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Applications of methylotrophs: can single carbon be harnessed for biotechnology?

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This review summarizes developments in the field of applied research involving microbial conversion of single carbon compounds (methane, methanol, CO_2). The potential of the microorganisms involved in biotechnological applications could be realized via engineering native C1 utilizers toward higher output of value-added compounds, including biofuels, or via production of value chemicals as parts of novel, heterologously expressed biochemical pathways. Alternatively, C1 metabolism could be implemented in traditional industrial platforms (*Escherichia coli*, yeast), via introduction of specific metabolic modules. Most recent research spanning both approaches is covered. The potential of C1 utilizers in biomining of rare Earth elements, as well as the potential of C1 consuming microbial consortia in industrial applications are discussed.

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Introduction

Specialized guilds of microorganisms are capable of consuming single carbon (C1) compounds, that is, compounds containing no carbon–carbon bonds, such as methane, methanol or CO_2. Microorganisms capable of oxidizing methane are known as methanotrophs, and, together with microorganisms that will not oxidize methane but will utilize other C1 compounds (methanol and so on), they belong to a broader guild of methylotrophs. Microorganisms capable of assimilating CO_2 are known as autotrophs. Some organisms are capable of both methylotropjy and autotrophy, which makes them autotrophic methylotrophs. The organisms utilizing C1 compounds are extremely attractive for biotechnological applications for two main reasons. On the one hand, value-added compounds could be produced from substrates such as methane (or derivatives) and/or CO_2, both of which are abundant in the environment. On the other hand, these compounds are also greenhouse gases, and their removal from the environment would prevent their escape into the atmosphere, decreasing their impact on global climate change. The potential of C1-utilizing microorganisms for industrial applications has been recognized early on. For example, a methylotroph-based single cell protein (SCP) production plant has been active in the 1980s [1]. Impressively, even in these early, pregenomic times, the industrial strain, *Methylophilus methylotrophus* was genetically modified for improved performance [2]. SCP production from methylotrophs is still alive and kicking [3•], and it is gaining further momentum trough employing novel prominent industrial platforms for SCP production, such as *Methylobacterium extorquens*, which, in addition to high biomass production, provides added benefits such as naturally synthesized anti-oxidant carotenoid compounds [4]. However, the future of harnessing C1 utilizing organisms for environmental biotechnology will likely involve metabolic engineering and synthetic biology. Most recent developments in this direction are discussed in this review.

Developing methylotroph catalysts

Native methylotrophs, some of which demonstrate impressively robust growth on C1 compounds, present natural platforms for biotechnology. Methylotrophs are available from different phylogenetic backgrounds, and with different temperature, salinity and pH optima. Thus, a strain with desired characteristics could be preselected as a starting platform.

Biofuels from methane

There has been renewed interest in converting methane into liquid biofuels by the way of microbial catalysis [3•], and thus a new search has been recently launched for microorganisms that could present attractive platforms for such applications. One such organism, *Methylomicrobium buryatense*, showed promise, having demonstrated attractive characteristics such as robust growth on methane and stress tolerance, including usage of natural gas of varying compositions [5]. This organism has been recently thoroughly investigated in terms of growth characteristics and responses to limitations of oxygen and methane [6], followed by the development of a metabolic model that predicts carbon distribution among different metabolic pathways during growth on methane [7]. The potential in biofuel production from this platform lies in naturally

high lipid content, typical of proteobacterial methanotrophs. The lipids, after extraction, could be catalytically converted into biodiesel, via esterification. While a promising platform, methanotrophs such as *M. buryatense* would require extensive engineering, to significantly increase lipid content [8], to make the platform commercially feasible. Recently, an enhancement in conversion of methane into lipids was reported for this organism, in an engineered strain with an increased flux through the phosphoketolase pathway [9].

Other products from methane

Meantime, the same organism has been tested as a platform for production of value-added compounds other than biofuels. *M. buryatense* was genetically modified, as a proof of principle, to ferment methane into lactate, by overexpressing a recombinant lactate dehydrogenase [10]. Importantly, lactate production, at least at the level achieved in the study, did not negatively affect cellular lipid concentration. Thus, in principle, methanotrophs could be used for co-production of biofuels and other value-added chemicals [10] (Figure 1).

Employing reverse methanogenesis

Not only bacteria but also archaea present potential industrial platforms for producing value-added chemicals from methane. A model archaeon *Methanosarcina acetivorans* has been engineered to produce acetate from methane, by reversing its native methanogenesis pathway, through the heterologously expressed methyl-CoM reductase (MCR) of an uncultivated methane-oxidizing archaeon of the ANME-1 type and linking methane oxidation to solid iron ($FeCl_3$) reduction, in anaerobic conditions [11•]. This strain was further engineered by expressing a gene for 3-hydroxybutyryl-CoA dehydrogenase from *Clostridium acetobutylicum*, resulting in secreted valuable product L-lactate, of sufficient optical purity for synthesizing the biodegradable plastic poly-lactic acid [12]. This work demonstrated the utility of anaerobic methane conversion, achieving an increased lactate yield compared to aerobic methane conversion to lactate [12].

