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Review

Harnessing the potential of microbial methane utilization for chasing sustainability

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Microbial methane utilization (known as methanotrophy) serves as a gatekeeper of methane emissions in numerous ecosystems. Methanotrophy became a platform for the production of biofuels, value-added chemicals, and novel molecules from natural or renewable gas. Methanotroph- driven Methanotroph-driven processes enable novel solutions for bioremediation, biomining of minerals, methane mitigation, and agriculture. All applications rely on in-depth understanding of methanotroph biochemistry, genetics, physiology, and ecological fitness.

Here, we review recent advances in the enzymology of methane utilization and methanotroph carbon assimilation pathways as well as progress toward engineering both native and synthetic methanotrophs. New bioreactor approaches to overcoming methane and oxygen mass transfer limitations are also described. Continued research in these areas is critical to future success in methanotroph optimization for industrial processes.

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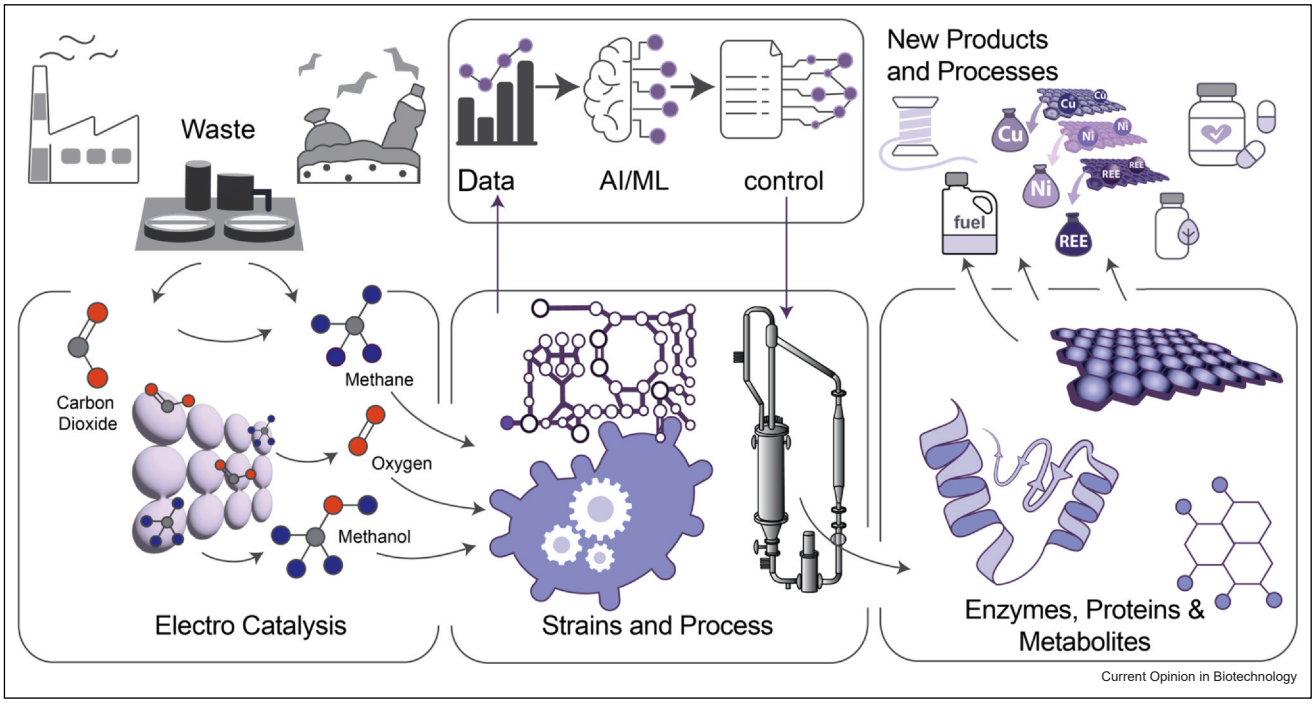
Introduction

Aerobic methanotrophy is the process by which methane (CH_4) is utilized as a source of both carbon and energy for microbial growth [1–3]. In nature, aerobic methanotrophs inhabit a variety of environments where they flourish at the intersecting gradients of O_2 and CH_4, including suspended water column habitats as well as soils, sediments, and geothermal vents. Much of the CH_4 produced biologically via methanogenesis or abiotically is oxidized by methanotrophs before its release into the atmosphere, providing an effective natural filter of CH_4 emissions [3]. Indeed, the combined activity of all types of methanotrophs makes up a significant global CH_4 sink, stifling emissions of this potent greenhouse gas to the atmosphere [2,3].

