Methanol Dehydrogenases as a Key Biocatalysts for Synthetic Methylotrophy

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One-carbon (C1) chemicals are potential building blocks for cheap and sustainable resources such as methane, methanol, formaldehyde, formate, carbon monoxide, and more. These resources have the potential to be made into raw materials for various products used in our daily life or precursors for pharmaceuticals through biological and chemical processes. Among the soluble C1 substrates, methanol is regarded as a biorenewable platform feedstock because nearly all bioresources can be converted into methanol through syngas. Synthetic methylotrophy can be exploited to produce fuels and chemicals using methanol as a feedstock that integrates natural or artificial methanol assimilation pathways in platform microorganisms. In the methanol utilization in methylotrophy, methanol dehydrogenase (Mdh) is a primary enzyme that converts methanol to formaldehyde. The discovery of new Mdhs and engineering of present Mdhs have been attempted to develop synthetic methylotrophic bacteria. In this review, we describe Mdhs, including in terms of their enzyme properties and engineering for desired activity. In addition, we specifically focus on the application of various Mdhs for synthetic methylotrophy.

Keywords: methanol dehydrogenase, synthetic methylotrophy, C1 gas, assimilation, formaldehyde

INTRODUCTION

One-carbon (C1) substrates are potential feedstocks and have recently gained attention and preference in industrial fields due to their natural abundance, low production cost, and availability as industrial by-products (Jiang et al., 2021). Among C1 chemicals, methanol is a potentially renewable feedstock for microorganisms as it is electron rich and can be derived from methane or CO₂ (Chen et al., 2020). In nature, methylotrophs, such as Methylobacterium extorquens and Bacillus methanolicus, can utilize methanol, and their biochemical function have been characterized (Brautaset et al., 2007; Bennett et al., 2018). However, so far, there are limitations in the engineering of native methylotrophs to produce heterologous products at high rates and titers due to the lack of genetic tools available. Recent advances in synthetic biology, integration of efficient methanol converting enzymes, genome engineering, and laboratory evolution are enabling the first steps toward the creation of synthetic methanol-utilizing microorganisms (Heux et al., 2018; Meyer et al., 2018; Bennett et al., 2020; Chen et al., 2020; Keller et al., 2020; Wang et al., 2020).

In the methanol utilization in methylotrophy, one of the key steps is the oxidation of methanol to formaldehyde by oxidoreductase (Zhang et al., 2017), and methanol dehydrogenases (Mdhs) are the main enzymes as they catalyze the oxidation of methanol to formaldehyde with two electrons.
and 2H⁺ (Le et al., 2021) (Figure 1). There are three native pathways of formaldehyde assimilation, that have been discovered and biochemically described for growth support of microorganisms in methanol, as follows: the ribulose monophosphate (RuMP) cycle, serine pathway, and xylulose monophosphate (XuMP) cycle (Zhang et al., 2017). The RuMP and serine cycles mainly occur in prokaryotes, the XuMP cycle is found in yeasts. Among them, the RuMP cycle of hexulose-6-phosphate synthases (HPS) and 6-phospho-3-hexulose isomerase (PHI) has been identified as the best combination because of its highest theoretical growth rate; thus, it has received the most attention (Heux et al., 2018; Claassens et al., 2019). Meanwhile, there have been a modified serine cycle in *Escherichia coli* was reported (Yu and Liao 2018) and only one study on XuMP in *Saccharomyces cerevisiae* (Dai et al., 2017).

Various hypotheses have been proposed regarding potential bottlenecks to efficient methanol assimilation. In particular, the concentration of Mdhs is a limitation, and poor kinetic and thermodynamic properties of methanol oxidation by nicotinamide adenine dinucleotide (NAD⁺)- Mdh is widely acknowledged (Whitaker et al., 2017; Woolston et al., 2018). The low activity and substrate affinity of Mdh fundamentally limits methanol assimilation flux, while a high NADH/NAD⁺ ratio negatively impacts the Gibbs free energy of methanol oxidation (Wang et al., 2020). Thus, the development of efficient Mdhs presents a significant challenge to synthetic methylotrophy. In this review, we summarize the current classifications, enzyme properties, and engineering of reported Mdhs. Additionally, we provide a comprehensive overview of recent advances in the use of Mdhs in engineering synthetic methylotrophy.

### Class of Methanol Dehydrogenases

Depending on the electron acceptors, Mdhs in methylotrophs are classified into three groups: NAD⁺-dependent Mdh, PQQ (pyrrolo-quinoline quinone)-dependent Mdh, and O₂-dependent AOX (alcohol oxidase) (Figure 2).

#### NAD⁺-dependent Mdh

NAD⁺-dependent Mdh in thermophilic Gram-positive methylotrophs uses NAD⁺ as the cofactor for the methanol oxidation. The first NAD⁺-dependent Mdh was reported in 1989 (Arfman et al., 1989). NAD⁺-dependent Mdhs also obtained from non-methylotrophic bacteria. To date, several NAD⁺-dependent Mdhs have been isolated from *Bacillus* sp. (such as *B. methanolicus* (Arfman et al., 1989; Arfman et al., 1991; Müller et al., 2015; Witthoff et al., 2015; Price et al., 2016) and *B. steaorthermophilus* (Whitaker et al., 2017)), *Lysinibacillus* sp. (such as *L. xylanilyticus* (Lee et al., 2020)), and *Cupriavidus* sp. (such as *C. necator* (Wu et al., 2016)). In particular, their NAD⁺-dependent Mdhs have been focused and reported for studies of recombinant *E. coli* as synthetic methylotrophs (Müller et al., 2015; Wu et al., 2016; Whitaker et al., 2017; Lee et al., 2020; Le et al., 2021). Three NAD⁺-dependent Mdhs have been found in *B. methanolicus* MGA3 (Mdh, Mdh2, and Mdh3). Moreover, the activity of all three Mdhs is modulated by an endogenous Mdh activator protein (ACT). In vitro studies suggest that ACT
enhances the methanol affinity, oxidation rate, and catalytic activity of Mdh; however, the detailed mechanism for activation is currently unclear (Hektor et al., 2002; Witthoff et al., 2015) and no effect has been shown in vivo in a synthetic methylotrophy (Müller et al., 2015) because detail research for the activator protein functions in native host has not been tested. To enable the assimilation of methanol as the carbon source in metabolic engineering, ACT-independent Mdh and their mutants from C. necator (Wu et al., 2016; Chen et al., 2018) and L. xylanilyticus (Lee et al., 2020; Le et al., 2021) have been reported and introduced into E. coli for methanol assimilation. As best candidate for synthetic methylotrophy, NAD\(^+\)-dependent Mdh that can perform its function under both aerobic and anaerobic conditions (Zhang et al., 2017). Besides, it uses NAD\(^+\), which is ubiquitous and can provide electrons for metabolite products, as the cofactor. Therefore, it may be the best candidate for recombinant-based synthetic methylotrophs (Zhang et al., 2017).