Electricity from methane

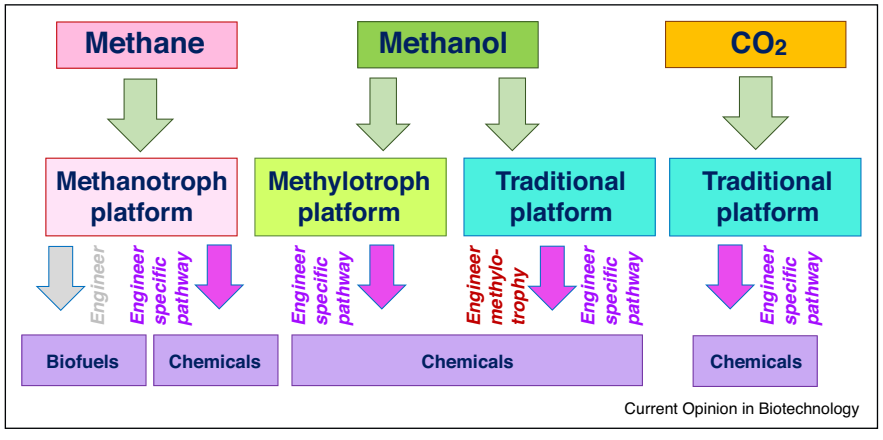
The same engineered host, *M. acetivorans* expressing MCR from ANME-1 archaeon, was employed as part of a synthetic consortium to form a microbial fuel cell converting methane into electrical current [13]. In this case, the engineered *M. acetivorans* needed to be paired with *Geobacter sulfurreducens*, a known exoelectrogen, and with a sludge adapted to methane, the latter replaceable by humic acids, proposed to act as electron shuttles [13,14].

Value-added products from methanol

One of the most developed platforms for value-added compounds from methanol remains to be the Gram-positive methylotroph *Bacillus methanolicus*. Results from experiments on engineering and validation of strains for production of L-glutamate, gamma-aminobutyric acid, L-lysine and cadaverine, through strain optimization and heterologous protein expression, have been reported over years, and these results have been recently summarized in a detailed review [15].

Another emergent bacterial platform is *M. extorquens* that has been recently engineered for *de novo* synthesis of the sesquiterpenoid α-humulene, by expressing heterologous proteins, combined with strain optimization [16]. *M. extorquens* has also been employed recently as a platform for production of 2-hydroxybutyric acid from methanol, by introducing a module that shuttles the natural intermediate of methylotrophic metabolism and polyhydroxyalkanoate synthesis, 3-hydroxybutyryl-CoA into the product of interest [17]. The same organism has also

Figure 1



A schematic summarizing general strategies in engineering microorganisms for applications in converting C1 compounds into value products. Grey arrow denotes an optional step.

been engineered to produce 3-hydroxypropionic acid, through implementing a methylmalonyl-CoA pathway dependent on heterologously expressed malonyl-CoA reductase, followed by promoter strength adjustment and by removal of a natural pathway that showed activity in product degradation [18]. *M. extorquens* has further been employed in expressing the mevalonate synthesis pathway, in which case the selectin for high mevalonate production was achieved through modification of a native regulator QscR that effects flux distribution among central pathways for carbon metabolism in this organism [19].

Improving yeast catalysts

Methylotrophic yeasts are also of interest for biotechnological applications. Recently, *Pichia pastoris* has been subjected to adaptive laboratory evolution, resulting in populations with improved growth on methanol and improved recombinant protein production, thus presenting a convenient platform for future engineering [20].

Engineering enzymes and pathways for synthetic methylotrophy

An alternative approach to harnessing the carbon and energy from C1 substrates using native methylotrophs is synthetic methylotrophy. As biochemistry of methylotrophy is well understood, and as many enzymes in methylotrophy are the enzymes also used in universally distributed pathways, this approach appears straight forward: introducing the missing modules into well-developed industrial hosts and fine tuning carbon/energy fluxes through transcriptional regulation and other controls.