In addition to being a greenhouse gas, CH_4 is an abundant and economical source of carbon for the microbial production of biofuels and other value-added products. Thus, deploying methanotrophs in industrial processes can produce economical materials while mitigating greenhouse gas emissions, especially from waste-derived biogas. With a number of innovations in electrocatalysis for CO_2 capturing and conversion into methane or methanol, biological methane utilization has entered a new era of development (Figure 1). However, for such processes to be commercially viable, the growth rate and substrate utilization efficiency of methanotrophs require optimization [4,5]. Besides native methanotrophs, there is a significant focus on engineering recombinant methanotrophs by incorporating the necessary oxidation/utilization genes into more 'lab-friendly' organisms such as *Escherichia coli*. The successful reconstruction of *E. coli* strains that can utilize methanol, along with the development of strains expressing active methane monooxygenases, brings the concept of synthetic methanotrophy closer to practical application [6–8]. Whether native or recombinant, a detailed understanding of aerobic methane utilization by methanotrophs is paramount to their successful engineering into industrial biocatalysts. Besides biocatalyst optimization, mass transfer limitations due to the very low solubility of methane in aqueous solution must be overcome [9]. How to supply the carbon substrate from the gas phase to biocatalysts in the liquid phase while minimizing energy consumption presents a significant engineering challenge.

In this review, we describe how aerobic methanotrophs oxidize methane, how they generate fixed carbon and energy from this process, how the physiology of methanotrophs can be optimized for use in industrial processes, and how to enhance mass transfer from gas to biocatalysts with an emphasis on recent findings in the field.

Figure 1



Overview of past and ongoing development in microbial methane utilization. Over the years, our knowledge has evolved from understanding methanotrophic pathways in native microbes to the efficient reconstruction of methylotrophy and some elements of methanotrophic metabolism in synthetic organisms. We also observe a dramatic expansion of applications for methanotrophic chassis.

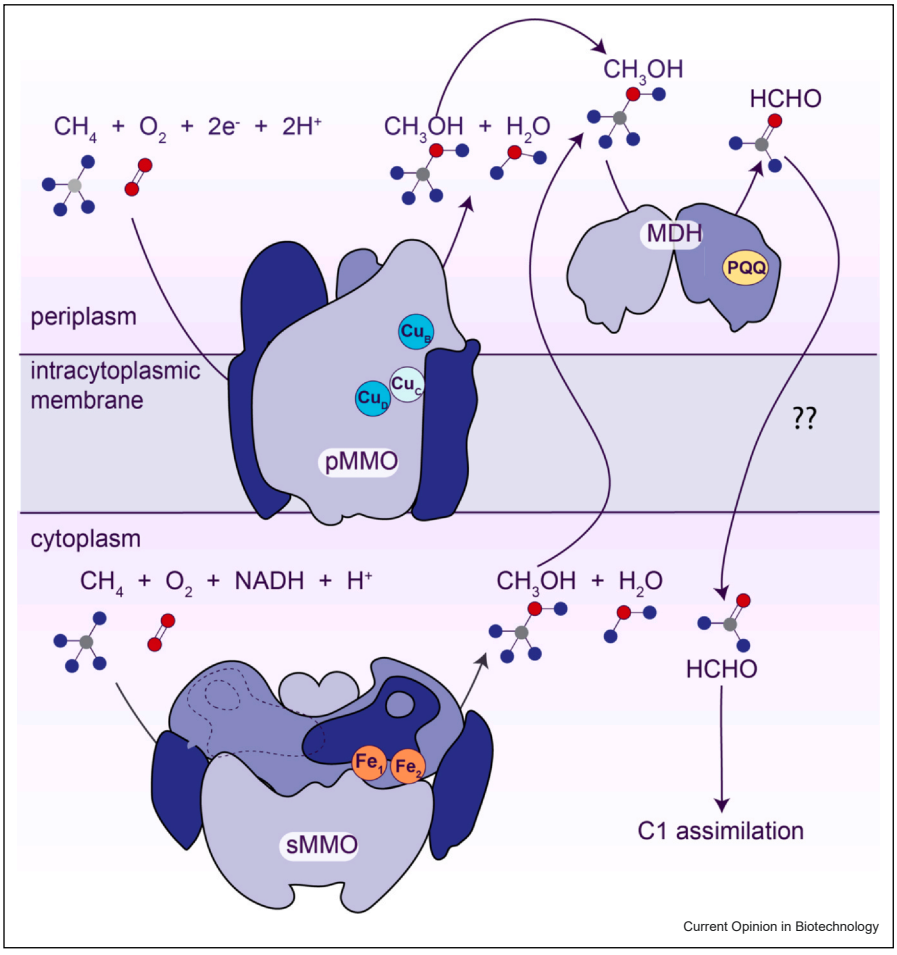
Biochemistry of oxygen-dependent methane oxidation

