with methanol and other carbon sources such as glucose and xylose. Hence, it is necessary to develop new strategies for engineering these strains that can grow solely on methanol. Engineering methanol-utilizing microbial cell factories requires novel tools of synthetic biology [64]. For instance, heterologous gene expression is an important tool, such as CRISPR/Cas9 technology, for introducing the genes of methanol utilization with high efficiency into industrial host strains [65]. In addition, promoter and protein engineering should be employed to regulate the expression of these heterologous genes and the function of their relative enzymes, respectively [66,67]. Finally, adaptive evolution strategy might be another important strategy to accomplish the goal that synthetic methylotrophs grow solely on methanol [3].

3. Microbial pathways for methanol utilization

Native methylotrophic microorganisms possess several methanol assimilation pathways, including the reductive acetyl-CoA (rAcCoA) pathway, the ribulose monophosphate cycle, xylulose monophosphate cycle, and the serine pathway (Fig. 3(A)). However, the relatively low efficiency of these pathways limits the production of value-added biochemicals in methylotrophs. Thus, researches on engineering methanol assimilation pathways are increasing attention (Fig. 3(B)).

3.1. Natural methanol-utilizing pathways

Methylotrophic microorganisms are a diverse microbiota that possess a great number of specialized enzymes that enable them to grow on the reduced one-carbon substrates, such as methanol [1]. To date, the natural pathways for methanol assimilation are found in methylotrophs, including the reductive acetyl-CoA pathway, the ribulose monophosphate (RuMP) cycle, the xylulose monophosphate (XuMP) cycle, the glyoxylate regeneration pathway (GLRP, also known as the ethylmalonyl-CoA cycle), and the serine pathway [12,68,69].

3.1.1. The reductive acetyl-CoA pathway

The rAcCoA pathway (also known as Wood-Ljungdahl pathway, WLP) is firstly occurred and elucidated in some acetogenic bacteria, such as the genera of *Clostridium* [70] and *Spirochaeta* [71]. This is a less energy-consuming pathway compared with other natural CO_2 fixation pathways, costing only one ATP equivalent [16]. The key enzymes in the rAcCoA pathway are carbon monoxide (CO) dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex and formate dehydrogenase (FDH) that are sensitive to oxygen. According to these key enzymes, the rAcCoA pathway can be divided into three essential steps: (i) one CO_2 molecule is reduced to form formate by FDH, and then formate is condensed with tetrahydrofolate (THF) to form 5-Methyl-THF via the THF cycle; (ii) another one CO_2 molecule is reduced to CO by bifunctional enzyme CODH/ACS; (iii) 5-methyl-THF transfers methyl to corrinoid iron sulfur protein (CoFeSP) by methyltransferase and then reacts with CO by CODH/ACS to form a molecule of acetyl-CoA. Acetyl-CoA is converted into acetate by phosphotransacetylase and acetate kinase. The rAcCoA pathway is the only reductive pathway that can capture one-carbon substrates and simultaneously generate ATP by transforming acetyl-CoA to acetate using acetate kinase [16]. For energy sources, H^+-ATPase can provide ATP and the energy-converting [Ni-Fe] hydrogenase can provide reduced ferredoxin (Fd_{red}^{2-}) [68]. In addition, some methanogens (e.g., *Methanobacteriales, Methanococcales*, and *Methanomicrobiales*) without cytochromes can also grow via another rAcCoA pathway with tetrahydromethanopterin (THMPT) as cofactors to capture CO_2, in which Na^+-ATPase can provide ATP and the flavin-based electron bifurcation can provide Fd_{red}^{2-} [68]. The rAcCoA pathway is presented in acetogens, but acetogens can produce a limited spectrum of final chemicals, such as acetate [16]. Due to the energy-saving profile of the rAcCoA pathway, it may be suitable for introducing this pathway into industrial chassis for the production of various biochemicals by entering into central metabolism with acetyl-CoA. In addition, the application of artificial energy systems such as light-driven photosynthesis systems [72] and nanomaterials [73] can replenish the “missing energy” for the heterologous rAcCoA pathway.

3.1.2. The RuMP and XuMP cycle

The RuMP cycle is originally discovered in methylotrophic bacteria, such as *Bacillus methanolicus* and *Methylophilus methylotrophus*, and methylotrophic yeasts such as *Pichia pastoris, Hansenula polymorpha*, and *Candida boidinii* can metabolize methanol through the XuMP cycle [69]. In the RuMP cycle, formaldehyde is condensed with ribulose 5-phosphate (Ru5P) to form hexulose-6-phosphate (H6P) by hexulose phosphate synthase (HPS). H6P is subsequently transformed into fructose 6-phosphate (F6P), which can enter into the central metabolism for the generation of biomass. Similarly, in the XuMP cycle, all carbon flux required for biomass formation is assimilated from formaldehyde. In this cycle, formaldehyde is initially condensed with xylulose 5-phophate (Xu5P) to form glyceraldehyde-3-phosphate (G3P) by dihydroxyacetone synthase (Das), and then G3P is converted into dihydroxyacetone (DHA). Finally, DHA can be further transformed into dihydroxyacetone phosphate (DHAP), which can enter into the central metabolism by dihydroxyacetone kinase (DAK) [69,74]. However, the RuMP and XuMP cycle are not the efficient pathways for methanol assimilation, due to carbon and energy loss. In the future, some strategies

