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REVIEW

Strategies and challenges with the microbial conversion of methanol to high-value chemicals

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Abstract

As alternatives to traditional fermentation substrates, methanol (CH_3OH), carbon dioxide (CO_2) and methane (CH_4) represent promising one-carbon (C1) sources that are readily available at low-cost and share similar metabolic pathway. Of these C1 compounds, methanol is used as a carbon and energy source by native methylotrophs, and can be obtained from CO_2 and CH_4 by chemical catalysis. Therefore, constructing and rewiring methanol utilization pathways may enable the use of one-carbon sources for microbial fermentations. Recent bioengineering efforts have shown that both native and nonnative methylotrophic organisms can be engineered to convert methanol, together with other carbon sources, into biofuels and other commodity chemicals. However, many challenges remain and must be overcome before industrial-scale bioprocessing can be established using these engineered cell refineries. Here, we provide a comprehensive summary and comparison of methanol metabolic pathways from different methylotrophs, followed by a review of recent progress in engineering methanol metabolic pathways in vitro and in vivo to produce chemicals. We discuss the major challenges associated with establishing efficient methanol metabolic pathways in microbial cells, and propose improved designs for future engineering.

KEYWORDS
energy supply, intermediate recycling, methanol, methylotrophs, redox cofactor

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1. INTRODUCTION

Rapid population growth and economic developments in the world are imposing an increased demand for food, materials, and energy. As traditional supply routes are unable to meet this demand, there is much interest in evaluating alternative supply routes for these resources. Production of biofuels and high-value commodity chemicals via microbial fermentation represents a promising strategy to provide materials and energy in the world market (Luo et al., 2019; Nielsen & Keasling, 2016; Nielsen et al., 2014). However, traditional fermentation substrates mainly comprise of sugars, such as glucose, which has raised concerns about food security and biodiversity (Naik et al., 2010) (Figure 1a). Therefore, exploring the use of renewable and nonfood carbon sources is becoming extremely important for industrial biomanufacturing. CO_2 and natural gas (consisting primarily of CH_4) are two of the most important and attractive carbon sources owing to their high abundance (Haynes & Gonzalez, 2014; Xu et al., 2018). The process of converting CO_2 (e.g., metal catalysation, Fujiwara et al., 2019; and electrocatalysis, Magdesieva et al., 2002) to value-added products is promising in the sense that it will not only relieve the greenhouse effect, but also shorten the cycle of organic compound production via plant-based photosynthesis (Liu et al., 2020; Xiaoding & Moulijn, 1996) (Figure 1b). In addition, CO_2/CH_4-based autotrophic microorganisms could directly convert CO_2/CH_4 into biofuels and chemicals (Long et al., 2018), which greatly promotes CO_2/CH_4-based microbial cell factory construction (Savakis & Hellingwerf, 2015). However, a major challenge in harvesting CO_2 and CH_4 as carbon sources for microbial fermentation is that they cannot be fixed efficiently by most microbes, owing to their gaseous form, thermodynamic instability (Naik et al., 2010), high energy-cost (Birdja et al., 2019), low catalytic activity of Rubisco (Erb & Zarzycki, 2016) and requirement for additional metabolic supplements such as ribulose-1,5-bisphosphate (RuBP) (Budzianowski, 2012; Erb & Zarzycki, 2018; B. Hu et al., 2013; G. Hu et al., 2018; Savakis & Hellingwerf, 2015). In contrast, methanol, a liquid one-carbon compound, is considered a promising C1 feedstock owing to its increasing availability (via conversion of natural gas) (Latimer et al., 2018; Linton & Niekus, 1987; Sperling, 2007), a predicted decrease in price (Bertau et al., 2014; Pfeifenschneider et al., 2017), and relatively low energy-cost compared with CO_2 and CH_4 (Schrader et al., 2009; Whitaker et al., 2015). Moreover, some methanol utilization pathways share similarities with CO_2 fixation pathways, such as the C5 regeneration pathway (Figure 1b) (Antonovsky et al., 2016; Cox & Quayle, 1975; Jakobsen et al., 2006). Therefore, a better understanding of methanol utilization pathways would not only benefit methylotrophic microorganism construction, but also provide a chassis for CO_2 and other C1 source utilization (Saeidi et al., 2014) (Figure 1b).

Native methylotrophs can grow on methanol as a single carbon and energy source, and include both methylotrophic bacteria, such as *Bacillus methanolicus* (Arfman et al., 1992; De Vries et al., 1992) and methylotrophic yeast, such as *Pichia pastoris* (Couderc & Baratti, 1980; Ellis et al., 1985). These native methylotrophs represent natural platforms for biotechnological engineering of C1 compound utilization, however the genetic tools for engineering these organisms are not as efficient as those for model organisms such as *Saccharomyces cerevisiae* and *Escherichia coli*. Moreover, drawbacks including insufficient accumulation of metabolic intermediates and

