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Review

Methane to bioproducts: unraveling the potential of methanotrophs for biomanufacturing

Justin N Tan[1,*], Keshav Ratra[1,*], Steven W Singer[2,3], Blake A Simmons[2,3], Shubhasish Goswami[2] and Deepika Awasthi[2,3]

With the continuous increase in the world population, anthropogenic activities will generate more waste and create greenhouse gases such as methane, amplifying global warming. The biological conversion of methane into biochemicals is a sustainable solution to sequester and convert this greenhouse gas. Methanotrophic bacteria fulfill this role by utilizing methane as a feedstock while manufacturing various bioproducts. Recently, methanotrophs have made their mark in industrial biomanufacturing. However, unlike glucose-utilizing model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, methanotrophs do not have established transformation methods and genetic tools, making these organisms challenging to engineer. Despite these challenges, recent advancements in methanotroph engineering demonstrate great promise, showcasing these C1-carbon-utilizing microbes as prospective hosts for bioproduction. This review discusses the recent developments and challenges in strain engineering, biomolecule production, and process development methodologies in the methanotroph field.

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Introduction

With the looming threat climate change poses, the search for a solution has become environmentally and economically desirable. The greenhouse gases such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide are classified drivers of climate change and have become the target of interest for researchers. These greenhouse gases primarily stem from anthropogenic activities of burning fossil fuels, electricity usage, and agriculture (Figure 1) [1]. Although CO_2 is the most emitted greenhouse gas, at 36.8 giga tons released in 2022 [2], CH_4 has also become a target greenhouse gas because of its stronger heat-trapping capabilities (> 25× stronger than CO_2) and is the second most emitted greenhouse gas at 793 million metric tons CO_2 equivalents released in 2021 [3]. Currently, CH_4 has contributed to 30% of global warming since the Industrial Revolution [4]. Thus, reducing CH_4 emissions is important for decreasing the earth's temperature significantly [5].

Efforts to mitigate CH_4 emissions have encompassed a diverse range of strategies. These include sealing off disused wells to prevent leaks and implementing microbiological techniques alongside dietary modifications to lower the CH_4 cattle produce [6,7]. One promising approach to mitigating CH_4 emissions is through the use of methanotrophs, organisms that consume CH_4 and act as an effective natural CH_4 sink [8]. Aerobic methanotrophs have been well studied and classified into two types, I and II, primarily based upon their CH_4 assimilation route. *Methylococcus capsulatus*, *Methylotuvimicrobium alcaliphilum*, and *Methylomonas sp.* DH-1 are well-characterized type 1 methanotrophs, whereas *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP are well-studied type II hosts [9]. Some of the recently isolated methanotrophs have been listed in Table 1. Methanotrophs also uniquely enable the biological conversion of CH_4 into eco-friendly and sustainable chemicals, demonstrating significantly higher efficiency than chemical methods [10]. For example, methanotrophs can convert CH_4 to methanol with a 78%