The biochemistry of CH_4 conversions has undergone substantial revision in the past two decades. The terms aerobic (AeMO) and anaerobic (ANME) methane oxidation are still commonly used to describe methane oxidation in prokaryotes compared to oxygen-independent conversions in archaea. However, we now know that AeMO pathways are more complex, and, in many cases, the fundamental definition of 'aerobic' has been challenged, as new methanotrophs are emerging that do not require oxygen for respiration, while still depending on oxygen for powering their methane oxidation machinery. In this review, we focus on this metabolic clade of methanotrophy (defined as O_2-dependent methane oxidation), which is found in numerous Proteobacteria and Verrucomicrobia, as well as the NC10 phylum. While most of the Proteobacterial and Verrucomicrobial methanotrophs still rely on external oxygen, the NC10 microbes have evolved a dedicated intracellular machinery for oxygen generation [3,4].

In O_2-dependent methane oxidation, methane is first oxidized to methanol either in the cytoplasm or within membranes by methane monooxygenase enzymes (MMOs) [10,11]. Methanol dehydrogenase (MDH) subsequently oxidizes the methanol to produce formaldehyde. Formaldehyde can then either enter central metabolism or be further oxidized to formate and finally to CO_2, the latter two steps yielding NADH. Methanotrophs likely originated from methylotrophic organisms through the lateral acquisition of the genes required to carry out methane oxidation, including enzymes, chaperones, and dedicated pathways, to uptake and process corresponding metal cofactors [12].

There are two types of MMOs, a cytoplasmic enzyme (sMMO) and a periplasmic and membrane-bound enzyme (pMMO; Figure 2). Besides cellular localization, the two systems differ in sequence, subunit composition, metal dependence, and chemical mechanism. Some methanotrophs can express both MMOs, with sMMO expression repressed by copper, while a large subset of strains only possesses the pMMO system, and a few strains only express sMMO [10–12]. In the presence of copper, and under sufficient methane levels, most methanotrophs produce extensive intracytoplasmic membranes (ICMs), which are densely packed with hexagonal arrays of pMMO, recently visualized by cryoelectron tomography (cryoET) [13] and cryoelectron microscopy (cryoEM) [14]. The molecular mechanisms

Figure 2



Biochemistry of methane oxidation. Methane is oxidized to methanol by metal-dependent MMOs in the cytoplasm or ICM. Methanol is then converted to formaldehyde by the periplasmic pyrroloquinoline quinone–dependent MDH. The mechanism of formaldehyde translocation to the cytoplasm for carbon assimilation remains unclear.

by which copper induces ICM formation and regulates sMMO expression are not well understood.