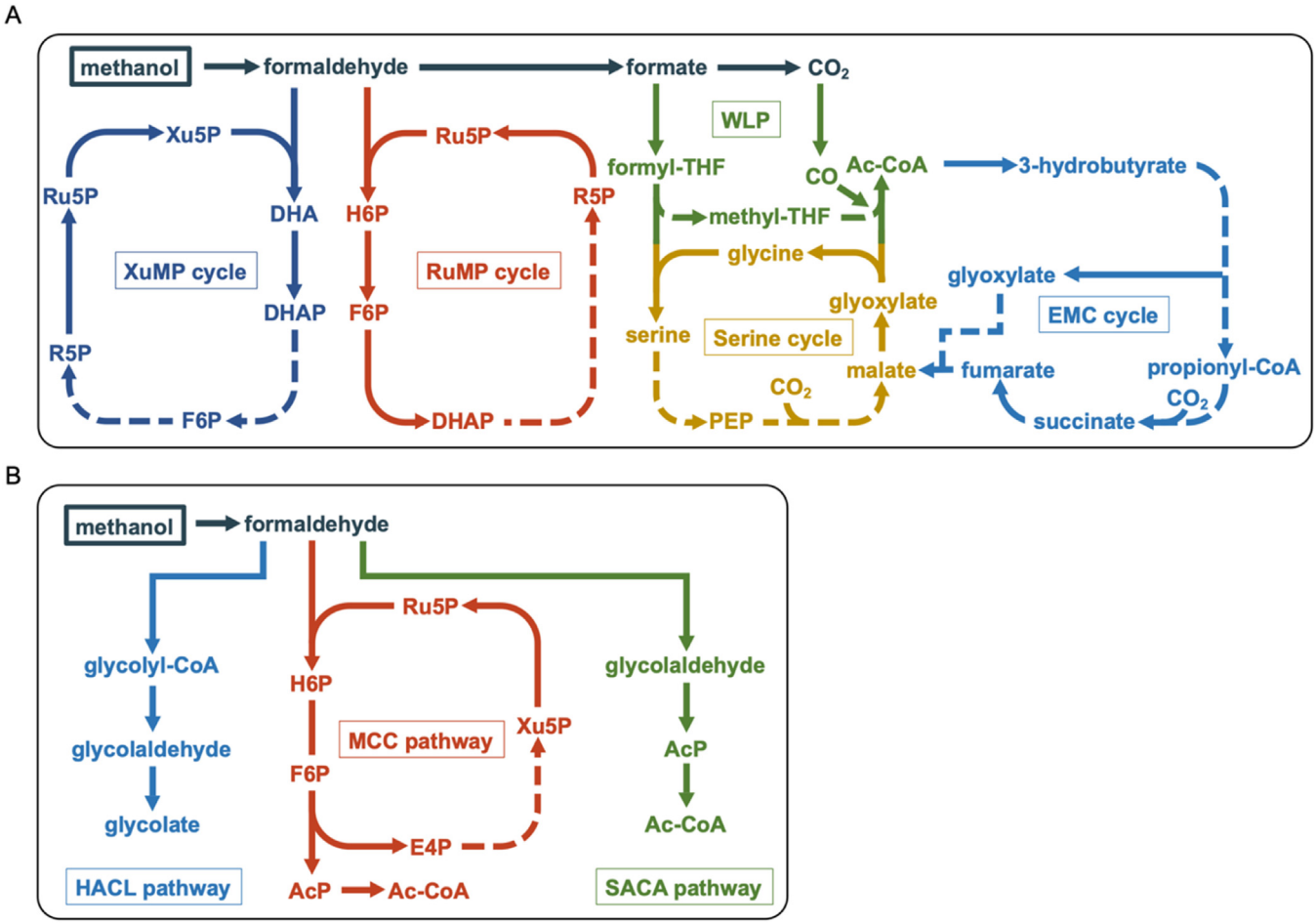

Fig. 3. Natural and synthetic methanol assimilation pathways. Natural methanol assimilation pathways (A), and synthetic methanol assimilation pathways (B). Dashed lines indicate the omitted reactions. Ac-CoA, acetyl-CoA; AcP, acetyl-phosphate; E4P, erythrose-4-phosphate; Xu5P, xylulose-5-phosphate; THF, tetrahydrofolate.

can be taken to solve these issues, such as discovering efficient methanol dehydrogenase [75,76], engineering key enzymes [77] to improve the methanol-utilizing rate, and constructing the minimized pathways to decrease carbon loss by combining CO_2 fixation with methanol assimilation pathways [78].

3.1.3. The serine pathway and ethylmalonyl-CoA (EMC) cycle

Some methylotrophic bacteria such as *Methylobacterium extorquens* can utilize formaldehyde through the serine pathway. In this pathway, formaldehyde is transformed into methylene-THF with THF as cofactor, involving two different ways: (i) condensing formaldehyde with THF to generate methylene-THF directly; (ii) oxidating formaldehyde to formate by methylene tetrahydromethanopterin-dependent enzymes and then assimilating formate to form methylene-THF by THF-dependent enzymes. After that, methylene-THF is condensed with glycine to form serine, and then undergoes a series of reactions to generate phosphoenolpyruvate (PEP). PEP is converted into oxaloacetate by PEP carboxylase and then to malate. Lastly, the serine cycle is completed by the glyoxylate regeneration pathway: the product of malyl-CoA cleavage, glyoxylate, is converted back into glycine, and the other cleavage product, acetyl-CoA, undergoes a series of reactions to regenerate the second molecule of glyoxylate [79] or the formation of biomass [1,80,81]. The ^{13}C-labeling experiment with *M. extorquens* AM1 indicates that at least 47% of the carbon of biomass comes from CO_2 [80]. In EMC cycle, malyl-CoA/methylmalyl-CoA lyase has the ability for cleaving methylmalyl-CoA into propionyl-CoA and glyoxylate. Then, propionyl-CoA is condensed with CO_2 to from methylmalonyl-CoA by propionyl-CoA carboxylase, eventually converting into succinate and then to malate for serine cycle [81]. However, hydroxypyruvate reductase may not be the best enzyme to operate the natural serine cycle in *E. coli*, as its side reactivity for producing glyoxylate reduces the efficiency of methanol assimilation.