Figure 1

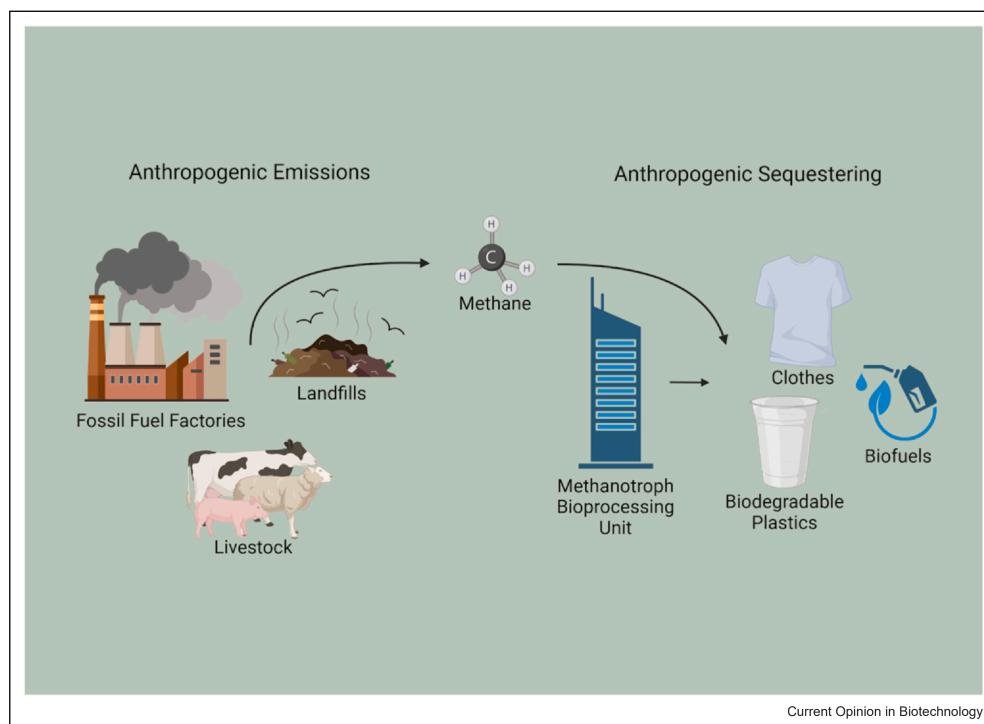


Illustration of anthropogenic CH_4 emissions being sequestered into products synthesized by a methanotroph-based bioprocessing unit. Agriculture, landfills, and fossil fuel emissions are the three largest anthropogenic CH_4 producers [21]. Illustration created using BioRender.com [22].

Table 1

List of recently isolated methanotrophs with their classification, isolation, and growth conditions. Additional information for more methanotrophs can be found elsewhere [9].

Strain	Growth (℃) conditions	Isolation	Source
Type I Gammaproteobacteria			
M. capsulatus MIR	40–50℃ Thermotolerant	Sludge of waste treatment plants in Russia	[23]
Methylmicrobium album BG8	25–30℃ Slightly acidophilic	Soda lakes in Southeast Siberia, Mongolia, Africa, and North America	[24,25]
Type 2 Alphaproteobacteria			
M. extorquens AWP227	25–30℃ Pink-pigmented facultatively methylotrophic	Soil in Poland	[26,27]
M. iwanois SD4	25–30℃ facultatively methanotrophic	Rhizosphere from rice in China	[28]

conversion efficiency, far surpassing the 25% efficiency achieved by chemical processes [11,12].

However, despite successful examples of bioconversion of CH_4 into valuable chemicals such as biofuels and bioplastics [13,14], there are challenges in working with different methanotrophs, as there are limitations in adaptable genetic toolboxes and low product titers [15,16]. One of the major hurdles that methanotrophs face is scaling up to industrial-level production because of the existing hurdles with lower gas-to-liquid conversion rate and lower gas solubility in liquid culture [17]. In addition, growth media in cultivating methanotrophs consists of many metals combined in an expensive trace element mix, making large-scale production expensive [18]. Nevertheless, research is ongoing to address the existing limitations through developing innovations such as CRISPR/Cas9 technology for genome editing in methanotrophs [19] and better bioreactor designs for enhanced gas and mass transfer [20] to improve the production of industrial chemicals. These recent and ongoing developments illuminate the scalable opportunities methanotrophs bring to the biotech industry. This article provides a comprehensive analysis of recent advancements and methodologies in bioengineering methanotrophs alongside a forward-looking perspective on the role and potential of methanotrophs in bioprocessing.

Biomolecule production using methanotrophs

Methanotrophs have garnered significant interest due to their potential to make a wide array of biomolecules, including polyhydroxyalkanoates (PHAs), polyisoprenoids, polyethers, polyurethanes, and polyesters [29]. These biomolecules have wide applications, underscoring methanotrophs' remarkable potential to provide sustainable alternatives to conventional materials [29]. Table 2 presents an extensive list of biomolecules made by both native and engineered strains of methanotrophs. As denoted, most chemicals synthesized are on the scale of milligrams, indicating the need for optimization for higher yield of these biomolecules.

Bioengineering strategies on methanotrophs for bioproduct synthesis

When manipulating the CH_4 metabolic pathways of a methanotroph, four strategies have been employed (depending upon the desired product): redesigning the Ribulose monophosphate pathway (type 1), enhancing the pyruvate pool, enhancing the acetyl-CoA pool, and manipulating the fatty acid biosynthesis pathway [54] (Figure 2).

