From nature to nurture: Essence and methods to isolate robust methanotrophic bacteria

Haritha Meruvu, Hui Wu, Ziyue Jiao, Liyan Wang, Qiang Fei

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A B S T R A C T

Methanotrophic bacteria are entities with innate biocatalytic potential to biofilter and oxidize methane into simpler compounds concomitantly conserving energy, which can contribute to copious industrial applications. The future and efficacy of such industrial applications relies upon acquiring and/or securing robust methanotrophs with taxonomic and phenotypic diversity. Despite several dramatic advances, isolation of robust methanotrophs is still a long-way challenging task with several lacunae to be filled in sequentially. Methanotrophs with high tolerance to methane can be isolated and cultivated by mimicking natural environs, and adopting strategies like adaptive metabolic evolution. This review summarizes existent and innovative methods for methanotrophic isolation and purification, and their respective applications. A comprehensive description of new insights shedding light upon how to isolate and concomitantly augment robust methanotrophic metabolism in an orchestrated fashion follows.

1. Introduction

Since ages, greenhouse gases like carbon dioxide (CO2), methane (CH4), nitrous oxide (N2O) and chlorofluorocarbons are perpetually being formed and fragmented apart through an interplay of chemical and biological processes. A balanced presence of these greenhouse gases is conducive to the earth's environment to mitigate global warming and nitrogen fixation, Xi'an Jiaotong University, Xi'an, Shaanxi, China

2. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China

3. Luoyang TMAXTREE Biotechnology Co., Ltd., Luoyang, China

4. Shaanxi Key Laboratory of Energy Chemical Process Intensiﬁcation, Xi'an Jiaotong University, Xi'an, China

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ABSTRACT

Methanotrophic bacteria are entities with innate biocatalytic potential to biofilter and oxidize methane into simpler compounds concomitantly conserving energy, which can contribute to copious industrial applications. The future and efficacy of such industrial applications relies upon acquiring and/or securing robust methanotrophs with taxonomic and phenotypic diversity. Despite several dramatic advances, isolation of robust methanotrophs is still a long-way challenging task with several lacunae to be filled in sequentially. Methanotrophs with high tolerance to methane can be isolated and cultivated by mimicking natural environs, and adopting strategies like adaptive metabolic evolution. This review summarizes existent and innovative methods for methanotrophic isolation and purification, and their respective applications. A comprehensive description of new insights shedding light upon how to isolate and concomitantly augment robust methanotrophic metabolism in an orchestrated fashion follows.

1. Introduction

Since ages, greenhouse gases like carbon dioxide (CO2), methane (CH4), nitrous oxide (N2O) and chlorofluorocarbons are perpetually being formed and fragmented apart through an interplay of chemical and biological processes. A balanced presence of these greenhouse gases is conducive to the earth's environment to mitigate global warming and other climatic extremities [1,2]. CH4 emissions have been reckoned from natural wetlands, rice paddies, animal husbandry, biomass burning, landfills, permafrost carbons of arctic lakes and fossils [3,4]. Human induced emissions in pretext of industrialized urbanization led to an augmented accumulation of atmospheric methane [5,6]. CH4 is a notorious greenhouse gas which leads to a substantial increase in radiative forcing with global warming potential of 80 over 20 years relative to CO2 [7–9]. Methanotrophs can oxidize and considerably mitigate environmental CH4 emissions and aerobic methanotrophic bacteria contributes to up to 10–20% of global CH4 obliteration [10–12].

Isolation, screening and cultivation of robust and/or unexplored methanotrophs from natural habitats is an attractive perspective to resolve global warming in the long-term [13,14]. Methanotrophs are generally resistant to laboratory cultivation, with typically diverse habitat preferences, including boiling acidic springs, alkaline lakes, permafrost thaw, volcanic geothermal/hydrothermal vents, seeps, farmlands, forest marshes, rice paddies, landfills, animal husbandry wastes, burned biomass, and effluent sludge from factories [4,15–17]. General cultivation techniques mimic natural habitat conditions in vitro, and include methanotroph culture in growth media within gas-tight vials or seal-tanked plates with provision of CH4 supply (20–50%) [18,19]. Apart from CH4 and air, incubation with supplementary gases like CO2, oxygen (O2), nitrogen (N2) and hydrogen (H2) could stimulate primary and intermediary metabolisms in methanotrophs like Methylocalidum, Methylococcus, Methylocella, Methyloferula, Methylocapsa, Candidatus Methylophorabilis oxyfera, Methylocaldum sp. and Verrucomicrobial species [20–22]. The compositions of various methanotrophic growth media like nitrate mineral salts (NMS), ammonium mineral salts (AMS), and other improvised/assorted media for inducing specific growth were briefly outlined in the compendium (Table 1). The compendium describes specific cultivation methods particularly designed for target methanotrophs of variable environs/habitats, which dictate acidophilic, neutrophilic, thermophilic or hydrothermophilic cultivation conditions [23–26].
Traditional and novel techniques for isolation, cultivation and purifying/enriching samples before streaking or pour-plating upon agar selection from chosen environment, its microscopic examination and designing comprehensive isolation approaches can be done by collecting selective environmental samples. Table 1 refers to enhanced valorisation of methane (methane-oxidation capacity) from peat soil was isolated through long periods of enrichment. Such enrichment isolation techniques of various methanotrophic strains from diverse sampling methods is presented (Table 2).