3.2. Synthetic methanol-utilizing pathways

To release metabolic burden and reduce energy consumption, many new artificial pathways for methanol assimilation have been developed so far, such as the methanol condensation cycle, 2-hydroxyacyl CoA lyase pathway, and synthetic acetyl-CoA pathway.

3.2.1. The methanol condensation cycle (MCC)

Natural methanol assimilation pathways are often restricted by many deficiencies, such as energy and carbon loss during the formation of acetyl-CoA. For example, in the RuMP cycle, formaldehyde is condensed with Ru5P to form pyruvate, and then pyruvate is decarboxylated into acetyl-CoA with CO_2 emission, resulting in the maximum theoretical carbon yield of 67%; CO_2 from methanol oxidation is fixed by the CBB cycle, requiring lots of ATP equivalents [82]; the serine pathway requires an external supply of ATP to drive thermodynamic unfavorable reactions [83]. To avoid these defects, methanol condensation cycle (MCC) is constructed, which is modified from the combination of the RuMP cycle and non-oxidative glycolysis, to convert methanol into high-chain alcohols with complete carbon conservation and ATP independence [82]. Condensing two formaldehydes with Ru5P to form

acetyl-CoA is the core portion of MCC, in which formaldehyde is assimilated by part of the RuMP cycle to form F6P and Xu5P. After that, phosphoketolases (F/Xpk) can cleave Xu5P (by Xpk) or F6P (by Fpk) to form acetyl-CoA, and then the associated sugar phosphate is transformed into Ru5P for the next cycle. Although the initial oxidation of methanol is thermodynamically difficult, it is possible to choose a set of enzyme concentrations such as Hps and F/Xpk to provide a significant driving force [82]. Moreover, in the first step of MCC, methanol oxidation by NAD^+-dependent methanol dehydrogenases can provide reducing equivalents, which can be used to drive the formation of high-chain alcohols. MCC allows the efficient conversion of methanol to high-chain alcohols or other compounds without carbon loss or ATP expenditure. Thus, MCC are attractive for industrial applications because of its oxygen-tolerant enzymes, high-carbon efficiency and favorable operation conditions [82].

3.2.2. The 2-hydroxyacyl CoA lyase (HACL) pathway

HACL, an enzyme involved in mammalian α-oxidation, can catalyze the ligation of carbonyl-containing molecules with formyl-CoA to produce C1-elongated 2-hydroxyacyl-CoA. Accordingly, the HACL pathway is designed for assimilating one-carbon substrates without additional energy input in a whole-cell biotransformation system. The key enzyme HACL from *Rhodospirillales bacterium* URHD0017 is expressed in supernatant of the bacteria lysate and acyl-CoA reductase (ACR) from *Listeria monocytogenes* is the best-performing variant for converting formaldehyde into formyl-CoA. HACL in this pathway can catalyze a previously unknown one-carbon condensation reaction between formyl-CoA and formaldehyde to form glycolyl-CoA. Glycolyl-CoA is subsequently reduced to form glycolaldehyde by ACR and then to glycolate by aldehyde dehydrogenase. Glycolate can serve as a substrate for cell growth, demonstrating the potential of HACL pathway to support synthetic methylotrophy [12,84].

3.2.3. The synthetic acetyl-CoA (SACA) pathway

Many methanol assimilation pathways described above largely overlapped with the known metabolic network, and thus it is challenging to distribute metabolic flux among the designed pathways and the original metabolic pathways. To avoid this problem, the SACA pathway is designed for biosynthesis of acetyl-CoA from methanol through only four steps without overlapping with the known metabolic network [83]. Firstly, methanol is oxidized to formaldehyde by methanol dehydrogenase. Secondly, two molecules of formaldehydes are condensed into glycolaldehyde by glycolaldehyde synthase (GALS), followed by acetyl-phosphate synthase (ACPS) for the formation of acetyl-phosphate (ACPS) using inorganic phosphate as cofactors. Eventually, ACPS is converted into acetyl-CoA by phosphate acetyltransferase (PTA) for generating biomass [85]. Moreover, the transformation of formaldehyde to acetyl-CoA in the SACA pathway is highly thermodynamically favorable [83]. However, the application of the SACA pathway in industrial hosts still remains a challenge due to the low specific activity of GALS and ACPS.

As many native or synthetic methanol assimilation pathways overlap with the central metabolic pathways, a trade-off between cell growth and metabolite production should be considered to maximize methanol utilization. Such overlaps will make researchers have to rewire cellular metabolic flux globally, which might disrupt cellular growth. Moreover, a deleterious overlap between native and synthetic pathways might strongly repress the synthetic pathway flux and limit the production of biochemicals. Although several orthogonal pathways for methanol assimilation have been developed, these pathways are not well introduced to industrial chassis, probably because the expression of heterologous enzymes triggers metabolic burden. An optimal solution for methanol assimilation is to design novel pathways without overlap with central metabolic network, favorable kinetics and high driving force [86]. In addition, a reasonable energy supply system should be designed for chassis cells to make the synthetic methanol assimilation pathways operate normally [9].

4. Microbial engineering strategies for methanol utilization

Despite significant efforts in developing synthetic methylotrophs in industrial chassis such as *E. coli, C. glutamicun* and *S. cerevisiae* for methanol assimilation, the efficiency of methanol utilization cannot satisfy the demands of industrialization. In addition, there are only a few of engineered strains can grow on methanol as the sole carbon source. Therefore, many strategies have been developed to enhance methanol assimilation, such as enhancing methanol oxidation (Fig. 4(A)), optimizing methanol assimilation pathways (Fig. 4(B)), and improving host compatibility (Fig. 4(C)).