A possible approach to improve the carbon conversion efficiency of CH_4 to products was recently shown by Henard *et al.* by genetically engineering methanotrophs to integrate phosphoketolase (*pka*) in the Ribulose monophosphate pathway [55]. It involves shuttling CH_4 in the form of formaldehyde to acetyl-CoA, bypassing the pyruvate decarboxylation by pyruvate dehydrogenase (PDH) to produce acetyl-CoA, thus preventing carbon loss as CO_2 [54]. This method also has the advantage of avoiding ATP usage [54]. Overexpressing *pka* resulted in a twofold increase in the concentration of acetyl-CoA in *M. buryatense* [55] (Figure 2).

An alternative strategy involves increasing the pyruvate pool, which subsequently enhances the production of pyruvate-derived compounds such as lactic acid (Figure 2). For example, Henard *et al.* examined the production of lactic acid through *M. alcaliphilum* 20Z PDH mutants, increasing the available pyruvate for lactate dehydrogenase (*ldh*), improving the flux of pyruvate to lactic acid [56].

Similar to pyruvate, enhancing acetyl-CoA production results in an increase in its derivatives, such as organic acids like crotonic acid and fatty acids [40]. Garg *et al.* engineered a reverse beta-oxidation pathway in *M. buryatense* 5GB1C with one turn to make the C4 product crotonic acid from acetyl-CoA [40]. They produced more butyric acid with more acetyl-CoA (Figure 2) using overexpressed acetyl-CoA