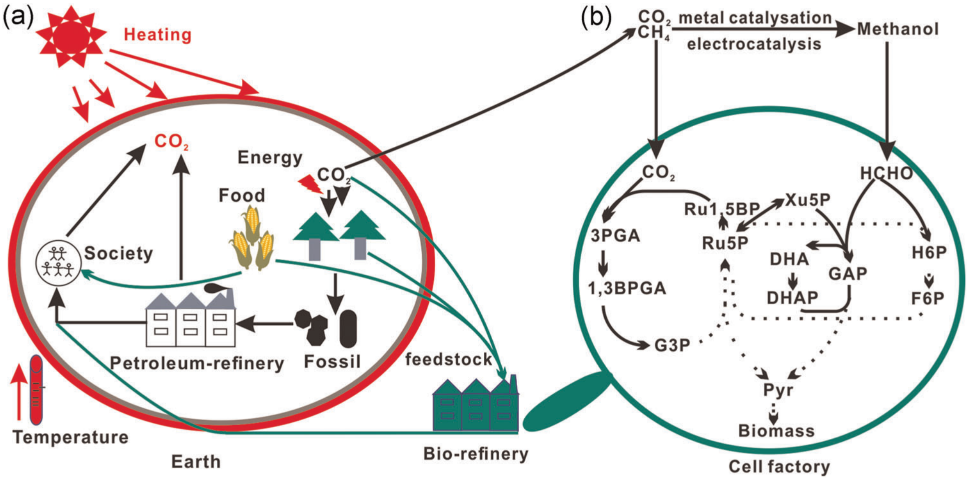

FIGURE 1 The traditional one-carbon cycle and one-carbon compound utilization for establishment of sustainable circular carbon economy. (a) The CO_2 cycle in nature. Plants convert CO_2 into different sources, that is, food and energy, to support human society development. Excessive human activity releases large amount of CO_2 exceeding the CO_2 sequestration speed by plants and leads to accumulation of CO_2 in atmosphere causing "greenhouse effect." (b) C1 sources (i.e., CO_2 and methanol) utilization by microorganisms in biorefinery for production of chemicals and biofuels, allowing for a sustainable circular carbon economy. One-carbon feedstock (CO_2-based or methanol-based) can be used by microorganisms in a biorefinery process to replace fuels and chemicals that produced today by petroleum-refinery process. 1,3BPGA, 1,3 bisphosphoglycerate; 3PGA, 3-phosphoglycerate; DHA, dihydroxyacetone;DHAP, dihydroxyacetone phosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde-3-phosphate; H6P, hexose 6-phosphate; Pyr, pyruvate; Ru1,5BP, ribulose 1,5-bisphosphate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate [Color figure can be viewed at wileyonlinelibrary.com]

low carbon yield due to insufficient methanol catabolism (Jorda et al., 2014), further prohibit the use of these organisms as cell factories to produce secondary metabolites. In addition, genes related to methanol utilization pathways are tightly regulated by methanol and repressed by other carbon sources in co-substrate media, and the molecular mechanism behind this is still unclear (Zhan et al., 2017). Energy generation from methanol as a substrate is insufficient to support all biological processes, as ^{13}C labeling results show that most of the methanol assimilated is used to produce energy for methanol assimilation itself (Jordà et al., 2012). All of these factors make it necessary to take a two-step strategy when using *P. pastoris* for fermentations, whereby biomass formation is supported in the first step by a traditional carbon source (e.g., glycerol), and methanol is only used as a substrate to maintain biomass in the second step (Bahrami et al., 2008; Zhan et al., 2017). These drawbacks indicate that methanol is not an ideal carbon and energy source for fermentations using *P. pastoris*.

An alternative approach to utilize methanol is to develop synthetic methylotrophs that can efficiently metabolize methanol either as a sole carbon and energy source, or as co-substrates with other carbohydrates. To do this, metabolic engineering and synthetic biology strategies were widely used in both native and synthetic methylotrophs to optimize methanol metabolic pathways. In this review, we first provide a comprehensive summary and comparison between different methanol metabolic pathways (Table 1). Then, we discuss recent efforts toward understanding and engineering native and synthetic methylotrophs to produce biofuels and high-value commodity chemicals. Finally, we discuss remaining challenges and propose modular strategies to improve methanol utilization by synthetic methylotrophs.

2. METHANOL METABOLIC PATHWAYS IN METHYLOTROPHS

Methanol metabolic pathways can be divided into two modules (Figure 2) in almost all methylotrophs. The first is a methanol oxidation module, in which methanol is enzymatically converted to formaldehyde (HCHO). The second is a formaldehyde assimilation module, where formaldehyde could be assimilated into central carbon metabolism via one of three pathways: (1) the xylulose monophosphate (XuMP) cycle, (2) the ribulose monophosphate (RuMP) cycle, or (3) the serine cycle. Of note, besides the main fate of assimilation, formaldehyde can also be oxidized to CO_2 in dissimilation pathways (W. Zhang et al., 2017). In this process, the carbon in formaldehyde is released as CO_2, but the proton generated is used to supply energy for biological processes.

The first module of methanol oxidation can be catalyzed by three methanol oxidoreductases, differentiated by their electron acceptor: (i) pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (PMDH), (ii) NAD^+-dependent methanol dehydrogenase (NMDH) and (iii) O_2-dependent alcohol oxidase (AOX). Among these, PMDH is the most complex as it requires the PQQ prosthetic group to transfer electrons to cytochrome *c*. In *Methylobacterium extorquens*, the expression of up to 15 genes are required to realize its function (M. Zhang & Lidstrom, 2003; W. Zhang et al., 2017). Even in *betaproteobacteria* with monomeric PMDH (Kalyuzhnaya et al., 2008), at least 4–7 additional enzymes are still required to synthesize PQQ, which additionally requires molecular oxygen (Velterop et al., 1995). Use of the PMDH circuit in synthetic methylotrophs construction has therefore been limited (Davidson, 2001).