4.1. Enhancing methanol oxidation

In all methylotrophs, the first step of methanol metabolism is the oxidation of methanol to formaldehyde, which is catalyzed by methanol dehydrogenases (MDH). NAD^+-dependent MDH has the highest electron retention rate in the catalytic process of methanol oxidation, which is conducive to the production of metabolites. However, this reaction is not conducive to the oxidation of methanol in terms of thermodynamics, and the affinity and catalytic activity of these enzymes to methanol are not high enough. The PQQ-dependent methanol oxidation process is complicated, and it is difficult to apply to exogenous chassis. In O_2-dependent methanol oxidation process, methanol is first oxidized by alcohol oxidase(AOD) to formaldehyde and produces H_2O_2, and then H_2O_2 is converted to O_2 and H_2O under the catalysis of catalase(CTA). However, this process produces neither ATP nor NADH, which is not conducive to the synthesis of target metabolites. To address these obstacles, many strategies are used to enhance the methanol oxidation process such as improving the efficiency of methanol dehydrogenase, designing enzyme complex for methanol oxidation and improving the regeneration of formaldehyde acceptors.

4.1.1. Improving the efficiency of methanol dehydrogenase

Currently, NAD^+-dependent methanol dehydrogenases (nMDHs) from natural methylotrophs are chosen for building synthetic methylotrophic cell factories, because they can oxidize methanol to produce electrons for metabolite production under aerobic and anaerobic conditions [2,3]. However, the main bottleneck of MDHs is their low catalytic activity for methanol oxidation in synthetic methylotrophs. Therefore, it is necessary to develop more efficient MDHs. There are two strategies for improving the catalytic efficiency of MDHs: screening more efficient MDHs from natural methylotrophs and modifying MDHs by directional mutation. Recently, enzyme screening has shown that MDHs from *B. methanolicus* or *Cupriavidus necator* N-1 have relatively high methanol consumption rate [87,88]. However, the specific activity of these MDHs still cannot meet the requirement of practical application. Mutation of MDHs is an effective method to further improve the rate of methanol assimilation. To engineer MDHs, automatic high throughput screening was used to mine MDHs with high affinity and activity for methanol. As a result, a chimeric variant CT4-1 of MDH from *C. necator* N-1 was created by recombining three mutations, A169V, A31V, and A26V. For this variant, the K_m value for methanol was further lowered to 21.6 mM, and the K_{cat}/K_m ratio was 6-fold higher than that of wild-type MDH [75]. Nevertheless, it is worth noting that enzyme screening and mutation should be taken into account in the physiological state of hosts, which may be one of valuable principles for improving methanol utilization efficiency of methylotrophic microorganisms.

4.1.2. Designing enzyme complex for methanol oxidation

Heterologous expression of multiple enzymes often leads to the imbalance of metabolic flux. To solve the limitation of specific heterologous enzymes, an ideal supramolecular enzyme complex, which can self-assemble into spatially defined architectures, is a promising approach to