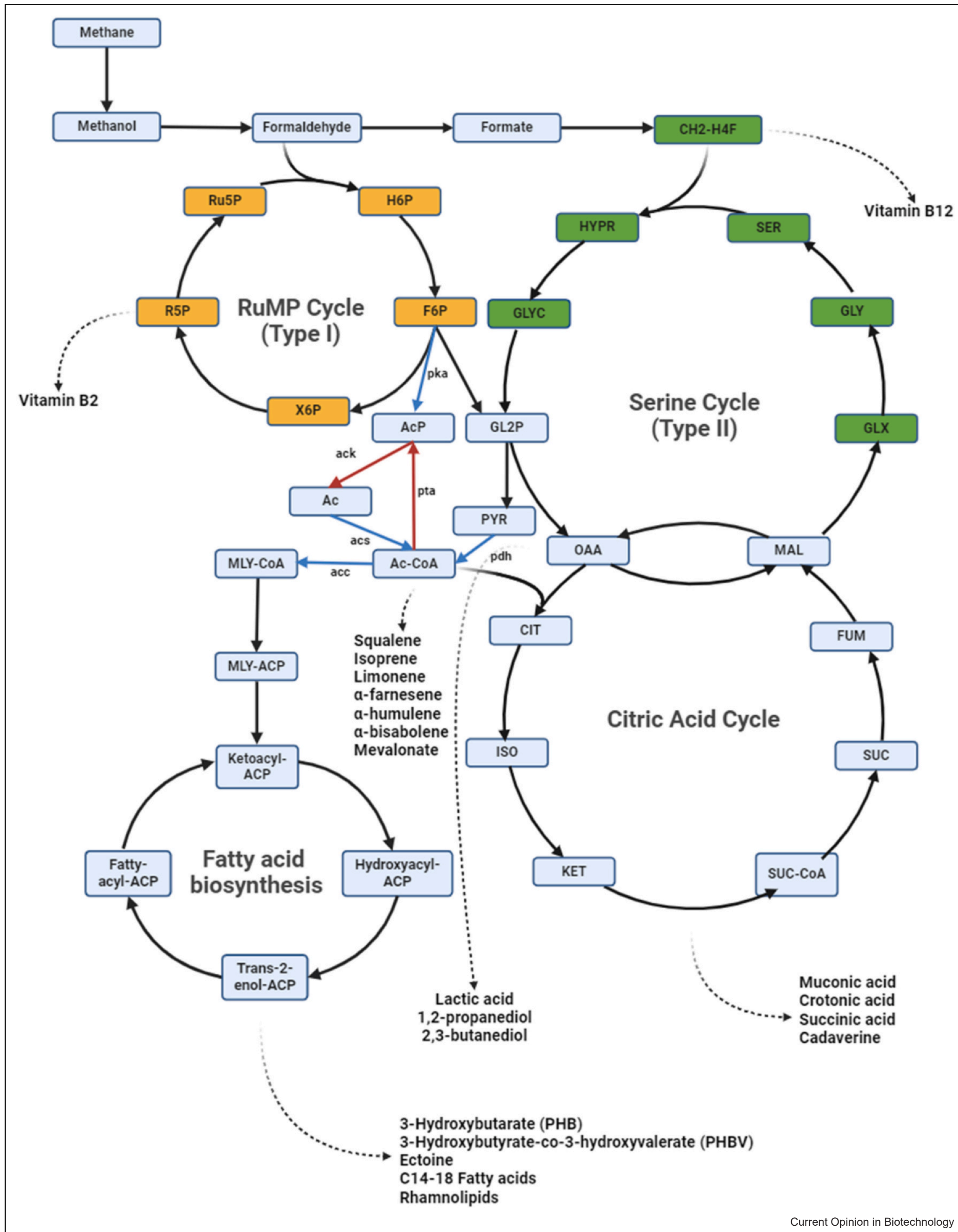
Table 2

Biochemicals and their titer/yield as produced by methanotrophs.

Organism	Bioproduct	Titer/yield	Reference
Native strains			
Methylosinus trichosporium OB3b	3-Hydroxybutarate (PHB)	500 mg/g DCW	Singh *et al.* [29]
M. alcaliphilum 20Z	3-Hydroxybutarate (PHB)	1853 ± 429 mg/g DCW	Chau *et al.* [30]
Methylosinus trichosporium 11131	3-Hydroxybutyrate	41.24%w/w	Mohammad *et al.* [31]
Methylocystis sp. MJC1	3-Hydroxybutyrate-co-3-hydroxyvalerate (PHBV)	8.9 g/l	Lee *et al.* [32]
Mixed methanotrophic cultures	3-Hydroxybutyrate-co-3-hydroxyvalerate (PHBV)	0.49 g/g-substrate	Gesicka *et al.* [33]
M. bryophila	Methanol	16.4 mmol/l	Patel *et al*. [34]
M. buryatense 5G1BC	Methanol	.9875 mM/hour	Priyadarsini *et al.* [35]
Methylobacterium sp. G-10	Cobalamin (vitamin B12)	800 ng/g biomass	Ivanova *et al.* [36]
Methylocystis sp. strain M	Riboflavin (vitamin B2)	0.116 ± 0.004 μM	Vasudevan *et al.* [37]
Engineered strains			
M. alcaliphilum 20Z	Ectoine	37.93 ± 3.27 mg/g DCW	Pham *et al.* [38]
M. alcaliphilum 20Z	Lactic acid	93 mg/l	Le *et al.* [39]
M. buryatense 5GB1C	Crotonic acid	70 mg/l mg/g CH_4	Garg *et al.* [40]
Methylomonas sp. DH-1	Succinic acid	134 mg/l	Nguyen *et al.* [41]
Methylomonas sp. DH-1	Squalene	31.3 mg/l	Kang *et al.* [42]
M. capsulatus Bath	Isoprene	228.6 mg/l	Emelianov *et al.* [43]
Methylomonas sp. 16a	Limonene	0.5 ppm	Dicosimo *et al.* [44]
M. alcaliphilum 20Z	α-Farnesene	91.55 mg/l	Pham *et al.* [45]
M. alcaliphilum 20Z	α-Humulene	0.04 mg/g DCW	Nguyen *et al.* [46]
Methylomonas sp. DH-1	Acetol	1.35 g/l	Chau *et al.* [47]
Methylosinus trichosporium OB3b	Cadaverine	283.63 mg/l	Nguyen *et al.* [48]
M. capsulatus	Mevalonate	2090 mg/l	Jeong *et al.* [16]
M. capsulatus Bath	Mevalonate	28.2 g DCW/l	Jang *et al.* [49]
M. alcaliphilum 20Z	Rhamnolipid	1 μM	Awasthi *et al.* [50]
M. alcaliphilum 20Z	2-Propanol	263 mg/l	Le *et al.* [51]
M. trichosporium OB3b	3-Hydroxybutarate (PHB)	191.41 mg/l	Kulkarni *et al.* [52]
M. trichosporium OB3b	Lipase	71.53 U/mg of activity	Kulkarni *et al.* [52]
M. trichosporium OB3b	1,2-Propanediol	251.1 mg/l	Park *et al.* [53]

DCW, dry cell weight; ppm: parts per million.
Titer: mg/l, μg/ml, and μM. Yield: ng/g biomass, mg/g DCW, g/g DCW, mg/l CH_4, mg/l biogas.