The second methanol oxidoreductase, NMDH, has a lower enzyme specificity towards methanol (K_m, 170 mM) compared to PMDH (20 μM) (Kalyuzhnaya et al., 2008; Sheehan et al., 1988; Wu et al., 2016), although incorporation of its activator protein (ACT) dramatically promotes its affinity towards methanol (9 mM) in vitro (Arfman et al., 1997; Ochsner et al., 2014). ACT acts by removing the nicotinamide mononucleotide (NMN) moiety of the NMDH-bound NAD^+ (Arfman et al., 1997; Wu et al., 2016), which increases the affinity of NMDH for methanol and NAD^+ (Arfman et al., 1991). Therefore, this simple two-component system is much easier to construct than the PMDH complex (Velterop et al., 1995). However, NMDH mainly exists in thermophilic Gram-positive methylotrophs (such as *B. stearothermophilus* and *B. methanolicus*) and has a high Gibbs free energy ($\Delta_r G' = +34.2\ \mathrm{kJ\ mol^{-1}}$) (Whitaker et al., 2015). This means that the recombinant strains harboring NMDH must be grown at very high temperatures (45–55°C) to facilitate methanol oxidation, which is not feasible for most microorganisms.

The third methanol oxidoreductase, AOX, is mainly found in eukaryotic methylotrophs such as *P. pastoris*. Although this oligomeric flavoenzyme alcohol oxidase has relatively low affinity for both methanol and oxygen, its Gibbs energy is much lower ($\Delta_r G' = -99.2\ \mathrm{kJ\ mol^{-1}}$) than the other two enzymes (Cereghino & Cregg, 2000; W. Zhang et al., 2017), which makes it more achievable for aerobic biological processes under standard conditions (Whitaker et al., 2015). To compensate for the low affinity towards methanol, *P. pastoris* expresses extremely high levels of AOX1, controlled by a strong methanol inducible promoter P_{AOX1}, which results in AOX accounting for more than 30% of total soluble protein (Inan & Meagher, 2001). However, as the overall standard enthalpy change for the reaction catalyzed by AOX is negative ($\Delta H_o = -112.41$ kJ/mol), heat would be released under aerobic conditions, which may be problematic during scale-up fermentation (Jungo et al., 2007; Krainer et al., 2012). Moreover, its proton utilization efficiency is lower than both PMDH or NMDH, owing to the formation of H_2O_2 instead of NADH.

Whereas methanol assimilation using PMDH and AOX are limited to aerobic conditions, NMDH can potentially be used in both aerobic and anaerobic conditions. Some studies have shown that the thermophilic (55°C) anaerobic conversion of methanol to acetate could be realized by the addition of bicarbonate (Paulo et al., 2003). Indeed, microorganisms using NMDH to oxidize methanol has been explored under anaerobic conditions for liquid fuel production (http://www.coskata.com). However, the thermodynamic constraints on NMDH would require an optimal growth temperature of

TABLE 1 Comparison of different methanol pathways

Module name[1]	Substrates	Products	ATP equivalents	NADH equivalents	CO_2 equivalents	Δ_rG'(kJ/mol)[ad]	Energetic efficiency (or microbial conversion) (Cotton et al., 2020)	Location	Cofactor	KM (mM)	Biomass productivity gCDW/l/h	Temperature optima (°C)
	First module											
NMDH	3 CH_3OH	3 HCHO	0	+ 3	0	+ 34.2 (Whitaker et al., 2015)		Cytoplasm	ACT, NAD^+	20	-	55
AOX	3 CH_3OH	3 HCHO	0	0	0	− 99.2 (Whitaker et al., 2015)		Peroxisome	PYC,O_2, FAD	0.47	-	37
PMDH	3 CH_3OH	3 HCHO	0	0	0	− 24.8 (Whitaker et al., 2015)		Cytoplasm	PQQ, Ca^{2+}	0.02	-	37
	Second module											
RuMP cycle	3 HCHO	1 Acetyl-CoA	+ 1	+2	+1[c]	−38.3[d]	37%-49%	Cytoplasm	RuBP	-	28.4 (Bozdag, 2013)	
XuMP cycle	3 HCHO	1 Acetyl-CoA	−1	+2	+1[c]	−78.4[d]	31%-36%	peroxisome (Rußmayer et al., 2015)	Xu5P	-	-	
Serine cycle	3 HCHO	3 Acetyl-CoA	−6	−6	−3[c]	−92.0[d]	32%-37%	Cytoplasm	Serine, $CH_2=H_4F$	-	1.2–3.6 (Bozdag, 2013)	

Note: All calculations in the first (second) module are based on converting methanol (formaldehyde) into formaldehyde (acetyl-CoA).

[a] Standard conditions are 25°C.

[b] The K_M values are obtained from BRENDA (https://www.brenda-enzymes.org/).

[c] CO_2 equivalents did not take dissimilation pathway into consideration.

[d] Δ_rG' in three different methanol utilization pathway were predicated by eQuilibrator (http://equilibrator.weizmann.ac.il/), pH 7, ionic strength = 0.1 M.