The sMMO system comprises three core components: a hydroxylase (MMOH), a reductase (MMOR), and a regulatory protein (MMOB) [11,15]. MMOH consists of three subunits arranged in an $\alpha_2\beta_2\gamma_2$ dimer with the α subunit housing a catalytic diiron active site. The two electrons required for methane oxidation are shuttled to the diiron center from NADH via a [2Fe-2S] cluster and an FAD cofactor in MMOR. The functional role of a fourth component that interacts with MMOH, MMOD, remains under investigation [16]. Structures of MMOH, the MMOH-MMOB complex, the MMOH-MMOD complex, MMOB, and individual domains of MMOR have been determined by a combination of crystallography, nuclear magnetic resonance, and cryo-electron microscopy [10]. These structures revealed the atomic details of the diiron active site, potential channels for CH_4 and O_2 access, and conformational changes upon MMOB binding, some of which modulate access to the active site [17,18]. The mechanism of CH_4 oxidation involves a series of iron-oxygen reactive intermediates, including the C-H bond-breaking intermediate Q, the molecular details of which are still under debate [19,20]. Binding of MMOR and MMOB, which are believed to occupy the same or similar sites on MMOH, and electron transfer are highly orchestrated to prevent unproductive reduction of intermediate Q before it reacts with substrate [21].

While similarly reactive intermediates and accompanying regulatory mechanisms likely exist in pMMO, studies are not as advanced due to difficulties isolating enzymatically active samples from the ICMs. pMMO consists of three subunits, PmoB, PmoA, and PmoC, arranged in an $\alpha_3\beta_3\gamma_3$

trimer [10,11]. Alphaproteobacterial pMMOs contain an additional 23-residue transmembrane helix, which was recently identified in *Methylocystis* sp. Rockwell pMMO [14]. Multiple monocopper-binding sites are modeled in pMMO crystallographic and cryoEM structures, including the Cu_B and bis-His sites in PmoB, the ligands to which are not strictly conserved in all methanotroph PmoB sequences, and the Cu_C and Cu_D sites in PmoC [11]. Additional sites in PmoB and PmoA have been reported [14], but the majority of spectroscopic, biochemical, and structural data are consistent with the presence of only these four sites.

Simultaneous occupancy of the Cu_C and Cu_D sites, which are separated by ~6 Å, has not been observed. In crystal structures of inactive, detergent-solubilized pMMO, Cu_C is occupied, and the region housing Cu_D is disordered. In cryoEM structures of active pMMO in lipid nanodiscs or native membranes, Cu_D is occupied, and Cu_C is empty [22–24]. These observations, along with a recent spectroscopic and cryoEM study of product analog binding to pMMO [25], strongly suggest that Cu_D is the active site. The source of electrons for pMMO remains unclear, with one possibility being ubiquinol that is reduced by a type 2 NADH:quinone oxidoreductase. In an alternative model, termed direct coupling, electrons from methanol oxidation by MDH are transferred back to pMMO via an MDH-associated cytochrome *c* [26].

Methanol produced by sMMO or pMMO is further converted to formaldehyde by the periplasmic pyrroloquinoline quinone-dependent enzyme MDH (Figure 2). The Mxa MDHs, which include two subunits, MxaF and MxaI, in an $\alpha_2\beta_2$ dimer, also require calcium, whereas the Xox MDH, also dimeric, consists of a single subunit, XoxF, and requires lanthanides. In strains that possess both the Mxa and Xox systems, expression is regulated by lanthanide ions [27–29]. Although MDHs are periplasmic, they are well known to associate with ICMs in methanotrophs, and transient interactions with purified pMMO have been detected [30,31]. Such interactions are supportive of the direct coupling electron transfer model as well as channeling of methanol directly to MDH from pMMO. However, stable complexes between pMMO and MDH have not been isolated or observed in the membranes by cryoEM or cryoET. In the case of sMMO, it is not known how methanol produced in the cytoplasm reaches MDH in the periplasm. Moreover, since the C_1-assimilatory pathways as well as the cofactor-linked oxidation enzymes (i.e. tetrahydromethanopterin, H_4MPT, and tetrahydromethanolofolate, H_4F, pathways) are located in the cytosol, the resultant formaldehyde, a toxic C_1-intermediate, must be transported from the periplasm (Figure 2). The formaldehyde transporters or any other elements that support this critical step are still unknown.