Figure 2



Targeted metabolic pathways for engineering methanotrophs. Gold box, carbon assimilation by the RuMP cycle in type I methanotrophs; Green box, SER cycle in type II methanotrophs; Blue box, overlapping metabolites in both types of methanotrophs. Red arrows, enzymes/genes knocked out; Blue arrows, enzymes overexpressed; Dotted arrows, end products of pathways involving multiple steps. Ac, acetate; Ac-CoA, acetyl-CoA; AcP, acetyl phosphate; CIT, citrate; F6P, fructose-6-phosphate; FUM, fumarate; GBP, glyceraldehyde-1,3-bisphosphate; GL2P, glycerate-2-phosphate; GLX, glyoxylate; GLY, glycine; GLYC, glycerate; H6P, hexulose-6-phosphate; HPYR, hydroxypyruvate; ISO, isocitrate; KET, ketoglutarate; MAL, malate; MLY-CoA, malonyl-CoA; MLY-ACP, malonyl-acyl carrier protein; OAA, oxaloacetate; PYR, pyruvate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; SER, serine; SUC, succinate; SUC-CoA, succinyl-CoA; X6P, xylulose-6-phosphate; Enzyme names: acs, acetyl-CoA synthetase; pkt, phosphoketolase. Illustration has been created using BioRender [22]. RuMP, Ribulose monophosphate.

synthetase [40,54]. Knockout of phosphate acetyltransferase (*pta*) is also a potential target for increasing the acetyl-CoA pool [54].

There have also been recent efforts to increase fatty acid production in methanotrophs [57]. Demidenko *et al.* produced excess fatty acids in the methanotroph *M. buryatense* 5GB1 by deleting acetate kinase (*ack*) and overexpression of acetyl-CoA carboxylase (*acc*) to increase fatty acid biosynthesis by 20% [57]. Recently, an indirect strategy was employed by Awasthi *et al.*, involving adaptive laboratory evolution on a biosurfactant, which improved malonyl-CoA cellular pool that directly improved fatty acid secretion ~5-fold, by a lab-adapted strain of *M. alcaliphilum* DSM19304 [50]. These direct and indirect approaches of bioengineering methanotrophs have been successful in demonstrating several proof-of-concept technologies that show promise in establishing methanotrophs as future industrial biocatalysts (Figure 2, Table 2).

Genetic engineering tools for methanotrophs

Effective genetic engineering methodologies for any host rely on robust DNA transfer methods, gene expression promoters, and precision gene editing tools. While these tools and techniques are abundantly available for sugar-utilizing model organisms such as *E. coli* and *S. cerevisiae*, their counterparts for methanotrophs remain limited and are still under development.

Conjugation has predominantly been the method of choice for foreign DNA transfer in methanotrophs due to its reproducibility and success. Due to the slow and time-consuming nature of this method [58], a faster alternative electroporation has been successfully tested in some strains, such as *Methylomicrobium buryatense* 5GB1C, *Methylomonas sp.* strain LW13, and *Methylobacter tundripaludum* 21/22 [15], indicating the potential for the development of more robust genetic engineering tools in methanotrophs. However, only recently Goswami *et al.* demonstrated a comprehensive analysis of parameters for a reproducible, robust, and fast electroporation method of DNA transfer in a type I host (*M. alcaliphilum*) [59]. More focused, comprehensive studies reporting the success or failure of gene transfer trials in other types and genera of methanotrophs are essential for advancing this field.

Heterologous gene expression often utilizes host-compliant promoters ranging from synthetic promoters (P_{tac}) to natural constitutive/inducible promoter (constitutive P_{sps}; methanol-induced, P_{mxaF}) [58,60] to drive gene expression [50]. For more inducible and constitutive promoters that have been successfully implemented in methanotrophs, see Table 3.

Table 3

List of inducible and constitutive promoters in methanotrophs.