**PQQ-dependent Mdh**

In Gram-negative methylotrophs, the oxidation of methanol occurs in the periplasmic space by PQQ-dependent Mdh (Skovran et al., 2019). Pure PQQ-dependent Mdh was first described in 1967 (Anthony and Zatman 1967). To date, PQQ-dependent Mdh has been isolated and purified from several different strains of microorganisms including Pseudomonas sp. (Anthony and Zatman 1965; Anthony and Zatman 1967; Patel et al., 1972), Methylococcus capsulatus (Patel et al., 1972), Hyphomicrobium denitrificans (Nijiri et al., 2006), Methylorubrum extorquens (formerly Methylbacterium extorquens) (Anthony 2004; Liu et al., 2006; Nakagawa et al., 2012), Methylversatilis universalis FAM5 (Kalyuzhnaya et al., 2008), Methylbium petroleiphilum (Kalyuzhnaya et al., 2008), Methylphaga petroleiphilum (Kalyuzhnaya et al., 2008), Methylobacterium nodulans (Kuznetsova et al., 2012), Methylibium petroleiphilum (formerly Methylomicrobium petroleiphilum) (Kalyuzhnaya et al., 2008), and Bradyrhizobium boryatense (Deng et al., 2018), M. fumariconicum (Jahn et al., 2018), and Bradyrhizobium diazefficients (Wang et al., 2019). The PQQ-dependent Mdh contains a PQQ prosthetic group. The chemical structure of the PQQ prosthetic group has been confirmed by two independent research groups using a wide range of chemical and physical techniques, such as X-ray, UV/Vis absorption spectra, and HPLC (Anthony 1982). The role of the PQQ prosthetic group is capturing electrons from methanol oxidation and passing them to the cytochrome (Anthony 2004). The biggest disadvantage is the requirement of molecular oxygen for PQQ bio-synthesis (Velterop et al., 1995), while some desired intermediates as precursors of value-added products such as lactate must be produced under anaerobic conditions. Therefore, this limits the application of PQQ-dependent Mdh.

In genomes of methylotrophs, PQQ-dependent Mdh are generally encoded by MxaF and XoxF. MxaF consists of small (MxaI) and large (MxaF) subunits, encoding PQQ-dependent Mdh using calcium (Ca\(^{2+}\)) as a cofactor (MxaF-Mdh) (Anthony 2004). Another PQQ-dependent Mdh, which uses lanthanides (Ln\(^{3+}\)) instead of Ca\(^{2+}\), is encoded by XoxF (XoxF-type Mdh) (Chistoserdova 2016; Skovran et al., 2019). XoxF-Mdh from M. extorquens AM1 is a representative of Ln\(^{3+}\)-dependent Mdh that it was studied carefully to show the biochemical characterization. XoxF of M. extorquens AM1 showed better activity when La\(^{3+}\) or Ca\(^{2+}\) and La\(^{3+}\) were added together than when Ca\(^{2+}\) was used alone as part of the cofactor complex (Vu et al., 2016; Good et al., 2020). In addition, other elements of lanthanide (Ce\(^{3+}\), Nd\(^{3+}\), Pr\(^{3+}\), Sm\(^{3+}\), Eu\(^{3+}\), or Gd\(^{3+}\)) were also found to be involved in the methanol oxidation activity (Pol et al., 2014). Lanthanides as important factor was suggested in regulatory and catalytic functions because the XoxF genes are required for transcription of the MxaFI (Vu et al., 2016).

**O\(_2\)**-dependent AOX

Unlike NAD\(^+\)-dependent and PQQ-dependent Mdh, O\(_2\)-dependent AOX is obtained from eukaryotic methylotrophs and is located in the peroxisome of yeasts (Egli et al., 1980). First, formaldehyde and hydrogen peroxide (H\(_2\)O\(_2\)), which are highly toxic chemicals for cells, are created from methanol oxidation by O\(_2\)-dependent AOX. To protect the cells, dihydroxyacetone synthase (DAS) and catalase (CTA) work to transform them into non-toxic chemicals (Zhang et al., 2017). O\(_2\)-dependent AOX only function under aerobic conditions and thus, has limitations similar to those of PQQ-dependent Mdh. In addition, another important limitation AOX’s is that the electrons from methanol are not captured as usable energy by the cell, but wasted in the generation of peroxide.

**BIOCHEMICAL CHARACTERIZATION OF METHANOL DEHYDROGENASES**

Among three classes of Mdh, enzyme properties of NAD\(^+\)- and PQQ-dependent Mdh are summarized in Table 1.