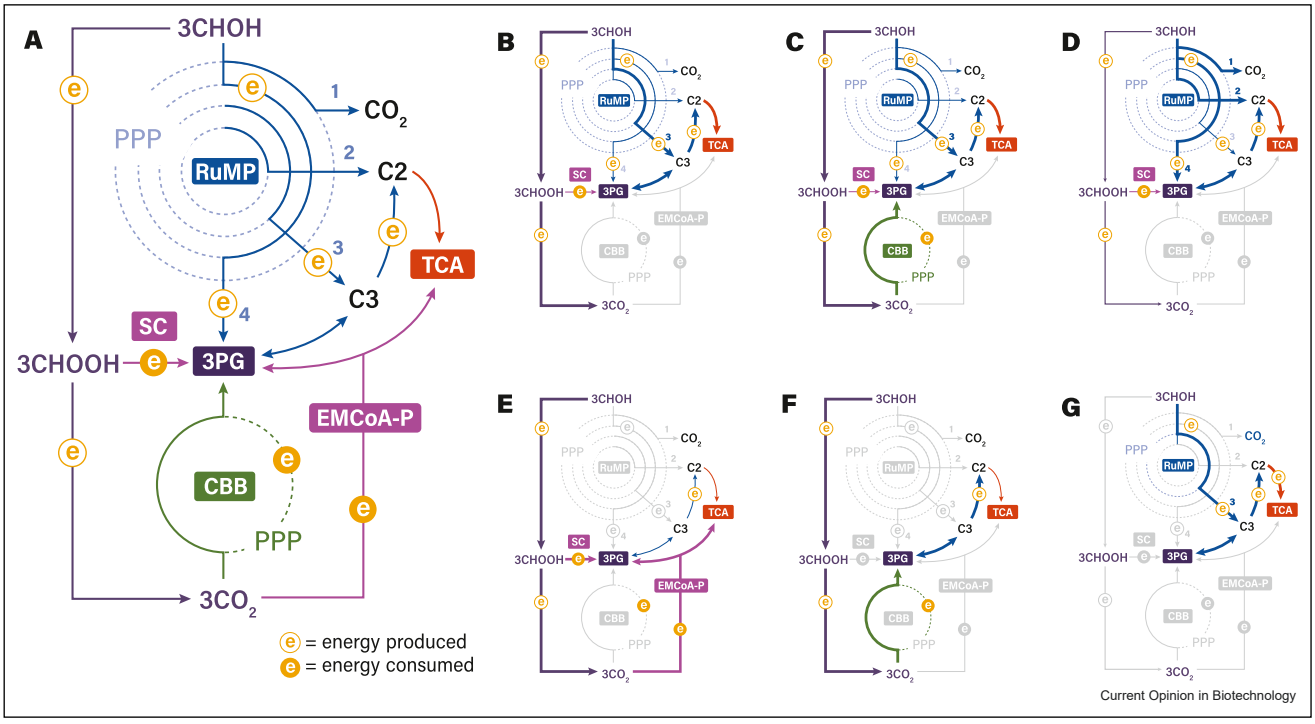
Methane assimilation: C_1-entry points

Once methanol is oxidized to formaldehyde, it can either be assimilated into biomass or oxidized to formate and eventually to CO_2. Thus, formaldehyde is a main branching point that controls the balance between energy production and carbon acquisition. Three main entry points for CH_4-derived carbon assimilation have been identified: (i) formaldehyde is integrated into phosphosugars via the ribulose monophosphate (RuMP) cycle, (ii) formate and CO_2 enter via the serine cycle (SC), and (iii) CO_2 is incorporated via the Calvin cycle (Figure 3). Since commonly described reactions for the SC pathways are not sufficient to drive C_1-anabolism, the SC pathway must operate in combination with additional metabolic modules. To complete the assimilation, the alphaproteobacterial (type II) methanotrophs regenerate glyoxylate by the ethylmalonyl-CoA pathway (EMCoA-P), which brings an equal amount of CO_2-derived carbon into the biomass [32,33]. It is now apparent that gammaproteobacterial methanotrophs (commonly described as type I or type X) also use the SC as a backdoor to reintegrate overflow of C_1-carbon into cellular biomass (Figure 3c) and it is predicted to serve as a supplementary route for serine or phosphoenolpyruvate biosynthesis and glycolate recycling [34,35].