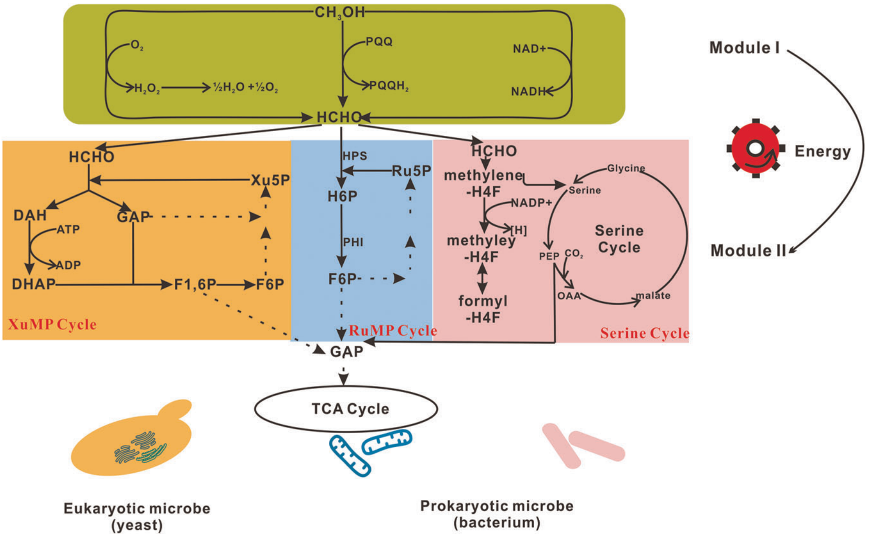

FIGURE 2 Methanol metabolic pathways in methylotrophs. AOX, alcohol oxidase; CAT1, catalase; DAK, dihydroxyacetone kinase; DAS, dihydroxyacetone synthase; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; EMP, Embden-Meyerhof-Parnas pathway; F1,6P, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; FBA, fructose-1,6-bisphosphate aldolase; FBP, fructose-1,6-bisphosphatas; GAP, glyceraldehyde-3-phosphate; H6P, D-arabino-3-hexulo-6-phosphate; HPS, hexulose phosphate synthase; MDH, methanol dehydrogenase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PHI, phosphohexulose isomerase; PPP, pentose phosphate pathway; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate [Color figure can be viewed at wileyonlinelibrary.com]

45–55°C, which is challenging for most microorganisms. Alternatively, HCHO must be kept at exceptionally low concentrations (<50 μM) (Woolston, King, et al., 2018). Such a low concentration of HCHO would however pose a challenge for one of the downstream enzymes in formaldehyde assimilation, which must then be engineered to have very strong substrate affinity towards HCHO.

The second module, HCHO assimilation, can occur via three possible routes: the serine pathway, the XuMP pathway, and the RuMP pathway. The serine pathway is the most carbon-efficient among the three pathways, as it can fix 3 mol of CO$_2$ along with 3 mol HCHO to form 3 mol acetyl-CoA. However, it is also the most energy-expensive pathway as it requires 2 mol of ATP and 2 mol of redox equivalents for each mole of acetyl-CoA produced. In contrast, the RuMP pathway is the most energy-efficient, because for each mole of acetyl-CoA produced, 2 mol of NADH plus one mole ATP are generated. Finally, the XuMP pathway generates 2 mol of NADH but consumes one mole ATP per mole of acetyl-CoA produced. These relationships are reflected by the specific maximum growth rate of bacteria expressing each of these three pathways: serine pathway (0.015–0.07 h^{-1}) < XuMP pathway (0.06–0.11 h^{-1}) < RuMP pathway (0.1–0.14 h^{-1}) (Cotton et al., 2020; Vartiainen et al., 2019).

Between all possible routes in the methanol oxidation and assimilation modules, four combinations are naturally occurring in methylotrophs. The first is the combination of NAD-dependent methanol dehydrogenase and ribulose monophosphate pathway (NMDH-RuMP pathway, NR) (Dijkhuizen et al., 1992), mainly found in thermophilic Gram-positive methylotrophic bacteria. The second is the oxygen-requiring alcohol oxidase followed by the xylulose monophosphate pathway (AOX-XuMP pathway, AX) (Schroer et al., 2010), which mainly exist in methylotrophic yeasts. The third is methanol dehydrogenase combined with the serine pathway (NMDH-serine pathway, NS) (Vuilleumier et al., 2009), also mainly found in bacteria. The last is pyrroloquinoline quinone (PQQ) dependent MDH followed by the ribulose monophosphate (PQQ-RuMP pathway, PR) pathway, mainly found in Gram-negative methylotrophic bacteria (Keltjens et al., 2014). Among those four native methanol metabolic pathways, the NR pathway incurs the lowest energy-cost, generating 1 mol ATP and 5 mol NADH (which could be converted to energy via respiration) when 3 mol methanol is fixed. However, this pathway has a positive Δ_rG' (+34.2 kJ/mol). The PR route is the second most efficient in terms of energetics, producing 1 mol ATP and 2 mol NADH when 3 mol methanol is fixed; followed by the AX pathway, producing 2 mol NADH but consuming 1 mol ATP when 3 mol methanol fixed. The AX pathway exhibits the lowest Δ_rG' (−99.2 kJ/mol), making it the most thermodynamically feasible in synthetic methylotrophs. Finally, the pathway with the highest energy cost is the NS pathway, which consumes 6 mol ATP and 6 mol NADH to fix 3 mol methanol.

Because of these energy generation characteristics, currently NR is the most common pathway to be engineered in the construction of methylotrophic microorganisms, followed by the AX and NS pathways (Dai et al., 2017; Gonzalez et al., 2018; Meyer et al., 2018; Yu & Liao, 2018).

3. ENGINEERING THE METHANOL UTILIZATION PATHWAYS

Although C–C bond construction from C1 compounds via chemical synthesis has been proven possible, this process remains challenging due to the requirement of high temperature, pressure, and energy

(Yu & Liao, 2018). Bioconversion is a promising alternative, with a relatively low energy cost and being generally an environmentally friendly process. The current use of native methylotrophic microorganisms, however, suffers from certain drawbacks such as poor genetic availability and low metabolic yield. Therefore, engineering nonnative methylotrophic microbes is a promising route to harvest C1 sources and convert them into value-added products. Below we review recent progress in engineering nonnative methylotrophic microbes both in vitro and in vivo.