Synthetic methanol utilization

So far, implementation of methylotrophy in the well-developed industrial platforms, *E. coli* and *Corynebacterium glutamicum* has followed similar schemes, introduction of a minimal set of enzymes enabling methylotrophy: a methanol dehydrogenase (Mdh) that converts methanol into formaldehyde, and hexulose phosphate synthase (Hps) and hexulosephosphate isomerase (Hpi) that catalyze sequential reactions converting formaldehyde into hexulose 6-phosphate and further into fructose 6-phosphate [21–23,24••] (Figure 1). Fructose 6-phosphate then serves as a common currency, feeding into the glycolysis pathway, the resulting pyruvate feeding into the tricarboxylic acid cycle. Obviously, methylotrophy is rather complex and cannot be fully recreated by adding three reactions to universal metabolic pathways. For example, a recent study that employed high density transposon mutagenesis in *M. extorquens* has identified a total of 147 genes indispensable for methylotrophy in this organism [25]. No such studies exist for methylotrophs utilizing the ribulose monophosphate cycle whose enzymes, Hps and Hpi, are employed in synthetic methylotrophy. Likely, methylotrophy in these species is also complex. Nevertheless, the three heterologously expressed methylotrophy genes (Mdh from *Bacillus methanolicus*, and Hps and Hpi from either *B. methanolicus* or *Bacillus subtilis*) have impacted metabolism of native non-methylotroph hosts, as demonstrated by increased methanol consumption and by labeling of intracellular metabolites with methanol-derived carbon [21,22]. Labeling of cadaverine in cadaverine-producing strain of *C. glutamicum* was also demonstrated [23]. Remarkably, when Mdh from *Bacillus stearothermophilus* was expressed in *E. coli*, along with Hps and Hpi from *B. methanolicus*, an increased flux from methanol to intracellular metabolites was observed, along with improved biomass yield [24••]. Further strain improvement was achieved when heterologously expressed methanol metabolism genes were placed under control of a specially-engineered formaldehyde-inducible promoter [26].

Meantime, an improved variant of Mdh has also been engineered, from a non-methylotroph *Cupriavidus necator*, through directed evolution [27]. The advantages of this new enzyme for further engineering of synthetic methylotrophs are its improved catalytic properties and its independence of a specific activator [27]. An alternative engineering approach, involving creation of a supramolecular enzyme channeling C1 substrates from methanol to fructose 6-phosphate, coupled with a NADH sink, has been reported, achieving improved methanol consumption by *E. coli* [28].

Development of a formaldehyde biosensor in *E. coli*, based on the native FrmR repressor protein and a cognate promoter, followed by optimization of the native repressor binding site and regulatory architecture has been recently reported [29]. This sensor has then been used to benchmark the *in vivo* activity of different Mdh variants, as well as to balance expression of *mdh*, *hps* and *hpi* genes, to minimize formaldehyde build-up while also reducing the burden of heterologous expression. This sensor holds promise for dynamic formaldehyde control strategies essential for establishing synthetic methylotrophy [29].

Synthetic methylotrophy has also been successfully conferred to naturally non-methylotrophic yeast, through expressing a methanol oxidase, a catalase, a dihydroxyacetone synthase and a dihydroxyacetone kinase from methylotrophic yeast [30]. In this case, engineered yeast strain could convert methanol into pyruvate. However, the engineered strain was not compared to native methanol utilizing strains in terms of growth or carbon turnover.

Synthetic CO_2 fixation

The holy grail of biotechnology, microbial conversion of CO_2 into sugars and other conventional molecules, has been approached recently using several original technical solutions (Figure 1). In one study, key enzymes of the Calvin–Benson–Bassham (CBB) cycle, ribulose bisphosphate carboxylase/oxygenase (RuBisCO) and

phosphoribulokinase (Prk) have been introduced into *E. coli*, along with carbonic anhydrase, to achieve flux through the synthetic CBB pathway [31]. In this case, the energy for CO_2 fixation was still generated from sugars, and thus growth of *E. coli* was heterotrophic. In another study, production of sugars from CO_2 alone has been achieved through decoupling the synthetic partial CBB cycle from the energy-generating pathways [32•]. The decoupling itself was originally insufficient for 'hemiautotrophic' growth of *E. coli*. However, such growth, along with sugar production from CO_2, has been gradually achieved through laboratory evolution, highlighting the importance of metabolic fine-tuning following the expression of enzymes and pathways non-native to the host, which, in this case, was demonstrated at a remarkably fast rate of 150 chemostat generations [32•].

A radically different approach toward improved CO_2 fixation was used by exploring the feasibility of a theoretically designed pathway [33••]. The pathway deemed promising for experimental validation was the so called crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle that shares many of its reactions with the naturally occurring ethylmnalonyl-CoA pathway that is important in both methylotrophy and in acetate utilization. In this *in vitro* study, the relevant enzymes, originating from all three domains of life, were tested for the expected performance, the bottlenecks were identified and corrected via enzyme evolution, and additional supporting modules, such as cofactor regeneration modules were added, culminating in a functional and efficient cycle [33••]. This proof of a principle study bears promise for future design of artificial photosynthetic processes or as a metabolic feature of artificial or minimal cells.