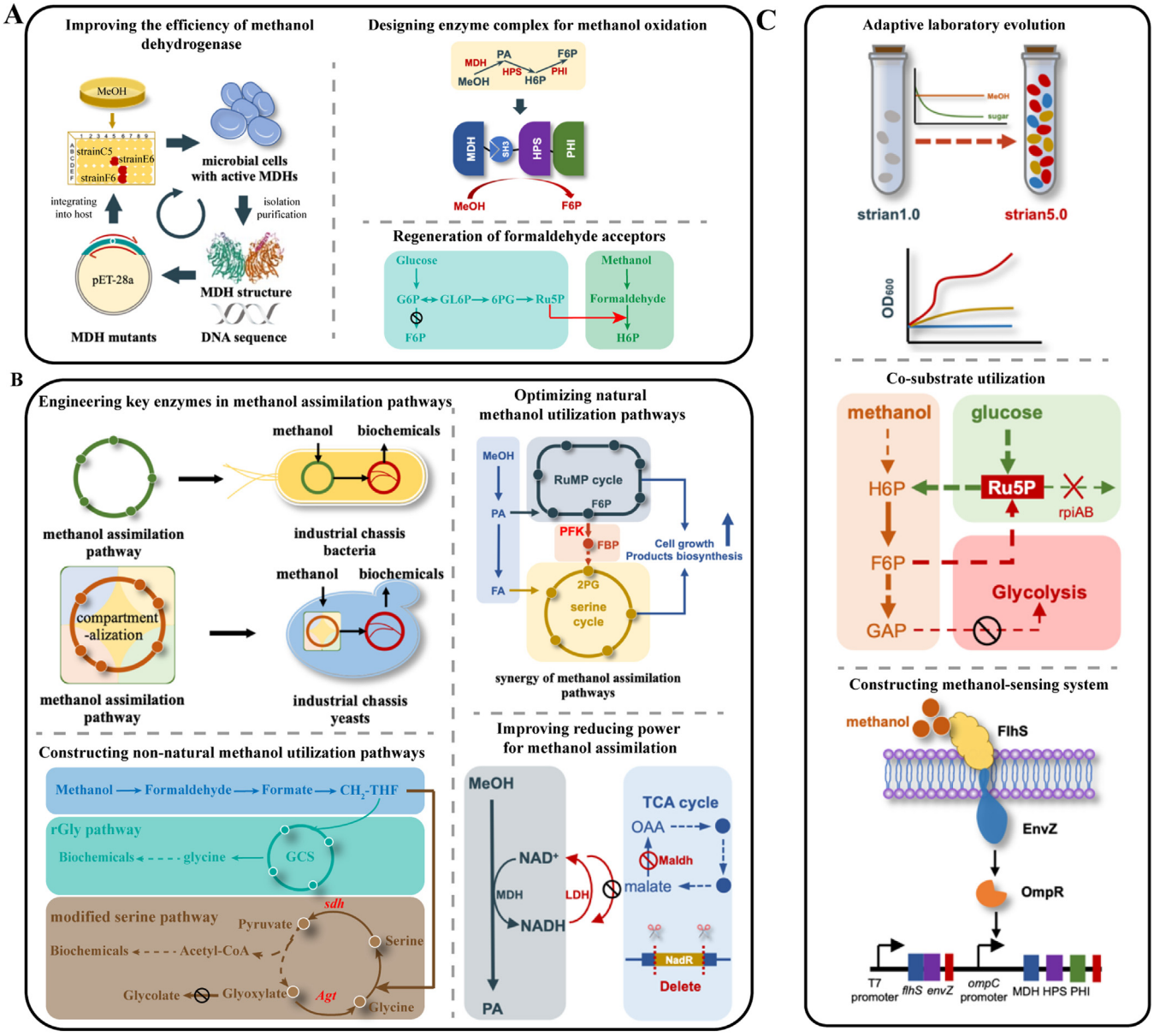

Fig. 4. Microbial engineering strategies for improving methanol utilization. (A) Enhancing methanol oxidation by improving the efficiency of MDHs, designing enzyme complex and improving the regeneration of formaldehyde acceptors. (B) Optimizing methanol assimilation pathways by engineering key enzymes in methanol assimilation pathways, optimizing natural methanol utilization pathways, constructing non-natural methanol utilization pathways and improving reducing power for methanol assimilation. (C) Improving host compatibility for methanol utilization by ALE, co-substrate utilization and constructing methanol-sensing system. MeOH, methanol; PA, formaldehyde; FA, formate; GAP, glyceraldehyde-3-phosphate; G6P, Glucose-6-phosphate; GL6P, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; F6P, fructose-6-phosphate; H6P, hexulose 6-phosphate; OAA, oxaloacetate; CH_2-THF, methylene-tetrahydrofolate; GSC, glycine cleavage system; *sdh*, serine dehydratase; *Agt*, alanine-glyoxylate transaminase.

improve the efficiency of methanol assimilation [89–91]. It is an effective way to construct enzyme complex using different linkers to connect different heterologous enzymes. Recently, two strategies are used to connect different enzymes. Strategy (i) is receptor-ligand interaction. By self-assembling of MDH (from *B. methanolicus*), HPS and PHI (from *Mycobacterium gastri*) into an enzyme complex by SH3-ligand, methanol consumption rate was increased by 2.3-fold in vitro, suggesting that key enzymes connected by SH3-ligand allow the high rate of cascade reaction [92]. Strategy (ii) is flexible peptides. For instance, the conversion of methanol to F6P was increased by 30% in vitro through fusing MDH from *Geobacillus stearothermophilus*, HPS and PHI from *B. methanolicus* via with flexible linkers $(GGGGS)_3$ and $(GGGGS)_6$ [93]. Engineering enzyme complex has been used to improve the efficiency of cascade reactions, and thus the formed substrate channeling makes this method desirable for pulling methanol toward F6P production. Therefore, this method has great potential for the construction of enzyme cascade reactions using flexible linkers or scaffold protein [94], opening new channels for the utilization of methanol and the production of chemicals.

4.1.3. *Improving the regeneration of formaldehyde acceptors*

One limitation of methanol assimilation in synthetic methylotrophs may be the insufficient formaldehyde acceptors, i.e., Ru5P or Xu5P. Thus, methanol assimilation can be accelerated by increasing the concentration of formaldehyde receptors. To date, two approaches are applied to regenerate formaldehyde acceptors for improving the efficiency of methanol utilization. Approach (i) is to regulate the non-oxidative

pentose phosphate pathway (PPP). Ru5P or Xu5P accumulation can be increased by regulating the non-oxidative PPP, because Ru5P and Xu5P are important intermediates in this pathway. For instance, carbon flux could be rerouted to the formation of Ru5P in *E. coli* by introducing the non-oxidative PPP from *B. methanolicus* and knocking out the phosphoglucose isomerase gene. Acetone production with *E. coli* 5PPP Δ*frmA*Δ*pgi* was increased by 110% compared to the control culture without methanol [95]. Approach (ii) is to inhibit the downstream glycolytic pathway. Increasing the concentration of F6P by inhibiting the downstream glycolytic pathway is beneficial to the regeneration of Ru5P. Accordingly, the engineered *E. coli* MG1655Δ*frmA*Δ*talA*Δ*talB*, in which the downstream glycolytic pathway was inhibited by iodoacetate and the *glpX* gene was overexpressed, could achieve a 4-fold increase in Ru5P, a 3-fold reduction in formaldehyde, and a 2-fold increase in methanol assimilation [96]. Further work can be carried out to improve the regeneration of formaldehyde acceptors through the following two aspects. For one thing, the introduction of heterogenous enzymes will cause metabolic burden, and thus it is important to explore the native transcription factors for fine-tuning the RuMP cycle systematically. For another thing, as one of important intermediates in methanol assimilation, F6P formation can be increased by strengthening the downstream of the RuMP or XuMP cycle to regenerate formaldehyde acceptors.

4.2. Optimizing methanol assimilation pathways

To promote the construction of cell factories that produce high-value chemicals, many strategies have been taken to construct methylotrophs and improve the oxidation efficiency of methanol to formaldehyde. However, in the process of methanol oxidation, a large amount of toxic formaldehyde will be accumulated in the cell, so it is necessary to quickly carry out the next reaction to avoid the non-specific reaction of formaldehyde with proteins and nucleic acids in the cell. Here, four strategies are summarized, mainly including: engineering key enzymes in methanol assimilation pathways, optimizing natural methanol utilization pathways, constructing non-natural methanol utilization pathways and improving reducing power for methanol assimilation.