3.1 In vitro engineering of the methanol utilization pathways

Cell-free methanol utilization systems have been constructed which side-step many complex cellular processes such as gene transcription, protein folding and assembly, and competing pathways within the cellular metabolic network (Price et al., 2016). The NR pathway is preferred for in vitro constructions of methanol metabolic pathways, due to its more efficient energetics in terms of ATP and NADH production (Bogorad et al., 2014). However, a significant energy barrier of NMDH must be overcome, which requires HCHO and NADH to be maintained at low concentrations to ensure that the reaction favors methanol oxidation. To improve NMDH activity and increase its affinity to methanol, two strategies have been tested. The first is to express an NAD-dependent but ACT-independent Mdh2 enzyme. Both directed evolution and protein engineering have been used to construct such a synthetic enzyme. For example, directed molecular evolution has resulted in a mutated version of CT4-1 (A126V, A31V, A169V) which has a 6-fold higher K_{cat}/K_m for methanol ($9.3\,M^{-1}S^{-1}$) and 10-fold lower K_{cat}/K_m for n-butanol ($48\,M^{-1}S^{-1}$) in vitro (Wu et al., 2016). In an independent study, a phage-assisted noncontinuous evolution (PANCE) method was adapted for Mdh2 evolution, resulting in an Mdh2 variant (Q5L, A363L) with up to 3.5-fold higher V_{max} (Roth et al., 2019). The second strategy to improve NMDH activity is to compress the reaction space. For example, by using a scaffoldless self-assembly methodology to organize Mdh, Hps, and Phi into an engineered supramolecular enzyme complex via SH3-ligand interaction pairing, in vitro production of fructose-6-phosphate (F6P) from methanol improved 97-fold (Price et al., 2016). Expression of an "NADH Sink" using the *E. coli* lactate dehydrogenase as an NADH scavenger, further improved methanol consumption in vitro.

This concept described above has also been tested in vivo using *E. coli*, however the improvement in methanol consumption here was smaller compared with that achieved in vitro. Although in vitro cell-free systems have shown outstanding characteristics in biofules production (Korman et al., 2017; Y.-H. P. Zhang, 2011), challenges remain such as stability of reaction system for multiple enzyme reactions (Bogorad et al., 2014) and toxic metabolites, for example, formaldehyde tolerance, to operate at large-scale.

Finally, to achieve the highest possible carbon conservation, a methanol condensation cycle (MCC) has been constructed by a combination of non-oxidative glycolysis (NOG) and the RuMP pathway. This synthetic approach completely avoids the complexity of NMDH from methylotrophs. MCC was first proven to be functional in vitro by conversion of methanol to ethanol or n-butanol. Using alcohol dehydrogenase from *S. cerevisiae*, the MCC was demonstrated to produce 610 mg/L ethanol from 6200 mg/L methanol, or 170 mg/L n-butanol from 6200 mg/L methanol in vitro (Bogorad et al., 2014). However, the use of MCC in vivo has not been reported so far.

3.2 Engineering nonnative methylotrophic microbes

Although progress have been shown in vitro, it is more challenging to achieve methanol utilization in vivo due to the complexity of the cellular environment. Engineering methanol metabolic pathways in nonnative methylotrophs have been mainly carried out in *E. coli*, *S. cerevisiae* and *Corynebacterium glutamicum* (Bennett et al., 2018) to produce high-value commodities (Balk et al., 2003; Fei et al., 2014; Haynes & Gonzalez, 2014).

In *E. coli*, since the cofactor PQQ required in PR pathway cannot be synthesized (Anthony, 2004; Davidson, 2001; Matsushita et al., 1997), the NR pathway is the most frequent choice to engineer (J. E. Müller et al., 2015; Rohlhill et al., 2017; W. Brian Whitaker et al., 2017). A number of NMDH enzyme candidates from different host organisms have been evaluated in *E. coli* for methanol utilization. ^{13}C labeling experiments showed that up to 40% incorporation of carbon in glycolytic and pentose phosphate pathway (PPP) intermediates were derived from methanol, when cells were incubated in co-carbon medium (J. E. Müller et al., 2015; W. Brian Whitaker et al., 2017). By expressing an NMDH homolog from *B. stearotermophilus* (W. Brian Whitaker et al., 2017), and with supplementation of yeast extract in the growth media, cells were able to grow on methanol (from $OD_{600} = 0.04$ to 0.08) (Gonzalez et al., 2018), and convert methanol to naringenin (W. Brian Whitaker et al., 2017).

In addition to *E. coli*, the NR pathway has also been integrated into *Corynebacterium glutamicum*. ^{13}C-labeling results indicated that more than 25% methanol was incorporated into glycolytic and PPP intermediates (Tuyishime et al., 2018). Moreover, supplying methanol as a co-substrate together with glucose or ribose further improved methanol consumption (with a methanol consumption rate of $1.7\,mM\,h^{-1}$) (Leßmeier & Pfeifenschneider, Carnicer, et al., 2015; Leßmeier & Wendisch, 2015; Witthoff et al., 2015).

In addition to the NR pathway, the AX pathway has also been tested for methylotrophic microbe construction. Results indicated that the AX pathway was more efficient than the NR pathway in *S. cerevisiae* (Dai et al., 2017). Lastly, a non-naturally occuring "hybrid" pathway (NMDH-Xu5P), which was rationally designed based on the observed advantages and disadvantages of natural methanol metabolic pathways, has also been introduced into *S. cerevisiae*. In this synthetic yeast strain, ^{13}C-labeling experiments showed that more than 30% CO_2 production was derived from methanol (Espinosa et al., 2019).