**Optimal Conditions for Methanol Oxidation Reaction by Mdh**

The most important factor, which has a considerable effect on the activity of Mdh, is cofactor binding. For NAD\(^+\)-dependent Mdh, a metal ion is involved in cofactor binding which may influence enzymatic activity (Hektor et al., 2002). Several metal ions have been examined for the effects on the methanol oxidation activity of Mdh, such as Fe\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), or Mg\(^{2+}\) ions (Sridhara et al., 1969; Arfman et al., 1991; Montella et al., 2005; Müller et al., 2015; Wu et al., 2016; Whitaker et al., 2017; Lee et al., 2020; Le et al., 2021). In general, the supplementation of Fe\(^{2+}\) or Mn\(^{2+}\) ions increase enzyme activity, and Mdh activity is inhibited by Cu\(^{2+}\), Co\(^{2+}\), or Zn\(^{2+}\) (Sridhara et al., 1969; Montella et al., 2005). In the case of Mdh from Lysinibacillus xylanilyticus (Lxmdh), Mn\(^{2+}\) or Fe\(^{2+}\) reduce its activity, whereas and Zn\(^{2+}\), Cu\(^{2+}\), or Co\(^{2+}\) inhibit Lxmdh activity (Lee et al., 2020). For almost all NAD\(^+\)-dependent
Mdh, Mg\textsuperscript{2+} increases the effect of enzyme activity (Arfman et al., 1989; Arfman et al., 1991; Müller et al., 2015; Whitaker et al., 2017; Lee et al., 2020; Le et al., 2021). For Mdh from *Cupriavidus necator* (Cnmdh), Ni\textsuperscript{2+} is typically the chosen cofactor (Wu et al., 2016).

For PQQ-dependent Mdh, Ca\textsuperscript{2+} plays a role in the active site (Anthony and Zatman 1967; Anthony 2004). The X-ray structure of Mdh from *M. extorquens, M. nodulans, Methylophilus* sp, and *P. denitrificans* has been determined to have one molecule of PQQ and one Ca\textsuperscript{2+} ion in each large α-subunit, which is encoded

| Enzyme                      | Source                                | Optimum tem. (°C) | Optimum pH | Molecular weight (kDa) | Association form | Metal ion | Refs              |
|-----------------------------|---------------------------------------|-------------------|------------|------------------------|------------------|-----------|-------------------|
| **NAD\textsuperscript{+}-Dependent Mdh** | *Bacillus methanolicus C1* | 57–59             | 9.5        | 4.3                    | Decamer          | Mg\textsuperscript{2+} | Arfman et al. (1991) |
| **Mg\textsuperscript{2+}** | *Bacillus methanolicus MGA3* | 37                | 7.4        | N.I.                   | Decamer          | Mg\textsuperscript{2+} | Müller et al. (2015) |
|                            | 45                                    | 9.5               | N.I.       | Decamer                | Mg\textsuperscript{2+} | Krog et al. (2013)    |
|                            | 50                                    | 9.5               | N.I.       | Decamer                | Mg\textsuperscript{2+} | Witthoff et al. (2015) |
|                            | 50                                    | 9.0               | N.I.       | Decamer                | Mg\textsuperscript{2+} | Ochsner et al. (2014) |
|                            | *Bacillus methanolicus PB1* | 37                | 7.4        | N.I.                   | Decamer          | Mg\textsuperscript{2+} | Müller et al. (2015) |
|                            | 45                                    | 9.5               | N.I.       | Decamer                | Mg\textsuperscript{2+} | Krog et al. (2013)    |
|                            | *Bacillus steareothermophilus* | 37                | 7.4        | N.I.                   | Decamer          | Mg\textsuperscript{2+} | Whitaker et al. (2017) |
|                            | *Lysinibacillus xylanilaticus* | 55                | 9.5        | N.I.                   | N.I.             | Mg\textsuperscript{2+} | Lee et al. (2020)     |
|                            | *Cupriavidus necator* N-1 | 30                | 9.5        | N.I.                   | N.I.             | Mg\textsuperscript{2+} | Wu et al. (2016)      |
|                            | *Pseudomonas sp. M27*                | N.I.              | 9.0        | α: 62, β: N.I.         | N.I.             | N.I.      | Patel et al. (1972) |
|                            | *Methylobacillus capsulatus* (Texas strain) | N.I.               | 9.0        | α: 62, β: N.I.         | N.I.             | N.I.      | Nojiri et al. (2006)  |
|                            | *Hyphomicrobium denitrificans* A3151 | 25                | 7.0        | α: 65, β: 9            | Heterotramer     | N.I.      | Anthony (2004)       |
|                            | *Methylorubrum extorquens* AM1    | 30                | 8.0        | N.I.                   | Heterotramer     | Ca\textsuperscript{2+} | Liu et al. (2006)     |
|                            | *Methylcobacterium nodulans* ORS 2060T | 50                | 9–10       | α: 60, β: 6.5          | Heterodimer      | No metal | Kuznetsova et al. (2012) |
|                            | *Methylphilus methanophilus* W9A1  | N.I.              | N.I.       | α: 62, β: 8            | Heterotramer     | Ca\textsuperscript{2+} | Leopoldini et al. (2007) |
|                            | *Paracoccus denitrificans*           | N.I.              | N.I.       | α: 67, β: 9.5          | Heterotramer     | Ca\textsuperscript{2+} | Xie et al. (2003)     |
|                            | *Methyllobacterium radiotolerans* NBRC15690 | N.I.             | N.I.       | α: 63, β: 8            | Homodimer        | La\textsuperscript{3+} | Hibi et al. (2011)    |
|                            | *Bradyrhizobium sp. MAFF211645*   | N.I.              | N.I.       | α: 68, β: N.I.         | Homodimer        | Ce\textsuperscript{3+} | Fitr-yanto et al. (2011) |
|                            | *Methylacidiphilum fumariolicum* SolV | 45                | 7.2        | N.I.                   | Homodimer        | Eu\textsuperscript{3+} | Jahn et al. (2018)   |
|                            | *Bradyrhizobium diaezoefficiens* strain USDA110 | N.I.             | N.I.       | α: 64, β: N.I.         | N.I.             | Ce\textsuperscript{3+} | Wang et al. (2019)    |