Promoter	Gene origin	Inducers	Host organism	Measured os	Source
Inducible					
P_{tac}	Synthetic combination of trp and lac promoters	IPTG	*M. alcaliphilum*	2,3-butanediol titer	[60]
P_m	*XylS*	3-Methyl-benzoate	*M. alcaliphilum*	GFP fluorescence	[59]
P_m	*XylS*	Sodium benzoate	*M. capsulatus* Bath	CRISPR-BE	[16]
P_{tetA}	Tetracycline	Anhydrotetracycline	*M. parvus* OBBP	CRISPR/Cas9	[64]
P_o	*Xyl* operon	Phenol	*M. capsulatus* Bath	dTomato fluorescence	[16]
P_{bad}	Arabinose operon	Arabinose	*M. buryatense* 5GB1C	xylE	[65]
Constitutive					
P_{hps}	3-hexulose-6-phosphate synthase	NA	*Methylomonas sp.* DH-1	GFP fluorescence	[66]
P_{aroK}	Shikimate kinase	NA	*Methylomonas sp.* DH-1	GFP expression	[66]
P_{hpi}	Hexulose phosphate isomerase	NA	*M. alcaliphilum*	NA	[67]
P_{pmoC}	Particulate CH_4 monooxygenase	NA	*M. capsulatus*	sfGFP reporter	[68]
P_{mxaF}	Methanol dehydrogenase	NA	*M. buryatense*	dTomato fluorescence	[58]
P_{sps}	Sucrose phosphate synthase	NA	*M. alcaliphilum*	Rhamnolipid production	[50]
P_{phaC}	Poly(R)hydroxybutyrate synthase	NA	*M. parvus* OBBP	CRISPR/Cas9	[64]
P_{rpmB}	Ribosomal protein L28	NA	*M. trichosporium* OB3b	GFP fluorescence	[66]
P_{tuf}	Elongation factor Tu	NA	*Methylomonas sp.* DH-1	GFP fluorescence	[66]
P_{rplU}	50S ribosomal protein L21	NA	*Methylomonas sp.* DH-1	GFP fluorescence	[66]
$P_{integrase}$	Integrase	NA	*M. trichosporium* OB3b	GFP fluorescence	[66]
$P_{(2Fe-2S)\text{-binding protein}}$	(2Fe–2S)-binding protein	NA	*M. trichosporium* OB3b	GFP fluorescence	[66]
P_{DnaA}	DnaA	NA	*M. trichosporium* OB3b	GFP fluorescence	[66]
$P_{Atp\text{-binding protein}}$	ATP-binding protein	NA	*M. trichosporium* OB3b	GFP fluorescence	[66]
P_{tal}	Transaldolase	NA	*Methylomonas* sp. DH-1	GFP fluorescence	[66]
P_{glnA}	Glutamine synthetase	NA	*Methylomonas sp.* DH-1	GFP fluorescence	[66]

NA: not applicable.

An essential aspect of host engineering is precise gene editing. Traditionally, genomic engineering in methanotrophs required a two-step process for site-specific recombination: (1) creating deletions in a host's chromosome while simultaneously adding an antibiotic resistance gene marker and (2) removing the selection marker with *sacB* counterselection [61], *Cre-loxP* [58] or the with creation of two variants [62]. However, these techniques are often time consuming and labor intensive and can leave unwanted DNA fragments. Therefore, there is a growing demand for more robust and efficient methods for engineering methanotrophs.

To advance the genetic engineering of methanotrophs, CRISPR/Cas9 system emerges as a promising alternative to potentially cut down the time required for genetic modifications and improve gene target efficacy. Furthermore, CRISPR/Cas9 functional capabilities could significantly enhance the genetic engineering potential of methanotrophs, extending beyond DNA cloning and enabling the rapid development of strains capable of producing recombinant biofuels, platform chemicals, and value-added compounds [19,63]. In this direction, Ruman *et al.* have developed the first efficient CRISPR/Cas9 system for methanotrophs (specifically *M. capsulatus* and *M. parvus* OBBP) [64] that generated scarless clean gene deletions and insertions in a methanotroph's genome [64]. This recent development of the CRISPR/Cas9 system by Ruman *et al.* allows for more optimized and efficient genetic engineering of methanotrophs. Moreover, Jeong *et al.* have developed a CRISPR-BE system to precisely edit individual DNA bases in *M. capsulatus* to shuttle acetyl-CoA and increase the production of mevalonate to a concentration of 2090 mg/l, the highest amount of synthetic biochemical produced from a methanotroph with CH_4 as its primary substrate [16]. These innovative base editing systems offer enhanced control over metabolic pathways of interest, leading to increased production of targeted products in methanotrophs.