4. CHALLENGES IN ENGINEERING SYNTHETIC METHYLOTROPHS

Despite significant efforts in developing synthetic methylotrophs in *E. coli*, *C. glutamicun* and *S. cerevisiae*, to date none of engineered strains can grow on methanol as the sole carbon source. Below we summarize some of the ground challenges that are limiting in methylotrophic microbe construction.

4.1 Tolerance of metabolic intermediates

While methanol is a promising carbon source, many metabolites of its catabolic pathways, including formaldehyde (Yurimoto et al., 2005), DHA (Molin & Blomberg, 2006), and H_2O_2 (Yurimoto, 2009), are toxic for cells (Table 2). Among these metabolic intermediates, formaldehyde is the most toxic to many microorganisms. Studies have shown that the growth defect of methylotrophic yeast (*Pichia methanolica*) on high methanol media is not caused directly by methanol toxicity, but rather by formaldehyde toxicity (Wakayama et al., 2016). To counter this toxicity, the formaldehyde dissimilation pathway can be activated to detoxify formaldehyde (Pfeifenschneider et al., 2017). These results suggest that the formaldehyde assimilation module may be the limiting step in methanol utilization (Papoutsakis et al., 1978). Therefore, the heterologous hosts must be engineered to either be tolerant to formaldehyde, or have efficient pathways to quickly detoxify it. Two approaches can be taken to reduce formaldehyde toxicity in methylotrophic strains. The first is to strengthen the efficiency of the formaldehyde dissimilation pathway. This strategy not only accelerates formaldehyde degradation, but also provides NADH which can be used for ATP generation. However, it leads to carbon loss in the form of CO_2. The second approach is to convert formaldehyde into a less toxic compound. For example, in the AX pathway the affinity of dihydroxyacetone synthase (DAS, Figure 2) towards formaldehyde is much higher (Km = 0.43) (Kato et al., 1982) than that of AOX for methanol (Km = 3) (Nichols & Cromartie, 1980), which indicates that DAS may be well-suited for quickly relieving formaldehyde toxicity in synthetic microbes utilizing AX pathway for methanol oxidation.

4.2 Cofactor balance

To overcome the thermodynamic constraint on methanol oxidation catalyzed by NMDH ($\Delta_r G' = 34.2 \pm 6.5\ \text{kJ mol}^{-1}$), an alternative method to promote this reaction is to accelerate NAD^+ regeneration. One way to achieve this is to weaken other NAD^+ utilization pathways. For example, Meyer et al. has constructed an *E. coli* strain wherein methanol is essential for growth, by knocking out NAD^+-dependent malate dehydrogenase and reducing the function of the NAD repressor nicotinamide mononuculeotide adenylyltransferase NadR (Meyer et al., 2018). These modifications led to a rebalance toward a high NAD^+/NADH ratio, which thermodynamically favored the oxidation of methanol. Another promising method is to build an NADH-ATP generation cycle. Since NADH can be transported into the mitochondria to generate ATP, enhancing the NADH shuttle system (i.e., malate-aspartate shuttle) (Eto et al., 1999) may be a good solution that is capable of not only promoting NAD^+ regeneration, but also providing additional ATP which can be used to further increase methanol utilization.

4.3 Energy supply

Based on the reactions shown in Table 1, it is clear that even though the NR pathway is energetically self-sufficient, excessive NADH will inhibit NMDH activity. This may be the reason why an NADH sink was necessary in a previous report of microbes expressing the NR pathway to metabolize methanol (Price et al., 2016). In contrast to the RuMP cycle, all other methanol-assimilating pathways have an energy demand. So far, two methods have been used to meet this energy demand. One is to strengthen the formaldehyde dissimilation pathway, which produces ATP from the generated NADH, although it can lead to > 40% carbon loss (Jorda et al., 2014). The formaldehyde dissimilation pathway generates 2 mol NADH in two enzymatic steps, which requires a very small proteomic investment and thus more proteomically efficient than glycolysis and the TCA cycle in generating protons for energy production. Moreover, enhanced formaldehyde dissimilation would relieve formaldehyde toxicity, which is likely why the TCA cycle was inhibited while the formaldehyde dissimilation pathway was up regulated in *P. pastoris* (Rußmayer et al., 2015).

The second method to meet the energy demand of methanol utilization is by adding other carbohydrates to the culture medium (Gonzalez et al., 2018; Meyer et al., 2018; Woolston, Roth, et al., 2018). For example, when the serine cycle was introduced into *E. coli*, the engineered strains were able to co-assimilate methanol together with a pyruvate source, i.e., xylose, thereby improving the production of acetyl-CoA derived C2 compounds (Yu & Liao, 2018). Dai et al. showed that when the AX pathway was introduced into *S. cerevisiae*, the recombinant strain consumed methanol up to 2.35 g/L, leading to 11.70% increase in cell growth (OD_{600}), when supplied with 1 g/L yeast extract (Dai et al., 2017). In *C. glutamicum*, biomass was improved by up to 30% in minimal medium containing both methanol and glucose (Witthoff et al., 2015), and up to 15.7% ^{13}C-labeled methanol was used for cadaverine production in minimal medium containing both methanol and ribose (Leßmeier et al., 2015). As additional carbohydrates are required to supply the energy necessary for methanol degradation, growth with NR independent pathway on methanol as the sole carbon source would be difficult.