R.T.—Room temperature; N.I.—No information.
by MxaF (Anthony and Williams 2003; Anthony 2004). Moreover, some types of Mdh s, which are encoded by XoxF, use Ln
−3 instead of Ca
2+ , which is a part of cofactor complex for Mdh s encoded by MxaF (Egli et al., 1980; Skovran et al., 2019). Ln
−3 was first suggested as a metal ion of the cofactor complex for PQQ-dependent Mdh s obtained from M. radiotolerans (Hibi et al., 2011) and Bradyrhizobium sp. (Fitiyanto et al., 2011) in 2011. Furthermore, Mdh s from M. extorquens AM1 (Nakagawa et al., 2012), M. aquaticum (Masuda et al., 2018), M. buryatense (Deng et al., 2018), M. fumaricola (Jahn et al., 2018), and B. diazoefficiens (Wang et al., 2019) have been observed to be Ln
−3-dependent Mdh s. Interestingly, the subunits of PQQ-dependent Mdh from M. aminosulfidivorans MPT are coordinated by an Mg
2+ ion instead of a Ca
2+ ion or Ln
−3 group (Cao et al., 2018). In addition, the activity of PQQ-dependent Mdh s under aerobic conditions with artificial electron acceptors in vitro requires the presence of an activator (e.g., ammonium salt) (Anthony 1982; Anthony and Williams 2003; Anthony 2004; Kuznetsova et al., 2012).

The other most important factors are the temperature and pH of the buffer in the enzyme assay. Almost all methanol dehydrogenases have high activity at high temperatures (−55°C) and high pH (9–10). Mdh s from M. nodulans (Mnmdh) exhibits maximal activity at pH 9–10, and it increases linearly with increasing temperature from 20°C to 50°C (Kuznetsova et al., 2012). The optimum pH for Mdh from Pseudomonas sp. M27 (Patel et al., 1972), M. capsulatus (Patel et al., 1972), and M. extorquens AM1 (Liu et al., 2006) are also 9. Similarly, an assay involving NAD
+/NADH-dependent Mdh s from thermodertertolent methylotrophic Bacillus strains is performed at 45–50°C, using glycine/KOH buffer at pH 9.5 (Arfman et al., 1991; Krog et al., 2013). Lxmdh and its mutant or Cnmdh also function better in buffers with a pH of 9.5; however, Lxmdh and its mutant exhibit high activity at 55°C (Lee et al., 2020; Le et al., 2021), while the temperature for testing Cnmdh activity is 30°C (Wu et al., 2016). On the other hand, the conditions for the Mdh s from M. methanolicus (Bmmdh) and B. stearothermophilus (Bsmdh) reactions are similar, at pH 7.4 and 37°C (Müller et al., 2015; Whittaker et al., 2017). When examining the activity of Mdh s obtained from L. xylanilyticus or Burkholderiales using spectrometer experiments to detect the changes in absorbance, room temperature is preferred (Kalyuzhnaya et al., 2008; Le et al., 2021). Moreover, buffer systems with a pH of 8.8 are used for Burkholderiales Mdh assays (Kalyuzhnaya et al., 2008). On the whole, the Mdh assay requires the presence of an ion as the binding cofactor. This depends on the type and source of Mdh. For PQQ-dependent Mdh s, the activator for enzyme activity is required under aerobic conditions.

**Molecular Weight of Methanol Dehydrogenases**

The molecular weight of most PQQ-dependent Mdh s has been identified as being between 112 and 158 kDa. The associated form of almost all PQQ-dependent Mdh s, which are Ca
2+ -dependent Mdh s, is a tetramer (α
3β
2). Therefore, it can be dissociated to α-subunits (56–76 kDa) and β-subunits (very small, ≤10 kDa) by a low pH or sodium dodecyl sulfate (SDS) (Anthony 1982), such as the Mdh from H. dendriticus (α: 65 kDa, β: 9 kDa) (Najiri et al., 2006), M. extorquens (α: 62–65 kDa, β: 7.5–8.5 kDa) (Anthony 2004; Liu et al., 2006), M. amnibusulfidivorans MP1 (α: 65–66 kDa, β: 7.5–7.6 kDa) (Kim et al., 2012; Cao et al., 2018), M. methylotrophus (α: 62 kDa, β: 8 kDa) (Leopoldini et al., 2007; Li et al., 2011), and M. radiotolerans (α: 60 kDa, β: 10 kDa) (Hibi et al., 2011). There are also some special cases with the heterodimer form (αβ), for example, Mdh s from M. nodulans (α: 60 kDa, β: 6.5 kDa) (Kuznetsova et al., 2012). Besides, the associated form of La
3+-dependent Mdh s is a homodimer (formed by two identical proteins), e.g., Mdh s from M. radiotolerans (120 kDa) (Hibi et al., 2011), Bradyrhizobium sp. (108 kDa) (Fitiyanto et al., 2011), M. extorquens AM1 (117 kDa) (Nakagawa et al., 2012), Methylacidiphilum fumarriolium SolV (63.6 kDa) (Jahn et al., 2018), and M. buryatense (Deng et al., 2018). On the other hand, the NAD
+/NADH-dependent Mdh with a single subunit has a molecular weight of around 40 kDa. For instance, the molecular weight of NAD
+/NADH-dependent Mdh from Bacillus sp. C1 (a thermodertertolent methylotrophic Bacillus) is 43 kDa (Arfman et al., 1989; Arfman et al., 1991). Other B. methanolicus strains (MGA3 and PB1) show a similar molecular weight at 43 kDa (Müller et al., 2015; Whittoff et al., 2015; Price et al., 2016). Moreover, Cnmdh from C. necator N-1 (Wu et al., 2016) or Lxmdh from L. xylanilyticus (Lee et al., 2020) show respective molecular subunits at 40.7 or 42.8 kDa (Table 1). According to the previous report, NAD
+/NADH-dependent Mdh s has decameric association structure (430 kDa) as native form (Vonck et al., 1991).