4.2.1. Engineering key enzymes in methanol assimilation pathways

The RuMP cycle in the natural methylotrophs mainly consists of three modules: formaldehyde fixation module that contains enzymes HPS and PHI; conversion module for converting F6P to pyruvate; carbon rearrangement module for the regeneration of formaldehyde receptor Ru5P. Since typical microbial chassis has the glycolysis pathway and TCA cycle, the key genes *mdh, hps* and *phi* from native methylotrophs are needed for constructing a complete pathway for methanol assimilation in non-methylotrophs. Therefore, many studies have successfully introduced the genes *mdh, hps* and *phi* from *B. methanolicus, B. stearothermophilus, C. necator* or *M. extorquens* into industrial chassis [50,87,85,97–99]. For example, a complete RuMP cycle was constructed in *E. coli* by integrating MDH, HPS and PHI, achieving up to 40% incorporation of methanol [45]. The XuMP cycle involved to the key enzymes AOX, DAS, and DAK from *P. pastoris* can be integrated into non-methylotrophic yeasts such as *S. cerevisiae* [62]. For instance, the synthetic methylotrophic *S. cerevisiae* TACDS2 with the heterologous expression of AOX, DAS and DAK has shown 1.04 g/L consumption in methanol and 3.13% increase in biomass compared with wild type strains when using methanol as the sole carbon source [61]. In the future, there are two aspects that need to be improved. Firstly, intact peroxisome and metabolic compartmentalization are crucial and advantageous for reducing the toxicity of intermediates to improve cell growth of native methylotrophic yeasts [41]. Thus, targeting formaldehyde and the associated metabolic reactions to peroxisome is one of the useful strategies for improving methanol-utilizing rate of synthetic methylotrophs [11,60]. Secondly, the RuMP and XuMP cycle are not the efficient pathways for methanol assimilation because of their carbon loss when the end product is acetyl-CoA. To avoid this problem, introducing CO_2 carboxylases such as pyruvate carboxylase can convert the product of RuMP or XuMP cycle to oxaloacetate which can re-enter into TCA cycle, thus reducing carbon loss [63].

4.2.2. Optimizing natural methanol utilization pathways

In the complex metabolic networks, one metabolite can be produced and catabolized in many different metabolic pathways. Therefore, precise regulation of metabolic flux can effectively improve substrate utilization and chemical production. Regulating carbon flux in methanol assimilation pathways can also improve the efficiency of methanol utilization. To date, two approaches have been used to rewire methanol assimilation pathways. Approach (i) is the synergy of native methanol assimilation pathways. Given that the RuMP cycle and the serine cycle have many advantages in energy efficiency and acetyl-CoA yield for methanol assimilation [69,100], a synergistic pathway for methanol assimilation was constructed in native methylotrophic bacterium *M. extorquens*. In this pathway, phosphofructokinase could catalyze the phosphorylation of F6P to form fructose-1,6-biphosphate, which could drive carbon flux from the RuMP cycle toward the serine cycle. As a result, this engineered *M. extorquens* AM1 increased cell growth rate and methanol consumption rate by 16.5% and 13.1%, respectively [101]. Approach (ii) is the synergy of heterologous and native methanol assimilation pathways. Based on the great efficiency of the RuMP cycle and the serine cycle in native methylotrophs, methanol assimilation rate was increased to 1.1 g/L/h by introducing a heterologous RuMP cycle into the methylotrophic yeast *Yarrowia lipolytica* Iso4 [102]. However, a trade-off between cell growth and metabolite production should be carefully considered, as the unbalanced metabolism may lead to cell death. To deal with this issue, it is desirable to design dynamic regulation pathway to redirect carbon flux toward target biochemicals, balance the production and consumption rates of intermediates, and inhibit the production of toxic compounds such as formaldehyde [103]. Another issue of concern is the maintenance of long-term cell activity for methanol assimilation after rewiring methanol assimilation pathways. Filtering toxic intermediates [104] or knocking out redundant genes [105] are beneficial to maintaining cell activity.

4.2.3. Constructing non-natural methanol utilization pathways

It is possible for non-native methylotrophs to grow on methanol using synthetic pathways. Thus, many synthetic assimilation pathways have been emerged. Frist, the reductive glycine(rGly) pathway is a synthetic pathway for methanol or formate assimilation, which is constructed by combining the methylene-tetrahydrofolate(CH_2-THF) synthesis pathway with a reversal of the glycine cleavage system(GCS) to synthesize glycine and pyruvate [88,106]. One-carbon substrates can be assimilated into pyruvate through the rGly pathway with the need of two ATP, enabling higher biomass yields than other natural aerobic methanol utilization pathways [107]. The rGly pathway has been used to engineer *E. coli* to assimilate formate that is from the conversion of methanol using MDH from *B. stearothermophilus* and the endogenous glutathione system. As a result, the biomass yield of this synthetic *E. coli* strain (gC_1M gC_2M gC_3M gEM) was 4.2 gCDW per mol-methanol, which was lower than that of native methylotrophs [94]. Similarly, the rGly pathway was also adopted in aerobic microorganisms such as *C. necator* [108] and *S. cerevisiae* [109] for methanol or formate assimilation. However, none of these studies obtained the desired results for methanol assimilation because of the relatively low specific activity of heterologous MDHs. Thus, in the future, more efficient MDHs need to be explored by high-throughput screening in native methylotrophs and enzyme engineering for large-scale mutant screening. On the other hand, all genes required for the core module of the rGly pathway are endogenous in *S. cerevisiae* [11]. Based on this, it may be convenient to construct a synthetic methylotroph *S. cerevisiae* for the production of high-value chemicals. Second, the modified serine cycle that utilizes a simple cyclic network has been integrated into *E. coli* for assimilating methanol efficiently. In this cycle, formaldehyde

dehydrogenase is used to simplify the oxidation of formaldehyde to formate, and alanine-glyoxylate transaminase and serine dehydratase are utilized to avoid hydroxypyruvate in the conversion of glyoxylate to PEP. As a result, methanol consumption rate of the engineered *E. coli* HY106 was shown up to 0.7 mM/h/OD_{600} with the supplement of 30 mM xylose and 200 mM methanol [47]. This study shows that building methanol assimilation pathways in non-methylotrophs is one of effective way to improve cellular tolerance and methanol utilization.