The spontaneous condensation of formaldehyde with H_4F was proposed initially, but has been debated, with undisputed proof that the nonenzymatic reaction cannot occur *in vivo* [36], and the pathway is now routed from formate into the H_4F-pathway [37]. Several studies hinted at the existence of H_4F-specific enzymatic systems that, similarly to formaldehyde activation, contribute to direct condensation of formaldehyde with H_4F [29]. From an energetic point of view, the existence of the enzymatic machinery that couples high-energy formaldehyde with H_4F is very plausible and would also mitigate formaldehyde toxicity. However, such an enzyme has not been identified. Furthermore, formate alone cannot support growth of methanotrophic bacteria, suggesting a significant gap in our understanding of this part of methanotrophic metabolism.

The RuMP pathway is generally assumed to be the most efficient route to C_1-carbon assimilation. In this pathway, three molecules of formaldehyde are combined with three molecules of ribulose-5-P to generate three molecules of hexulose-6-P. The hexulose-6-P is further converted to three fructose-6-P molecules. Fructose-6-P then enters central metabolism via different and often co-occurring pathways, including oxidative or non-oxidative pentose phosphate pathways, glycolytic pathways (EMP and EDD), and the phosphoketolase shunt (Figure 3) [34,37]. The essentiality of each pathway as well as their contributions to carbon flux differ in even closely related methanotrophs [38–40]. While the underlying regulatory machinery is not fully understood, it

Figure 3



C_1-assimilation pathways in native and synthetic microbes. **(a)** Summary of described assimilatory pathways, including the ribulose monophosphate pathway (RuMP) variants (blue): including the oxidative pentose phosphate pathways (oxPPP) (1), phosphoketolase pathways, XFP-RuMP [33], also described as bifidobacterium shunt [39] and methanol-condensation cycle [57] (2), EDD-RuMP (3), and EMP-RuMP (4); the SC integrated with the EMCoA-P pathway(pink); and the Calvin cycle, CBB (green). **(b)** Carbon assimilation flux in Type I methanotrophs with the EDD-RuMP, initially predicted as the main methanotrophic pathways [43] but observed only in some strains during growth on methanol [37,39]; **(c)** Carbon flux in Type X methanotrophs, such as *Methylococcus* [78–80]; **(d)** Carbon flux in Type I methanotrophs, such as *Methylotuvimicrobium*, the EMP-RuMP variants enables the high efficiency of methane utilization, and commonly observed during growth on methane [38,40]; **(e)** Carbon assimilation in Type II methanotrophs, such as *Methylosinus/Methylocystis* [31,32]; **(f)** Autotrophic pathways for C_1–assimilation found in some alphaproteobacterial and Verrucomicrobial [51] methanotrophs. **(g)** Synthetic C_1 assimilation pathway, reconstructed in *E.coli* [58]. Connection line thickness indicates the predicted carbon flow based on -omics data analysis or direct ^{13}C-carbon measurements. Gray lines represent pathways that are absent in the type of microorganism described.

is now recognized that the flexibility of C_1-metabolism is at least partially driven by the respiratory and the primary oxidation machinery inventory (summarized in Refs. [26,41]). New ^{13}C-carbon flux data in combination with mutagenesis uncovered a more significant contribution of the CO_2 fixation even in well-studied methanotrophs such as *Methylococcus capsulatus* (Bath) [42,43]. The essentiality of the Calvin cycle not only overturned longstanding theoretical predictions that the CO_2-assimilation pathway is not energetically feasible [44] but also suggests that the energetics of methanotrophic metabolism are far from being understood. Novel studies suggest the possibility of mixotrophy as a route to overcome the energy limitation in methanotrophs that rely on autotrophy for carbon assimilation [45,46].

Harnessing methanotrophs for biotechnology

Methanotrophs are an extremely attractive option for biotechnological applications since methane is an abundant, inexpensive, and easily accessible source of carbon, both as natural gas and in a mixture with CO_2 in biogas [47]. Unlike sugar upgrading processes, which compete with the agricultural industry for expensive starting materials, the majority of methane emissions are currently flared or vented to the atmosphere, rendering methane a huge untapped market. Strategies to engineer efficient use of methane to produce value-added chemicals fall into two main research efforts: augmentation of native methanotrophs toward desired outputs using established or new systems biology toolboxes for non-conventional chassis and reconstructing synthetic methanotrophy in model bacterial systems such as *E. coli*.