**Substrate Affinity Toward Methanol of Wild-type or Engineered NAD-Mdh**

Although, MxaFI-Mdh s from M. extorquens AM1, with a high efficiency (kcat/KM) of methanol production, has been suggested as the best choice for engineering E. coli (Anthony and Williams 2003), it requires at least 11 gene products for its functional assembly (Chistoserdova et al., 2003). In addition, XoxF-Mdh s from M. extorquens AM1 would be required only three genes with high catalytic efficiency (Keltjens et al., 2014), PQQ-dependent Mdh s are not suitable for synthetic methylotrophy using engineered E. coli. Because, PQQ as critical cofactor is critical limit that specially E. coli is not able to synthesize PQQ (Anthony 2004). In the case of O2-dependent AOX, its product, H2O2, is also challenging because it is the highly toxic to most hosts. Therefore, only NAD
+/NADH-dependent Mdh has been considered as the best candidate for synthetic methylotrophs (Zhang et al., 2017), which requires only one gene for functional production and can generate the reducing equivalent (NADH) to promote strain growth under both aerobic and anaerobic conditions. To successfully achieve methanol assimilation, the Mdh kinetics, including substrate affinity and catalytic activity, should be improved for methanol assimilation through directed evolution or rational approach based engineering. Various NAD
+/NADH-dependent Mdh s from B. methanolicus (Vonck et al., 1991; De Vries et al., 1992; Hektor et al., 2002; Krog et al., 2004).
### TABLE 2 | Summary of substrate affinity for methanol by NAD⁺-Dependent Mdhs.

| Enzyme type | Strain | Type of enzyme | Vₘₐₓ (U/mg) | kₗ (s⁻¹) | Kₘ (mM) | Evolution method | Refs |
|-------------|--------|----------------|-------------|----------|--------|-----------------|------|
| Wild type Mdh | B. methanolicus MGA3 | Mdh | 0.06 ± 0.002 | N.I. | 170 ± 20 | WT | Krog et al. (2013) |
|             | Mdh 2 | 0.09 ± 0.003 | N.I. | 360 ± 30 | WT |               |      |
|             | Mdh 3 | 0.07 ± 0.005 | N.I. | 200 ± 70 | WT |               |      |
|             | Mdh + ACT | 0.4 ± 0.02 | N.I. | 26 ± 7 | WT |               |      |
|             | Mdh 2 + ACT | 0.2 ± 0.008 | N.I. | 200 ± 20 | WT |               |      |
|             | Mdh 3 + ACT | 0.4 ± 0.008 | N.I. | 150 ± 10 | WT |               |      |
|             | Mdh | 0.151 ± 0.008 | N.I. | 150 ± 25 | WT | Ochsner et al. (2014) |
|             | Mdh 2 | 0.151 ± 0.012 | N.I. | 416 ± 97 | WT |               |      |
|             | Mdh + ACT | 0.474 ± 0.032 | N.I. | 9 ± 2 | WT |               |      |
|             | Mdh 2 + ACT | 0.394 ± 0.016 | N.I. | 96 ± 12 | WT |               |      |
| B. methanolicus PB1 | Mdh | 0.03 ± 0.001 | N.I. | 220 ± 30 | WT | Krog et al. (2013) |
|             | Mdh 1 | 0.015 ± 0.001 | N.I. | 170 ± 60 | WT |               |      |
|             | Mdh 2 | 0.09 ± 0.004 | N.I. | 330 ± 0.05 | WT |               |      |
|             | Mdh + ACT | 0.2 ± 0.003 | N.I. | 10 ± 1 | WT |               |      |
|             | Mdh 1 + ACT | 0.06 ± 0.002 | N.I. | 5 ± 1 | WT |               |      |
|             | Mdh 2 + ACT | 0.38 ± 0.04 | N.I. | 110 ± 50 | WT |               |      |
| C. necator N-1 WT | Mdh 2 | 0.32 ± N.I. | 0.22 ± 0.01 | 132 ± 15.4 | WT | Wu et al. (2016) |
| B. steatheromophilus | Mdh | 2.1 ± N.I. | N.I. | 20 ± N.I. | WT | Whitaker et al. (2017) |
| L. xylanilyticus | Mdh 2 | 0.3027 ± 0.0169 | 0.21 ± 0.01 | 3.23 ± 1.05 | WT | Lee et al. (2020) |
| Engineered Mdh | B. methanolicus MGA3 S98G | Mdh | 0.44 ± 0.053 | 0.35 ± N.I. | 1,151 ± 274 | Rational approach | Ochsner et al. (2014) |
|             | B. methanolicus MGA3 S98G + ACT | Mdh | 0.819 ± 0.082 | 0.59 ± N.I. | 847 ± 190 | Rational approach | Ochsner et al. (2014) |
|             | C. necator N-1 CT4-1 | Mdh 2 | 0.29 ± N.I. | 0.20 ± 0.01 | 21.6 ± 1.5 | Directed evolution | Wu et al. (2016) |
|             | L. xylanilyticus Mdh -S1011V | Mdh 2 | 0.3423 ± 0.02167 | 0.24 ± 0.01 | 10.35 ± 3.87 | Rational approach | Lee et al. (2020) |
|             | L. xylanilyticus Mdh -T141S | Mdh 2 | 0.4629 ± 0.0576 | 0.33 ± 0.04 | 51.24 ± 23.95 | Rational approach |      |
|             | L. xylanilyticus Mdh -A164F | Mdh 2 | 0.4753 ± 0.05072 | 0.33 ± 0.03 | 36.83 ± 3.56 | Rational approach |      |
|             | L. xylanilyticus Mdh -E396V | Mdh 2 | N.I. | 0.020 ± 0.002 | 0.010 ± 0.003 | Directed evolution | Le et al. (2021) |
|             | L. xylanilyticus Mdh -K318N | Mdh 2 | N.I. | 0.027 ± 0.005 | 0.048 ± 0.0072 | Directed evolution |      |
|             | L. xylanilyticus Mdh -E396V + K318N | Mdh 2 | N.I. | 0.022 ± 0.002 | 0.233 ± 0.107 | Directed evolution |      |
|             | B. methanolicus (WT) | Mdh 2 | 0.0365 ± 0.0017 | N.I. | 636 ± 74 | Directed evolution | Roth et al. (2019) |
|             | B. methanolicus QSL E123G | Mdh 2 | 0.0386 ± 0.0016 | N.I. | 615 ± 66 | Directed evolution |      |
|             | B. methanolicus QSL M163V | Mdh 2 | 0.050 ± 0.0031 | N.I. | 627 ± 89 | Directed evolution |      |
|             | B. methanolicus QSL A164P | Mdh 2 | 0.0754 ± 0.0023 | N.I. | 440 ± 39 | Directed evolution |      |
|             | B. methanolicus QSL A363L | Mdh 2 | 0.127 ± 0.0035 | N.I. | 432 ± 32 | Directed evolution |      |
|             | B. methanolicus QSL A164P | Mdh 2 | 0.0885 ± 0.0023 | N.I. | 329 ± 28 | Directed evolution |      |