4.4 Intermediate recycling

The last but not least challenge is the regeneration of intermediates such as ribulose-5-phosphate (Ru5P) or xylulose-5-phosphate

TABLE 2 Tolerance for different intermediates

Compounds	Organism	Max tolerance concentration	KM (mM)[a]	Characteristics
Formaldehyde (HCHO)	*Yarrowia lipolytica*	1 mM (Vartiainen et al., 2019)	Dihydroxyacetone synthase (DAS): 0.43 (Kato et al., 1982) −1.86 (Ro et al., 1997), hexulose phosphate synthase (HPS): 0.98 (Rozova et al., 2017)−1.4 (R. Müller & Babel, 1980)	Damage proteins, nucleic acid and lipids and inhibit cell grow (Nian et al., 2013; Pilat & Prokop, 1975; Songur et al., 2010)
	S. cerevisiae	IC_{20} = 0.6 mM (North et al., 2016)		
	Pichia methanolica	0.3 mM (Wakayama et al., 2016)		
	Candida boidini 11 Bh	3.6–4.0 mM (Pilat & Prokop, 1976)		
	Pichia pastoris	5 mM (Sunga & Cregg, 2004)		
Dihydroxyacetone (DHA)	*S. cerevisiae*	200 mM (Molin & Blomberg, 2006; Molin et al., 2003)	Dihydroxyacetone kinase (DAK): 0.005 (Molin et al., 2003) −0.008 (Gutknecht et al., 2001)	Carbon-based toxicity (Molin et al., 2003) and the toxicity of DHA appears to be due to its intracellular conversion to an aldehyde compound, presumably methylglyoxal (Subedi et al., 2008)
Hydrogen peroxide (H_2O_2)	*S. cerevisiae*	2–3 mM (Hasan et al., 2002; Poljak et al., 2003)	Catalase (CAT1): 0.65 (Hochman & Goldberg, 1991) – 15 (Santoni et al., 2004)	Result in DNA and membrane damage that cause mutagenesis and kill cells (Brunk et al., 1995; Imlay et al., 1988)
	Candida albicans	50 mM (González-Párraga et al., 2003)		
	Yarrowia lipolytica	120 mM (Biryukova et al., 2006)		

[a] The KM values are obtained from BRENDA (https://www.brenda-enzymes.org/).

(Xu5P). The most common solution to this is to use another carbohydrate as a co-substrate to sustain a pool of these intermediates. Several feedstocks such as gluconate and xylose have been used as precursors for these intermediates to support methanol utilization. Vorholt et al. showed that gluconate or glucose could provide Ru5P through the PPP, which significantly accelerates HCHO fixation (Meyer et al., 2018). Woolston, Roth, et al. (2018) showed that xylose is a promising co-carbon source for HCHO fixation owing to its capability of forming Xu5P or Ru5P directly. Although such co-carbon strategies could promote methanol utilization, biomass formation is not mainly supported by methanol. Engineering of alternative pathway for regeneration of C5 sugar phosphates in bacteria, as demonstrated by activating sedoheptulose bisphosphatease variants, may provide some hints for the eventual resolution of this problem (Woolston, Roth, et al., 2018). Hannes et al. have shown that the Xu5P regeneration pathway has been duplicated in the evolution of *P. pastoris*, which has been compartmentalized into the peroxisome (Rußmayer et al., 2015). This indicates that engineering of enzyme compartmentalization may be an alternative strategy to improve the recycling of metabolic intermediates in methanol utilization pathways.

5. CONCLUSION REMARKS

In recent years, much attention has been focused on the development of methylotrophic organisms due to the significant advantages of methanol fermentation and its potential application in conversion of methane. However, it is clear that most of the engineered methylotrophic strains showed poor growth capability in methanol medium (Cantera et al., 2018; Duan et al., 2015; Schrader et al., 2009), which limits the engineering of methanol metabolic pathways to produce high-value products in both native and nonnative methylotrophic microorganisms. To promote the construction of methylotrophic microbial cell factories, the reiterative design–build–test–learn (DBTL) cycle of metaboic engineering (Chen et al., 2017; Nielsen & Keasling, 2016) may be employed as described below.

5.1. Design

In the process of engineering native and nonnative methylotrophy, most efforts have been focused on introducing natrual pathways into model microbes. However, each natural pathway presents a different set of drawbacks, therefore different synthetic novel pathways may be better suitable for different applications. For example, computational analysis of thousands of metabolic reactions to address energy consumption and carbon loss, have led to several nonnatural formaldehyde assimilation pathways (Yang et al., 2019). Also, some synthetic or 'hybrid' pathways like variants of reductive glycine pathway (Cotton et al., 2020) have been found to outperfome the native pathway. For each application, to achieve the optimal design of a methanol-utilizing microbial cell factory, three modules should be considered: module I (methanol-degradation module)—methanol adsorption and fixation; module II (energy-producing module)—energy generation to support methanol degradation; and module III (Intermediate recycle module)—supplying sufficient intermediates for HCHO assimilation and promoting intermediate recycle (Figure 3). In addition, a balance between the characteristics of the final products, the efficiency of the designed pathway(s), and the biomass yield should be carefully considered during the design phase.

5.2. Build

Novel synthetic biology modules and tools should be employed for the construction and optimization of methanol-utilizing microbial cell factories. For instance, one key tool is for heterologous gene expression, whereby methanol utilization genes are identified and introduced into host species, for example using CRISPR/Cas9-based methodologies (Caspeta et al., 2014). To finetune the expression of these heterologous genes, regulatory elements such as gene promoters may be targeted for engineering, as many studies have shown that most genes involved in methanol utilization pathways are tightly regulated by methanol or its metabolites (Rußmayer et al., 2015; Yurimoto et al., 2000). Other enzyme characteristics can also be optimized, for example by improving substrate affinity and reducing substrate/metabolites inhibition, via directed evolution, rational protein engineering or a combination of enzyme engineering methodologies (Eriksen et al., 2014). The use of advanced biosensors, that is, the recently developed formaldehyde biosensor (Woolston, Roth, et al., 2018), should also be incorporated in the process of building methanol-utilizing microbial cell factories, to dynamically control the methanol dissimilation pathway, and thus supporting energy generation and reducing carbon loss via this route.