4.2.4. Improving reducing power for methanol assimilation

Introducing heterologous MDHs in non-native methylotrophs not only generates formaldehyde, but also produces nicotinamide adenine dinucleotide (NADH). The accumulation of NADH increases the Gibbs free energy of catalytic reaction and is detrimental to the first step of methanol oxidation. To alleviate the negative effect of NADH on methanol assimilation, three strategies are adopted to decrease NADH/NAD^+ ratio. (i) Weakening the activity of TCA cycle. The TCA cycle produces a large number of NADH when microorganisms grow on sugars. However, the inefficient TCA cycle in native methylotrophs favors intracellular NADH homeostasis for facilitating their methanol assimilation [110]. Accordingly, knockdown of MDH in native methylotrophs *M. extorquens* AM1 or *B. methanolicus* MGA3 could impair the TCA cycle and increase NAD^+ accumulation [111,112]. (ii) Reducing the activity of NADH-regenerating enzymes. To further accelerate NADH regeneration to overcome the thermodynamic barrier on methanol oxidation, the evolved strain *E. coli* MeSV2.2, in which malate dehydrogenase was knocked out and the activity of NADH homeostasis/biosynthesis was decreased, achieved a higher NAD^+/NADH ratio than that of *E. coli* MeSV2. Finally, up to 24% methanol was incorporated into core metabolites [97]. (iii) Introducing heterologous enzymes to promote NADH consumption. The introduction of some heterologous enzymes may lead to the consumption of NADH, and thus the generation of NAD^+ could enhance the activity of NAD^+-dependent MDHs. For example, when lactate dehydrogenase with NAD^+ regeneration was introduced into *E. coli*, the activity of NAD^+-dependent MDHs was improved, thereby increasing the rate of methanol assimilation in the RuMP cycle [92]. The following two points should also be noted for generating NAD^+ in the future research. Firstly, cell growth may be affected with NADH reduction by weakening the TCA cycle and NADH-regenerating enzymes. Based on this, introducing NADH consumption enzymes to replace the less active ones may be desired for further studies. Secondly, it is worth noting that if MDH is not active enough, increasing NAD^+ concentration will lead to the imbalance of intracellular NADH/ NAD^+ ratio. Thus, the properties of MDHs need to be considered initially when increasing intracellular NAD^+ accumulation.

4.3. Improving host compatibility for methanol utilization

Two major modules, methanol oxidation and methanol assimilation pathways, have been used for the construction of methylotrophs. However, the compatibility between host strain and methanol utilization module remains a challenge. To improve the methanol utilization efficiency and productivity of the synthesized methylotrophs, many strategies have been applied such as adaptive laboratory evolution (ALE), co-substrate utilization and constructing methanol-sensing system.

4.3.1. Adaptive laboratory evolution (ALE)

ALE is one of frequently used methods that provide comprehensive understanding of molecular mechanisms to the adaptive genetic changes in microorganisms during long-term evolution under specific conditions. Recently, ALE can be used to maintain intracellular metabolic flux and redox homeostasis by adaptively optimizing cellular metabolism [113]. To obtain more efficient synthetic methylotrophs with low-active glycolytic pathway or TCA cycle, two different evolutionary approaches have been adopted: methanol-dependent ALE and methanol/sugar-dependent ALE. For example, the engineered *E. coli* CFC526.0Δ*pfkA*Δ*gapA: gapC* was evolved in methanol to obtain synthetic methylotrophs [46], but the evolved strain *E. coli* CFC680.1 still grew slowly in methanol. To further improve the efficiency of ALE, the genes encoding phosphogluconate dehydratase, ribose-5-phosphate isomerase and NAD^+-dependent malate dehydrogenase were knocked out in *E. coli*, and then was evolved by continuously reducing sugars in glucose and methanol medium. As a result, the evolved strain *E. coli* SM1 had a high methanol-utilizing rate of 13 mM/gCDW/h similar with the native methylotrophs [97]. Overall, ALE is a very effective method for enhancing substrate tolerance and chemical production. However, the relatively long periods of time are still needed due to the low rate of spontaneous mutations in ALE. In the future, the limitation of ALE can be overcome from the following aspects. Firstly, the development of gene editing tools such as CRISPR/Cas9 [114] can compensate for the limitation of ALE, thus improving the efficiency of ALE. Secondly, the ability of eVOLVER with a scalable do-it-yourself framework can precisely be carried out through high-throughput experiments, which can save a lot of time for ALE [115].

4.3.2. Co-substrate utilization

In general, synthetic methylotrophs cannot grow well solely on methanol, and thus other carbon sources are also needed to supply additional energy and support cellular metabolism in methanol medium [3]. To date, three available methods are developed for co-substrate utilization. Method (i) is to supply available sugars. In general, supplementation with the suitable amounts of sugars (such as glucose, xylose, and ribose) can effectively increase the growth of methylotrophs. For example, as one of common substrates, glucose could be supplied to improve the growth of the engineered yeast *Ogataea polymorpha* HpFA01, and thus the final production of fatty acid was increased to 0.98 g/L on methanol medium [116]. Method (ii) is to change the preference of carbon sources. Except for glucose, other sugars can also be used to improve the efficiency of microbial methanol assimilation. For example, the evolved methylotrophic strain *E. coli* CFC65, in which the genes *ripAB* and *rpe* were knocked out and the heterologous enzymes from the RuMP pathway were partially expressed, have shown a 11-fold enhancement in methanol consumption compared to that of *E. coli* BL21 [117]. Method (iii) is to add non-sugar substrates. In addition to gluconic acid, yeast powder, glycose, ribose, and xylose, leucine could be used as auxiliary substrates to improve the efficiency of methanol assimilation, and thus deleting the leucine-responsive regulatory protein was able to further improve the rate of methanol-utilization in *E. coli* BW25113 Δ*lrp* [118]. Although it is easy to provide additional carbon sources to support the synthetic methylotrophs in methanol medium, future research is to construct synthetic methylotrophs that can use methanol as the sole carbon and energy sources. Thus, more efforts are needed to explore new methylotrophs by high-throughput screening approaches [119] and make a deep understanding in the metabolic mechanism of methylotrophs.