| Wild type ADH | C. glutamicum R AdhA | Class I | 0.29 ± N.I. | 0.20 ± 0.01 | 97 ± 9.8 | WT | Wu et al. (2016) |
| L. sphaericus C3-41 | N.I. | 0.0029 ± N.I. | N.I. | N.I. | N.I. | WT | Wu et al. (2015) |
| L. fusiformis ZC1 | N.I. | 0.0038 ± N.I. | N.I. | N.I. | N.I. | WT |      |
| B. coagulans 36D1 | N.I. | 0.0058 ± N.I. | N.I. | N.I. | N.I. | WT |      |
| D. hyrniensis YS1 | N.I. | 0.0018 ± N.I. | N.I. | N.I. | N.I. | WT |      |

N.I.—No information.

2013; Ochsner et al., 2014; Müller et al., 2015; Witthoff et al., 2015), **C. necator** (Wu et al., 2016), **B. steatheromophilus** (Whitaker et al., 2017), **L. xylanilyticus** (Lee et al., 2020) were reported in methanol conversion. Researchers are searching for NAD⁺-dependent Mdhs with higher activity and lower Kₘ from different microorganisms and improving their characteristics by a rational approach and directed evolution (Hektor et al., 2002; Ochsner et al., 2014; Roth et al., 2019; Lee et al., 2020; Le et al., 2021) (**Table 2**). Specially, the improvement of substrate affinity toward low concentration methanol is focused in the development of Mdh-driven synthetic methylotrophy because of the high toxicity of methanol for E. coli (Dyrda et al., 2019).

NAD⁺-dependent Mdhs from **B. methanolicus** that has been studied a lot (Vonck et al., 1991; De Vries et al., 1992; Hektor et al., 2002; Krog et al., 2013; Ochsner et al., 2014; Müller et al., 2015; Witthoff et al., 2015). They support cell growth and methanol uptake with high speed in native **B. methanolicus**. However, the catalytic activity of Mdhs from **B. methanolicus in vitro** and **in vivo** are limited because of the unclear mechanism of ACT (Hektor et al., 2002; Witthoff et al., 2015), even though...
ACT significantly improve the $K_M$ value of Bmmdh (reduced from 1.8- to 14.0-fold) (Krog et al., 2013; Ochsner et al., 2014). Second, an ACT-independent Mdh from *C. necator* was developed and characterized for the kinetics and substrate specificity on 2016 (Wu et al., 2016). It showed the low affinity to methanol (132 mM for $K_M$) compared to that of Mdhs from *B. methanolicus* (170-360 mM for $K_M$) (Krog et al., 2013; Ochsner et al., 2014; Müller et al., 2015; Wu et al., 2016). Another study showed an Mdh from *B. stearothermophilus*, which shares 21–23% amino acid identity with the Mdh from *B. methanolicus* (Whitaker et al., 2017). The affinity of Mdh from *B. stearothermophilus* showed a lower value than that from Bmmdh and Cnmdh (20 mM for $K_M$), thus, it had superior performance *in vivo* than previously published Mdhs. In particular, Lee et al. found an Mdh from *L. xylanilyticus*, that had higher substrate specificity towards methanol than Bmmdh, Cnmdh and Bsmdh (Lee et al., 2020). In addition, it is also an ACT-independent Mdh with an impressively low affinity (3.23 mM for $K_M$).

To improve the activity of Mdhs, site-directed (Hektor et al., 2002; Ochsner et al., 2014), site-saturation (Wu et al., 2016) or random mutagenesis (Le et al., 2021) is used for creating Mdh mutants. In 2002, Hektor et al. used site-directed mutagenesis to confirm the role of various amino acid residues in the NAD(H) binding site in Mdh from *B. methanolicus* C1 (Hektor et al., 2002). All mutants are impaired in cofactor NAD(H) binding, though, some mutants (G95A, S97G, and S97T) retained Mdh activity. To improve the activity of Mdhs, site-directed (Hektor et al., 2002; Ochsner et al., 2014), site-saturation (Wu et al., 2016) or random mutagenesis (Le et al., 2021) is used for creating Mdh mutants. In 2002, Hektor et al. used site-directed mutagenesis to confirm the role of various amino acid residues in the NAD(H) binding site in Mdh from *B. methanolicus* C1 (Hektor et al., 2002). All mutants are impaired in cofactor NAD(H) binding, though, some mutants (G95A, S97G, and S97T) retained Mdh activity. Finally, only the S97G mutant displayed as “fully activated” in Mdh reaction rates. Another study from Ochsner et al. investigated the effect of site-directed mutations in the predicted active site of Mdh from *B. methanolicus* MGA3 (Ochsner et al., 2014). The $V_{max}$ of Bmmdh S98G increased two-fold compared with that of its wild-type (WT), yet its $K_M$ value also increased in the absence of ACT. Even upon adding ACT, the catalytic efficiency of Bmmdh S98G was similar to that of WT (a doubling of $V_{max}$ with a slight reduction in $K_M$). Meanwhile, Bmmdh2 S101G lost the activity on methanol. For Mdh from *C. necator*, the site-saturation mutagenesis on the Mdh2 A169 site was constructed (Wu et al., 2016). In the first round of screening, eight possible positive variants with over 50% activity improvement (based on the Nash reaction) were selected from 2000 screened variants, and, finally, CT1-2 was used as the template for another error-prone PCR library in the second round of screening. Afterward, CT4-1, the recombinant of three mutations (A169V, A31V and A26V), which showed a low $K_M$ (21.6 mM) and an unchanged $k_{cat}$ (0.2 s$^{-1}$) compared with WT Mdh2, was created by various rounds of high throughput screening (HTS). For studying the activity of Mdh from *L. xylanilyticus*, eight residues within 4.5 Å of the center of the docked substrate were selected to contribute toward site-directed mutagenesis (Lee et al., 2020). Finally, the mutations S101V ($K_M = 10.35$), T141S ($K_M = 51.24$) and A164F ($K_M = 36.83$) improved the enzyme’s specific activity towards methanol compared to that of the Lxmdh WT. Furthermore, a random mutant library of *L. xylanilyticus* Mdh was constructed and high throughput screened by an formaldehyde detectible biosensor (Le et al., 2021). As a result, several mutants were characterized by high catalytic efficiency and low $K_M$ compared with Lxmdh WT and its published mutants. Thus, mutant Lxmdh E396V, which has the highest catalytic efficiency (79-fold that of WT catalytic efficiency) and an impressive $K_M$ value (0.01 mM), was found. Moreover, the $K_M$ value of another Lxmdh mutant, K318N, was also impressive (0.046 mM). Nevertheless, the recombinant of two mutations (E396V and K318N) had a higher $K_M$ value compared with each mutant (0.233 mM).