5.3. Test

The purpose of this step of the DBTL cycle is to characterize the constructed cell factories, which provides crucial information for the learning phase and to guide the next iterative DBTL cycle. A number of evaluation criteria can be applicable as an evaluation index, of which the cell growth rate on methanol is the most intuitive and most commonly used. In addition to the cell growth rate, adaptability to methanol can be evaluated via verification of the utilization efficiency of methanol as a carbon source, and tolerance to methanol and its downstream metabolites. Pathway efficiency and omics-based anlayses can also be used in the testing phase to evaluate the microbial cell facotires, for example, through transcription network analysis and metabolic flux analysis (-Figure 3). As these evaluations are often costly and time-consuming, strategies to shorten the testing phase and improve evaluation efficiency, such as mechanic/robotic high-throughput

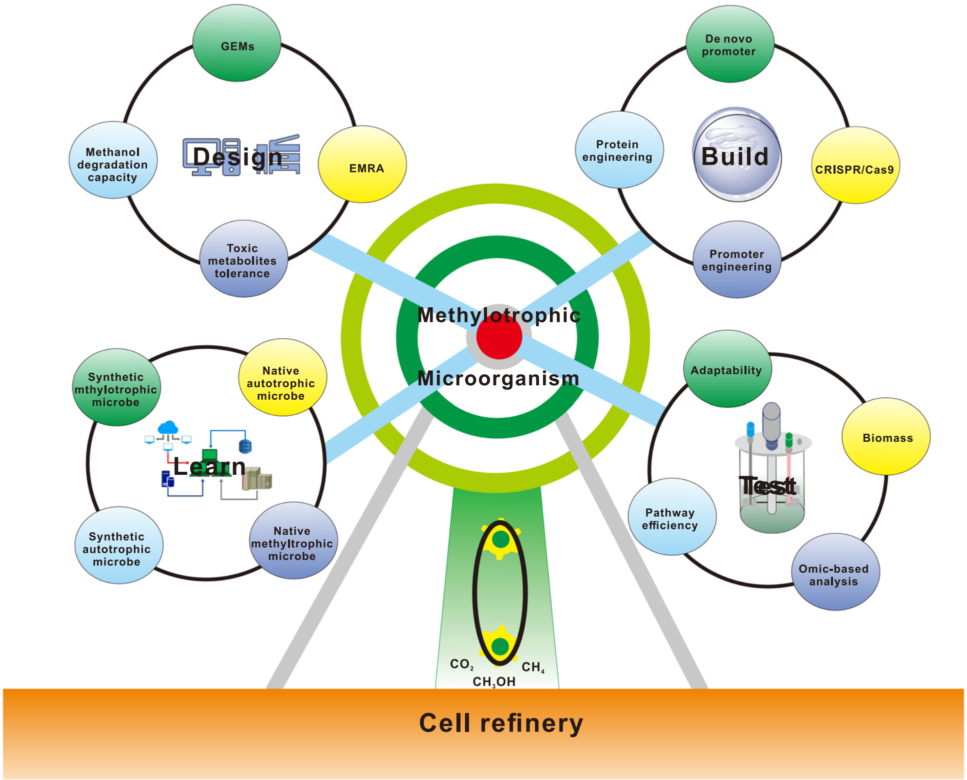

FIGURE 3 The use of design–build–test–learn cycle for methylotrophic microorganism construction. Design: Based on available knowledge and computational-aided modeling to guide the design of efficient metabolic pathways for methanol carbon utilization. Build: using novel synthetic biology tools and modules to construct metabolic pathways in different microbial hosts for methanol utilization. Test: detailed characterization of constructed strains in terms of cell growth, product formation, and even omics-based phenotyping of strains. Learn: the generated data will be combined with computational based integrative analysis to find further engineering targets for the next cycle, to reach finally the desired methylotrophic microbial cell factory [Color figure can be viewed at wileyonlinelibrary.com]

approaches, careful selection of specific test characteristics, and/or rapid omics-based analyses would lead to significant improvements in the DBTL cycle to construct methanol-utilizing cell factories (Srivatsan et al., 2020).

5.4. Learn

A better understanding of the metabolic pathways in native methylotrophs is an important step towards constructing more efficient synthetic methylotrophic organisms. For example, based on a deep analysis of the AX pathway in *P. pastoris*, combined with lessons learned while constructing nonnative methylotrophic microbes, it is now understood that a sufficient supply of metabolic intermediates, high-efficiency energy transformation from NADH, as well as the ability to dissimilate formaldehyde, are three of the most important characteristics for efficient AX pathway construction. In addition, more and more synthetic methylotrophic constructions and characterizations through the iterative design–build–test–learn (DBTL) cycle of metabolic engineering will generate more useful data and provide new knowledge for future methylotrophic microorganism construction.

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AUTHOR CONTRIBUTIONS

Chunjun Zhan wrote the manuscript. Xiaowei Li provided related papers. Yankun Yang gave some useful suggestions. Jens Nielsen, Zhonghu Bai, and Yun Chen revised this paper and gave lots of great suggestions. All authors read and approved the final manscript.

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