Current challenges in industrializing methanotrophs

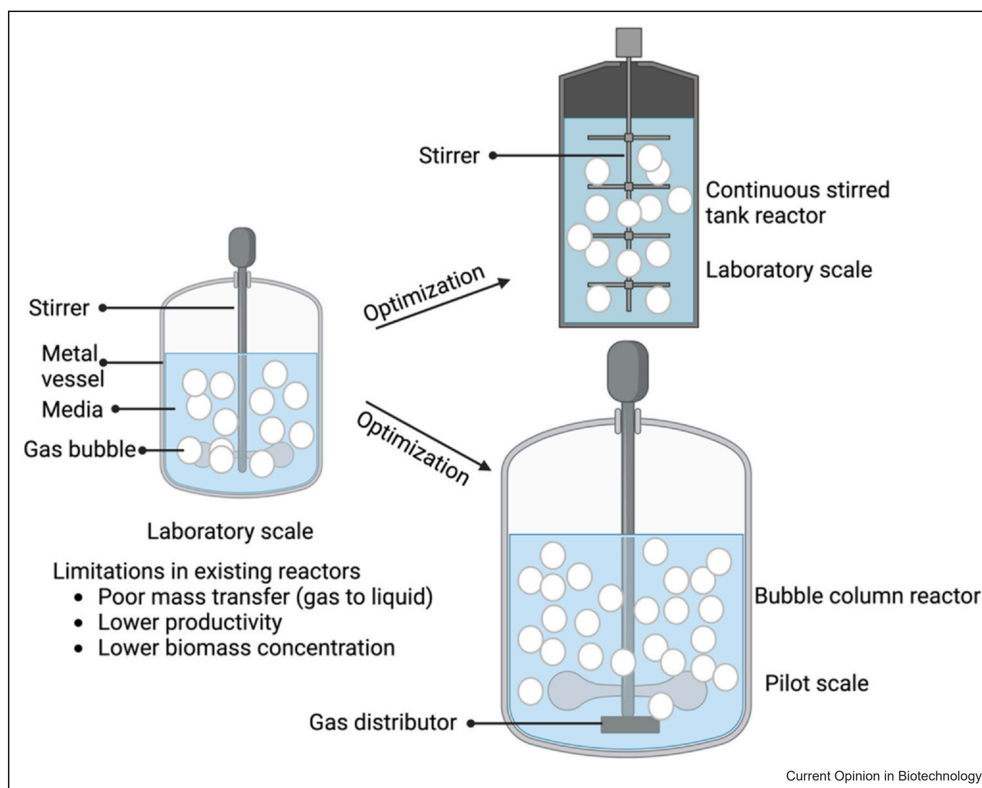
Methanotrophs have shown potential at the laboratory scale for biomolecule production. However, scaling methanotrophs from benchtop experiments to industrial applications requires careful optimizations and practical implementation [17]. Methanotrophs require specific growth conditions like pH (5–9) and temperature (20–35°C) [69], making ongoing monitoring and adjustments important to sustain optimal conditions for their proliferation. Another challenge when scaling methanotroph production is the lack of efficient conversion of CH_4 from its gaseous state to a liquid form that is readily accessible to methanotrophs at larger scales [17]. To address these issues, using a bioreactor designed for efficient temperature regulation, mixing, and other parameters can significantly improve the CH_4-to-liquid conversion efficiency (mass transfer rate) [70]. Two of the potential strategies for designing an optimized reactor for gas fermentations at lab and pilot scale have been depicted in Figure 3 [71]. Moreover, the buildup of by-products or the target product can inhibit cell growth and decrease their productivity. However, approaches like extracting the product or developing genetically engineered strains with enhanced tolerance to these products are effective solutions to this challenge [72]. Other obstacles include maintaining the genetic fitness of engineered strains at a larger scale while increasing production [73]. One of the most difficult challenges lies in the economic viability, resulting from the expenses associated with methanotrophic culture media and equipment. Therefore, optimizing methanotrophs for industrial-scale applications needs a multidisciplinary strategy, involving collaboration across microbiology, systems biology, bioprocess engineering, and other related fields.