Efforts to engineer native methanotrophs into efficient biocatalysts have been accelerated by recent studies of methanotrophs on a systems biology level. Whole cell transcriptomic, proteomic, and metabolomic data have been incorporated into whole genome-scale metabolic models [48,49]. As a result, new models for methanotrophy have been established and used to inform more

efficient genetic engineering of methanotrophs to control carbon and energy flux. In particular, biomass yield has been increased by the overexpression of the phosphoketolase pathway [50] and the generation of a xylose-consuming methanotrophic strain [51].

Decades-long implementation of methanotrophy for bioremediation of organic compounds or metal biomining has entered a new era of interest. The discovery that methanotrophic metal-chelating molecules, such as methanobactins, can improve copper sequestration in animal systems has driven a new focus on methanotroph-based pharmaceuticals [52,53]. Insights into copper and lanthanide sequestration pathways in methanotrophs have led to bioinspired opportunities for biomining of critical materials [54,55].

Efforts to engineer a synthetic methanotroph have focused on *E. coli*. Generation of a methane-oxidizing strain has proven difficult, likely due to the complexity of the MMOs and their requirement for reducing equivalents. Several designed MMO mimics have been reported to oxidize methane in *E. coli* [56,57], but it is not clear that these proteins contain properly assembled metal active sites. Furthermore, these studies lack direct comparisons to native MMO activity using identical methods. In contrast, recent advances have been made in engineering *E. coli* to utilize methanol [58–60]. The RuMP cycle has been the preferred pathway for these studies, as it uses many of the pentose phosphate enzymes already present in *E. coli*. Targeted genetic engineering followed by experimental evolution resulted in an *E. coli* strain that is capable of utilizing methanol with a growth rate comparable to natural methylotrophs [6,57–59]. Furthermore, the possibility of chemical production from methanol using a synthetic host has been demonstrated [7]. The successful expression of sMMO has been reported as well [61].

Methanotrophic consortia are a long-thought strategy to improve the limitations of C_1-derived biosynthesis and gas fermentation. Specifically for biogas (~60% CH_4, 40% CO_2) conversion, synthetic methanotroph-photoautotroph cocultures have shown significant promise [61,62]. The coculture strategy is inspired by the prevalence of metabolic coupling of CH_4 oxidation to oxygenic photosynthesis [63–65]. Using the principles that drive the natural consortia, various synthetic methanotroph-photoautotroph cocultures have been studied for biogas valorization and exhibit significantly increased methane consumption and biomass production rates [61,62,66–68].

Overcoming mass transfer limitations

In addition to biocatalyst optimization, viable commercial applications require delivery of methane to the biocatalysts with minimum energy consumption. Methane has a very low solubility in water, only 0.0014 mol/(kg·bar) at 25°C [69], which presents a significant barrier in substrate supply. To improve throughput and reduce reactor volume, industrial strains for methane conversion should have a high specific affinity for methane, a_A^0 (expressed as the fraction of $V_{\max\,(app)}$, the whole cell maximum methane oxidation rate, and $K_{m(app)}$, the whole cell Michaelis-Menten constant). For example, *Methylotuvimicrobium buryatense* 5GB1C, one of the promising industrial methanotrophic strains, has a specific affinity of 1.101×10^{-9} l cell^{-1}h^{-1} [70]. Therefore, biological methane conversion processes are mass transfer limited in general, that is, the consumption of methane is limited by how fast methane can be transported from the feeding gas stream to the biocatalysts, instead of how fast the catalysts can convert the methane. The low solubility of methane in water not only requires a higher energy input (for agitation) but also results in lower biocatalyst concentration in the reactor, which limits the methane conversion rate and, therefore, the reactor throughput, necessitating large bioreactor sizes.

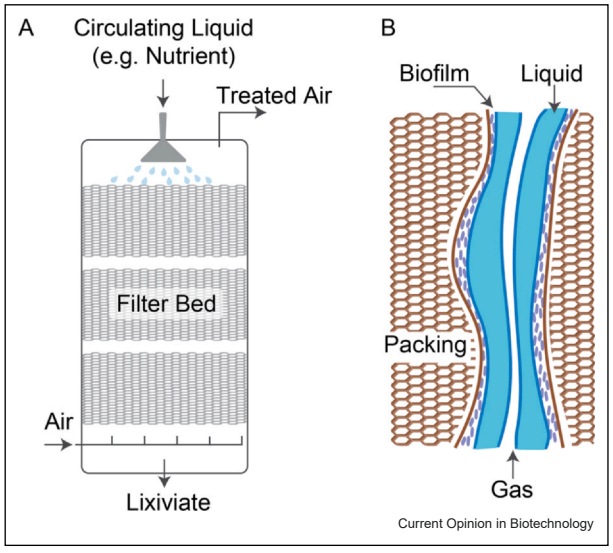
The methane transfer rate is governed by the equation:

$$\textit{methane transfer rate} = k_L a\left(\frac{C_G}{H} - C_L\right) = \frac{\textit{driving force}}{\textit{resistance}} = \frac{\frac{C_G}{H} - C_L}{\frac{1}{k_L a}}$$

Here, the mass transfer rate can be enhanced either by reducing the mass transfer resistance (i.e. increasing $k_L a$) or by increasing the driving force (i.e. by increasing $\frac{C_G}{H}$).

Existing strategies to reduce mass transfer resistance all focus on increasing the specific surface area of the gas–liquid interface [71–76]. For high methane concentration substrates (e.g. natural gas), enhancing mass transfer involves bioreactor designs that can create and maintain small gas bubbles to maximize interface area. Reactor types include tubular loop reactors, external loop airlift reactors, bubble columns, and continuous stirred-tank reactors. These systems typically require substantial energy input to ensure effective mixing and re-circulation of the gas and liquid phases. For low methane concentration substrates (e.g. 1–5% CH_4 in waste gas), bioreactor design usually focuses on maximizing interfacial area without active mixing by immobilizing cells on porous solid carriers. These include biofilters and biotrickling filters (Figure 4). In these porous packed bed reactors, aerobic methanotrophs (communities) grow and form a biofilm on the surface of the porous packing material to provide a huge gas–liquid interface. These systems drastically reduce energy

Figure 4



Schematic diagram of the conventional biofilter **(a)** and illustration of mass transfer in biofilter **(b)**.

consumption by avoiding continuous gas–liquid mixing. However, they come with trade-offs such as slow startup times, limited reactor lifespan, and challenges in biomass recovery.

Strategies to increase the driving force usually modify Henry's constant (H) by introducing agents with higher methane affinity, such as vectors, polymers, nanoparticles, and electrolytes [72]. While these agents can improve methane solubility and thus the mass transfer driving force, they are currently only suitable for high-methane concentration scenarios. Furthermore, this approach remains largely limited to laboratory studies due to technical challenges in separating and recycling the additives in practical systems.

In general, studies to determine mass transfer coefficients in bioreactors for gas treatment are limited, and no study has addressed the influence of biomass on the partitioning coefficient of methane in gas–liquid and gas–biofilm interfaces. A two-film theory [77] suggests that the dominant mass transfer resistance of transporting gas substrates to biocatalysts lies in the bulk liquid phase/layer. Therefore, reducing the bulk liquid phase could significantly improve mass transfer, an idea supported by a recent attempt to immobilize cells through hydrogel surface absorption [78].

Conclusions and future directions

Recent investigations of methanotrophy, from molecular to single cell to microbial community levels, have delivered several novel scientific discoveries. Progress has been made toward engineering native methanotrophs or methanotroph cocultures for bioprocessing.

Significant progress has been made in engineering native methanotrophs or methanotroph co-cultures for bioprocessing applications. While synthetic methanotrophy in *E. coli* shows promise, it requires further validation. To advance the field, a deeper understanding of the cellular structures and metabolic pathways that facilitate energy-efficient methane transformations in native biological systems is essential.

Additionally, innovations in methane fermentation process designs — covering everything from reactors to microbial strategies — are urgently needed to promote mass transfer and enable large-scale implementation. This is crucial for addressing environmental challenges associated with methane emissions. We also anticipate the use of machine learning and artificial intelligence approaches to enhance the stability of gas fermentation processes. Continued collaborations among microbiologists, biochemists, and engineers are vital for transforming fundamental knowledge into commercially viable biotechnologies.

Author Contributions

MGK, ACR, and JW compiled literature and wrote the manuscript.

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Data Availability

No data were used for the preparation of this article.

Declaration of Competing Interest

The authors declare no competing interest.

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- • of special interest
- •• of outstanding interest

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