Many alcohol dehydrogenases (ADHs), which can catalyze methanol oxidation, may be treated as Mdhs. Although, the catalytic efficiency of methanol oxidation by ADHs is low, it is another good candidate for synthetic methylotrophy. As an example, the AdhA from *Corynebacterium glutamicum* R has shown a low $K_M$ value of methanol activity (97 mM) compared with Bmmdh and Cnmdh (Kotrbova-Kozak et al., 2007; Wu et al., 2016). A number of ADH enzymes has been tested for the methanol oxidation activity without kinetic values, such as ADHs from *Lysinibacillus sphaericus*, *Lysinibacillus fusiformis*, *Bacillus coagulans* and *Desulfitobacterium hafniense* (Müller et al., 2015).

Furthermore, critically, improving methanol oxidation rates by kinetically improved Mdh variants would only be enabled in cells where there is sufficiently fast of formaldehyde assimilation (Woolston et al., 2018). This is important for the development of Mdh-directed evolution approaches. This is covered in the synthetic methylotrophy section of this review.

**APPLICATION OF MDHS IN SYNTHETIC METHYLOTROPHY**

C1 feed stocks are inexpensive abiotic resources for microbial bio production. Among all C1, the soluble C1 substrates, such as methanol, may be more suitable feed stocks because of the avoidance of mass transfer limitation (Claassens et al., 2019). Synthetic methyiotrophy using the integration of Mdhs for the assimilation of methanol as a carbon source into non methylotrophs such as *E. coli* and *C. glutamicum* has been investigated further in recent studies.

For the design of synthetic methylotrophy, a number of biochemical and practical considerations should be considered. Compared to PQQ-dependent Mdhs and O2-dependent Aods, NAD-dependent Mdhs require only enzyme for its functional assembly in both aerobic and anaerobic conditions. Although, PQQ-dependent Mdhs has very high substrate affinity and activity toward methanol, PQQ biosynthesis requires molecular oxygen (Veltrop et al., 1995), which will restrict the applications of PQQ-dependent Mdhs as some of metabolites must be produced only under anaerobic conditions. Unfortunately, there are no PQQ biosynthesis pathway in *E. coli* and *C. glutamicum* as candidate for synthetic methyiotrophy. NAD-dependent Mdhs can be utilize a ubiquitous cofactor (NAD) that can be generate reducing equivalents in the form of NADH and used to provide electron for metabolite production under both aerobic and anaerobic conditions and generate reducing equivalents (NADH), which can help promote strain growth. In this...
point, NAD-dependent MDHs may be the best candidates for synthetic methylotrophy (Zhang et al., 2017).

For instance, introducing NAD$^+$-dependent Mdhs is the simplest way to engineer methanol oxidation for all reasons mentioned above. Many researchers are also trying to improve the methanol bioconversion efficiency of synthetic methylotrophy by searching for the NAD$^+$-dependent Mdhs with better characteristics from different organisms via directed evolution (Table 3). The Mdhs from *B. methanolicus* (Müller et al., 2015; Witthoff et al., 2015; Dai et al., 2017; Meyer et al., 2020), *B. stearothermophilus* (Whitaker et al., 2017; Bennett et al., 2018; Tuyishime et al., 2018; Bennett et al., 2020; Rohllhill et al., 2020), and *C. necator* (Chen et al., 2018; Tuyishime et al., 2018; Woolston et al., 2018; Chen et al., 2020; Keller et al., 2020) have been used for synthetic methylotrophy in recent studies with *E. coli* as the most popular host (Müller et al., 2015; Whitaker et al., 2017; Bennett et al., 2018; Chen et al., 2018; Meyer et al., 2018; Woolston et al., 2018; Bennett et al., 2020; Chen et al., 2020; Keller et al., 2020), besides *C. glutamicum* (Whitthoff et al., 2015; Hennig et al., 2020; Tuyishime et al., 2018) and *S. cerevisiae* (Dai et al., 2017). In vitro system to mimic synthetic methylotrophy using scaffold system by enzyme assembly for enhancement of methanol utilization have been also attempt (Price et al., 2016).