Future directions

For methanotrophs to become effective industrial biocatalysts, extensive research is needed to develop advanced synthetic biology tools for strain engineering and the synthesis of recombinant molecules. Additionally, innovative bioreactors designed for gas-based bioprocesses are crucial for efficient process scale-up. Advancements in synthetic biology tools include rapid DNA transformation techniques, such as electroporation, which offer advantages over traditional methods like conjugation [15]. This also includes the use of specialized gene expression promoters for fine-tuning metabolic pathways and bioproduct synthesis, broadening the application of CRISPR/Cas9 technology, and exploring innovative approaches like Serine Recombinase-Assisted Genome Engineering (SAGE) for precise gene editing beyond preliminary proof-of-concept stages [16,19,64,74]. Innovative reactors include continuously stirred reactors or bubble column reactors for gas fermentation and scale-up [71].

Another promising approach for methanotroph-based biomanufacturing is the development of new growing conditions that can allow methanotrophs to create more products and thrive longer. Recent research highlighted by Gęsicka *et al.* has explored the innovative approach of co-culturing methanotrophs alongside other micro-organisms, such as hydrogenotrophs, to enhance the removal of toxic by-products and excess metabolites. This synergistic interaction has led to a significant increase in the accumulation of valuable products such as PHA, thereby boosting the efficiency of methanotrophs. These advancements are pivotal in scaling methanotrophic processes to industrial levels, offering promising avenues for sustainable biotechnological applications [14]. Another study by Weiblen *et al.* shows that upregulation of the oxygen-binding protein gene *bar* in *Methylomonas denitrificans* FJG1 improved the survivability of the

Figure 3



Schematic of gas bioreactors, existing design, and optimized solutions for laboratory and pilot scale [71]. Illustration has been created using BioRender [22].

methanotroph under hypoxic conditions, which could open opportunities for increased efficiency of bioprocesses that require primarily anaerobic conditions [75].

Companies such as Calysta, NatureWorks, and LanzaTech are investing in methanotroph research. These companies are pushing for large-scale production of PHA, lactic acids and polylactic acids, and isobutanol [76]. As companies advance the field of methanotroph engineering, these micro-organisms are evolving into biofactories, mirroring the trajectory previously set by ethanol production. Companies are also employing metabolic engineering targets from model organisms such as *E. coli* and translating them into methanotrophic bacteria. This approach is currently being utilized by Intrexon Co. to potentially produce 1,4-butanediol in methanotrophs [54,77,78]. Continued research is needed to establish and improve metabolic engineering tactics from other organisms into methanotrophs.

Conclusion

Methanotrophs reduce CH_4 emissions by consuming the potent greenhouse gas, thereby acting as an effective carbon sink. Despite their environmental benefits, the potential of methanotrophs as industrial biomanufacturing hosts remains largely untapped. Recent strides in enhancing genetic toolkits, refining metabolic pathways, and designing specialized gas fermentation bioreactors have highlighted the promise of methanotrophs as viable host organisms for future biotechnological applications. To fully harness their potential, a concerted effort toward scaling up these engineering solutions to facilitate large-scale production of biomolecules is essential.

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Author contribution

J.T. and K.R. investigated the methodologies, prepared the first draft, illustrations, and updated revisions. S.G. and D.A. conceptualized the idea and revised and edited the manuscript. S.G. conceptualized the format of the review. D.A. secured funding and provided supervision. S.W.S. and B.A.S. reviewed the manuscript. All authors read, commented, and approved the manuscript.

CRediT authorship contribution statement

Justin N. Tan, Keshav Ratra, Subhasish Goswami, Deepika Awasthi, Blake Simmons: Writing, reviewing, editing. **Steven W. Singer** – Conceptualization, methodology. **Deepika Awasthi, Subhasish Goswami**: Supervision.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

B.A.S. has a financial interest in Illium Technologies, Caribou Biofuels, and Erg Bio. The remaining authors declare that they do not have any conflict of interest.

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- • of special interest
- •• of outstanding interest

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