Yet another approach for innovative CO_2 fixation has been tested in *E. coli*, by implementing a so-called 4-hydroxy-2-oxobutanoic acid (HOB) cycle [34]. In this pathway, CO_2 is fixed by propionyl-CoA carboxylase and is processed via native *E. coli* enzymes involved in L-homoserine biosynthesis, combined with novel activities evolved through selective evolution, in a mutant background lacking key C1 transfer functions, and with an addition of a heterologously expressed gene *panB*. This pathway's designated product is HOB, whose cleavage into pyruvate and methylene-tetrahydrofolate results in a common C1 currency, produced in a novel synthetic pathway [34].

Computational protein design

Synthetic methylotrophy can also be enabled by computationally designed enzymes that do not exist in nature. Such a novel enzyme, named formolase, has been described, that performs a carboligation reaction, directly converting formaldehyde into dihydroxyacetone that then feeds into central metabolism of *E. coli* [35]. Combining this enzyme with a Mdh has produced the shortest, liner pathway for methanol conversion into multicarbon compounds [36].

Other applications

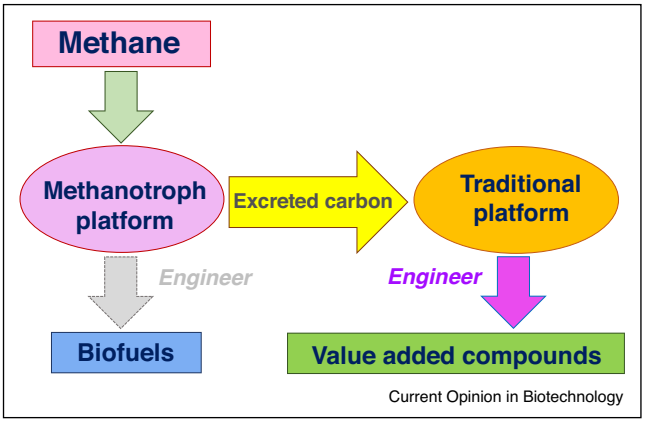
Recently, and rather unexpectedly, methylotrophs have emerged as organisms with a potential in biomining, especially with respect to rare Earth elements lanthanides (REEs). A general propensity of bacteria for binding REEs has been recognized, though the specific mechanisms of binding are not well understood [37,38]. *E. coli* has been successfully engineered for enhanced binding of REEs, by functionalizing an outer membrane protein [39]. However, methylotrophs may have special properties in REE biomining as they tend to encode and express REE-dependent enzymes, specifically, a novel type of REE-dependent Mdh [40,41], as well as a novel ethanol dehydrogenase [42]. The latter enzyme has also been identified recently in a non-methylotrophic organism *Pseudomonas putida* that itself is an industrially prominent strain [43]. As the REE-dependent enzymes have been shown to be regulated at the transcriptional level, responding to low concentrations of REEs [41], specific mechanisms for sensing, transport and binding are likely to exist, and these await to be discovered. Elucidation of these mechanisms, as well as genes and proteins involved will form a base for engineering microbes for binding and concentrating REEs for industrial purposes.

Future directions

The significance of C1 compounds for modern humanity continues to increase [44]. Both methane and CO_2 are practically unlimited on this planet. The removal of these gases, steadily produced by both natural and anthropogenic sources, and their conversion into value added compounds, including biofuels, would present the most practical solution to both greenhouse effect mitigation and to harvesting these abundant and sustainable carbon compounds. While microorganisms naturally capable of utilizing C1 compounds present attractive biotechnological platforms, many challenges exist that so far limited their broad use on large and commercially feasible scales. A reverse approach, of engineering some of the well-developed commercial platforms, such as *E. coli* or yeast, to consume C1 compounds with an output of value-added compounds has also been challenging so far. However, the outlook is rather bright for both approaches, as the tools and technologies are now in place to push the technical boundaries of these limitations, including genomic knowledge and the computational power available.

One novel aspect that has not been explored yet in terms of biotechnological use is the communal nature of C1 metabolism that appears to be common not only in anaerobic but also in aerobic settings [45]. Analogously to photoautotrophs, methanotrophs release carbon that can be consumed by non-methanotroph satellites [45].

Figure 2



A schematic depicting carbon flow in a designed synthetic consortium for converting methane into value products. Grey arrow denotes an optional step.

Thus, it is possible that commercially feasible platforms will involve communities rather than single microorganism cultures (Figure 2). Synthetic photosynthetic consortia have been already explored as production platforms [46], and use of synthetic communities in place of single strains, for a variety of biotechnological applications is gaining momentum [47]. Early work with synthetic methanotroph/non-methanotroph communities holds promise [48,49], but these are yet to be explored in terms of carbon conversion/product synthesis relevant to industrial needs. The potential of methylotrophs in applications to REE mining also awaits experimental testing.

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Conflict of interest

The author declares no conflicts of interest.

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