4.3.3. Constructing methanol-sensing system

In addition to methanol bioconversion, the ability of cells to sense environmental methanol is equally important. Native methylotrophs can sense methanol, and then some key genes are induced for methanol utilization. Thus, microbial cells can make better use of methanol by regulating these genes that respond to methanol [120,121]. Recently, two approaches have been used to construct methanol-sensing synthetic methylotrophs. Approach (i) is chimeric two-component system. Two-component system, a basic stimulus-response mechanism, allows microorganisms to sense changes in environment. The integration of FlhS/EnvZ (FlhSZ) chimeric histidine kinase, which was formed by fusing the sensing domain of FlhS from *Paracoccus denitrificans* with the transmitter domain of EnvZ from *E. coli*, enabled *E. coli* BL21 to sense methanol, and thus activated OmpR to induce *gfp* expression [121]. When *gfp* was replaced with methanol assimilation-related genes, the synthetic strains were able to initiate methanol assimilation

pathways under a certain concentration of methanol. Approach (ii) is formaldehyde-sensing system. As methanol is always oxidized to form formaldehyde in methanol assimilation pathways, it is necessary to construct a system that responds to formaldehyde for reducing formaldehyde toxicity and improving its assimilation efficiency. *E. coli* has a native formaldehyde-inducible promoter P_{frm} at the upstream of the *frmRAB* operon [122,123]. Thus, P_{frm}-FrmR toolkit can be constructed to regulate methanol assimilation pathways for improving the efficiency of methanol utilization. For example, intracellular formaldehyde accumulation in *E. coli* K-12 Δ*frmA*Δ*pgi* could be avoided by a P_{frm}-inducible gene expression cassette (*mdh-hps-phi*) to optimize the expression levels of *mdh, hps*, and *phi*, resulting in the yield of biomass on methanol up to 0.35 gCDW/g methanol [124]. The development of methanol-sensing systems can help synthetic methylotrophs to dynamically regulate methanol assimilation pathways, enabling them to maintain cell growth.

5. Conclusions

Industrial manufacturing has made great strides in recent years in converting methanol into high-value chemicals, which has maximized the application value of methanol [125]. As an alternative strategy, sustainable methanol biotransformation has great potential for expanding product spectrum. Construction of methanol metabolic pathways in native methylotrophs sheds light on the reaction mechanism of methanol assimilation and offers guides for engineering synthetic methylotrophs. Although numerous metabolic engineering strategies have been developed to optimize methanol assimilation, the efficiency of methanol assimilation cannot fully meet industrial needs. Therefore, there still exists many challenges that need to be overcome.

Developing microbial hosts with excellent ability for methanol utilization. (i) Although native methylotrophs can assimilate methanol for cell growth, the metabolic activity of methanol is still not high enough for efficient production of chemicals. To obtain more efficient native methylotrophs, it may be useful to screen methylotrophs from extreme environment with methanol as the sole carbon and energy source [126]. (ii) Systematic mechanism for methanol assimilation in native methylotrophs still remain unclear. Multi-omics data can provide comprehensive understanding of methanol metabolic network in native methylotrophs, which will facilitate subsequently genetic regulation to improve methanol-utilizing rate [127]. (iii) The methanol-utilizing efficiency of synthetic methylotrophs still remain below the expected level. To address this issue, one strategy is to investigate new microbial hosts such as *B. subtilis* for integrating methanol assimilation pathways [56]. Another strategy is to search new or high-efficiency enzymes for methanol assimilation, which can be further introduced into specific microbial hosts for the construction of synthetic methylotrophs [128].

Optimizing methanol assimilation pathways. (i) Methanol assimilation by the rAcCoA pathway can only obtain a narrow product spectrum such as acetate. To address this issue, the rAcCoA pathway can be integrated into anaerobic bacteria with excellent metabolism and then acetyl-CoA can be regulated as precursors for the synthesis of a wide range of biochemicals. (ii) Some methanol assimilation pathways such as the RuMP cycle are not efficient enough to produce biochemicals because of carbon loss during the conversion of pyruvate to acetyl-CoA. Introducing carboxylases such as pyruvate carboxylase can convert pyruvate to oxaloacetate rather than acetyl-CoA, thereby reducing carbon loss [63]. (iii) Although many synthetic pathways have been developed, only a few of them can successfully operate *in vivo*. Thus, new methanol assimilation pathways should be further developed in vivo. For example, the rGly pathway has the potential to assimilate methanol since methanol can be oxidized to formate with the generation of reducing power [94,98]. In addition, the modified serine cycle can avoid hydroxypyruvate as an intermediate, which can improve methanol assimilation of *E. coli* HY106 [129].

Improving methanol assimilation of synthetic methylotrophs. (i) Due to the limited metabolic flux of methanol assimilation pathways such as the RuMP cycle, the synthetic methylotrophs [130] are difficult to grow solely on methanol. A practical way for the desired synthetic methylotrophs growing on methanol alone is to combine genetic modification with ALE to weaken the native carbon metabolic pathways and enhance methanol assimilation pathways [46]. Another efficient strategy is to design orthogonal methanol assimilation pathways that operate independently [86]. (ii) Methanol and formaldehyde are toxic for microorganisms when accumulating in certain range of concentrations. Multienzyme supramolecular complexes that is assembled via scaffold engineering have been adopted to prevent the accumulation of toxic intermediates [114,115]. Moreover, the genome-scale CRISPRi technology can be used to explore the mechanism of methanol or formaldehyde tolerance, and then key functional genes may be obtained for improving the tolerance of toxic substrates [131].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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