Although, NAD-dependent MDHs are their favored MDHs for synthetic methylotrophy according to the recent study, the PQP MDH XoxF has revealed novel activities, such as the oxidation of formaldehyde *in vivo* (Pol et al., 2014; Good et al., 2019). This shows that these enzymes also can generate new activities for synthetic methylotrophy, even if PQP must be added; and further, these enzymes may yet reveal undiscovered activities that cannot be generated by NAD-dependent MDHs that would be of great interest to the field.

| Host | Carbon source/substrate | Used Mdhs | Refs |
|------|------------------------|-----------|------|
| *E. coli* | 0.4% glucose and 1 M methanol | Mdh from *B. methanolicus* MGA3 and PB1 | Müller et al. (2015) |
| | 5 mM sodium gluconate, 20 mM sodium pyruvate, 0.1 g/L yeast extract and 250 mM methanol, 10 g/L glucose | Mdh from *B. methanolicus* PB1 | Meyer et al. (2018) |
| | 500 mM methanol | Mdh from *B. stearothermophilus* Whitaker et al. (2017) |
| | 60 mM methanol and 1 g/L yeast extract | Mdh from *B. stearothermophilus* Bennett et al. (2018) |
| | 60 mM methanol and 0.5 g/L yeast extract or 4 g/L glucose | Mdh from *B. stearothermophilus* Bennett et al. (2020) |
| | 100 mM methanol and 0.5 g/L yeast extract | Mdh from *B. stearothermophilus* Rohllhill et al. (2020) |
| | 6 g/L xylose and 250 mM methanol | Mdh 2 from *C. necator* N-1 | Woolston et al. (2018) |
| | 250 mM methanol, 50 mM ribose or xylose, 0.05% casamino acids | Mdh 2 CT4-1 from *C. necator* N-1 | Chen et al. (2018) |
| | 400 mM methanol and 20 mM xylose | Mdh 2 CT4-1 from *C. necator* N-1 | Chen et al. (2020) |
| | 500 mM methanol and 20 mM pyruvate | Mdh 2 CT4-1 from *C. necator* N-1 | Keller et al. (2020) |
| *C. glutamicum* | 120 mM methanol and 55 mM glucose | Mdh and MD3 from *B. methanolicus* MGA3 | Witthoff et al. (2015) |
| | 500 mM methanol and 20 mM co-substrates (ribose, xylose or gluconate) | Mdh from *B. methanolicus* | | |
| | 96.90 mM methanol and 25.32 mM xylose | Mdh from *B. stearothermophilus* | | |
| *S. cerevisiae* | 10 g/L methanol, 20 g/L glucose, 10 g/L yeast extract and 20 g/L peptone | Mdh from *B. methanolicus* MGA3 | | |

This consideration could be extended to other steps for engineering synthetic methylotrophy. As mentioned, the speed of formaldehyde assimilation has a big effect on improving methanol oxidation rates. For example, Whitaker et al. combined NAD$^+$-dependent Mdh from *B. stearothermophilus* and RuMP pathway enzymes from *B. methanolicus* to engineer *E. coli*, which can grow with methanol as the carbon source. Through their engineered *E. coli* strain (BW25113 ΔfrmA expressing *B. stearothermophilus* Mdh and *B. methanolicus* RuMP), the amount of biomass derived from methanol was determined to be 0.289 ± 0.028 gCDW/gMeOH in media, including 60 mM methanol and 1 g/L yeast extract. A similar increase of biomass in the presence of yeast extract and methanol at a larger scale was confirmed by bioreactor experiments (0.344 ± 0.012 gCDW/gMeOH) (Whitaker et al., 2017).

**SYSTEM BIOLOGY BASED PATHWAY OPTIMIZATION**

System-wide consideration of engineering strategies is necessary. To address the complexity and identify the best combination of genes for a given host, several computational tools have been developed for the *in silico* design of metabolic pathways (Medema et al., 2012; Vieira et al., 2014; Carbonell et al., 2016). They help identify the best combinations of genes and pathways and optimize the host metabolism, such as transport, cofactors, C1 acceptor regeneration, and chemical toxicity. Müller et al. used the OptFlux software for *in silico* modeling approaches to test the preferred choice of enzymes and pathways by modifying a stoichiometric genome-scale *E. coli* model. A model containing 1,271 gene products and reactions with 1,676 metabolites was established and modified to find a solution for efficient methanol metabolism as a carbon source with a maximal μ of 0.88 h$^{-1}$. |
In methylo trophs, the absence of methanol (or formaldehyde) controls the expression of genes involved, so microorganisms can adapt to the changing of carbon sources (Selvamani et al., 2017). For this reason, regulating the gene expression of methanol and the formaldehyde response is also important. Another important factor is the efficient regeneration of formaldehyde acceptors for methanol assimilation (Woolston et al., 2018). In this regard, it is worth mimicking native methylotrophs (Wang et al., 2019). Five enzymes of the nonoxidative pentose phosphate pathway (PPP) from \textit{B. methanolicus} were introduced into \textit{E. coli} (Bennett et al., 2018). The whole PPP is usually kept for formaldehyde acceptor regeneration, however, it prevents methanol consumption in the absence of a cosubstrate (such as glucose). Therefore, synthetic methanol-dependent strains are engineered for methanol as a co-consumption regime. This leads to the cell growth being bound to methanol assimilation to improve methanol utilization via adaptive laboratory evolution (ALE) (Chen et al., 2020; Wang et al., 2020).

**CONCLUSION**

In this review, the enzymatic properties of various reported Mdhs and their applications in synthetic methylotrophy were discussed. Protein engineering and molecular modifications using site-directed mutagenesis, random mutagenesis, HTS, and direct evolution can potentially advance further studies in this field by improving the properties (i.e., activity, thermos ability, and substrate-binding affinity) of existing Mdh enzymes and discovering new Mdh enzymes. The proposal for engineering Mdh-based synthetic methylotrophy is providing value-added products from methanol. Until now, several useful metabolites of methanol have been produced, proving the potential of methanol-based bio-manufacturing. Therefore, we may take advantage of Mdhs for the utilization of methanol as feedstock for high value chemicals, which is a methanol-based bio-economy.

**AUTHOR CONTRIBUTIONS**

T-KL, GH, and S-JY initiated the project. T-KL and Y-JL searched the data base. T-KL wrote the first drafts of the manuscript and Y-JL, GH, and S-JY contributed to further revisions and the final version. All authors have made a direct intellectual contribution to the work and approved it for publication.

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