2. Traditional isolation techniques for methanotrophs

Bacterial isolation steps commonly include specimen sample collection from chosen environment, its microscopic examination and diluting/enriching samples before streaking or pour-plating upon agar plates for spatial segregation. Enrichment of collected samples in fluids is done to activate the constricted growth of requisite bacteria. Physical isolation of bacteria from their natural abodes and laboratory cultivation can be done providing optimal growth conditions, and methanotrophs require the most finicky cultivation requisites. Since many decades extensive pioneering researches based on methanotroph isolation, characterization, classification and taxonomic frameworks have been carried out by various scientists. Isolation of methanotrophs can be done by collecting selective environmental samples and enriching them in supporting growth media periodically inducing methanotrophy and inhibiting methylotrophy. A spirillar microaerophilic Candidatus Methylospira mobilis sourced from peat soil was isolated through long periods of enrichment. Such enrichment isolation techniques of various methanotrophic strains from diverse sampling methods is presented (Table 2).

Table 1

| Medium               | Major Components (g/L)                                                                 | Target methanotrophs                          | Ref     |
|----------------------|----------------------------------------------------------------------------------------|------------------------------------------------|---------|
| Nitrate mineral salts (NMS) medium | KNO₃ 1g; MgSO₄·7H₂O 1g; Na₂HPO₄·12 H₂O 0.717g; KH₂PO₄ 0.272g; CaCl₂·6H₂O 0.2g; Fe(II) EDTA 0.004g; distilled water 1000 mL; pH 6.8 | Neutrophilic methanotrophs from freshwater and marine environments | [24,97] |
| Dilute nitrate mineral salts (DNMS) medium | NaN₃·6H₂O·Na₂HPO₄ buffer; pH 5.5; -7.0; | Mild acidophiles and neutrophiles from freshwater and saline-terrestrial environments | [98] |
| Ammonium mineral salts (AMS) medium | NH₄Cl 0.5g; MgSO₄·7H₂O 1g; Na₂HPO₄·12 H₂O 0.717g; KH₂PO₄ 0.272g; CaCl₂·6H₂O 0.2g; ferric ammonium EDTA 0.005g; distilled water 1000 mL; pH 6.8 | Neutrophilic methanotrophs from freshwater environments | [97] |
| Fortified ANMS media | NH₄Cl 0.5g; MgSO₄·7H₂O 0.27g; ferric ammonium EDTA 0.005g; distilled water 1000 mL; NaCl 0.15 M; KNO₃ 0.5 g; FeCl₃ 0.15 g; KH₂PO₄ 0.15 g; CaCl₂·6H₂O 0.04 M; pH 7.0 | Marine methanotrophs found in lathanade rich environments | [25] |
| Low-salt mineral medium (LMM) | KNO₃ 0.1g; MgSO₄·7H₂O 0.02g; CaCl₂·2H₂O 0.15g; KBr 0.01g; distilled water 100 mL; 100 ml iron stock solution (Fe-Na EDTA 4.5 g/L), optional supplements (NH₄Cl (LMM-AC) or (NH₄)₂SO₄) FeNaEDTA, 100 nM lanthanides (LaCl₃, CeCl₃.7H₂O/NdCl₃.6H₂O/PrCl₃), pH 7.8. | Thermophilic methanotrophs of alkaline hot springs | [99] |
| Assorted medium | 0.2 mM MgCl₂·6 H₂O; 0.2 mM CaCl₂·2 H₂O; 1 mM Na₂SO₄; 2 mM K₂SO₄; 2 mM (NH₄)₂SO₄; 0.717 g Na₂HPO₄·12 H₂O; 0.272 g KH₂PO₄; 1000 mL distilled water, pH 3.0 | Acidoiphiles methanotrophs of volcanic environments | [100] |
| Medium 3.9C10.2 | NH₄Cl 0.2g; KH₂PO₄ 0.05g; MgSO₄·7H₂O 0.02g; CaCl₂·6H₂O 0.011g; Fe-EDTA powder 0.005g; 1000 mL distilled water, pH 3.9 | Acidiphiles from geothermal soils | [59] |
| Medium M2 | KNO₃ 0.25g; KH₂PO₄ 0.1g; MgSO₄·7H₂O 0.05g; CaCl₂·2H₂O 0.011g; NaCl 0.02g; 1000 mL distilled water, pH 5.5 | Methanotrophs from freshwater wetlands and mildly acidic soils | [101] |
| Medium N | KH₂PO₄ 0.002g; KNO₃ 0.001g; NaCl 0.008g; NaCl 0.003g; CaCl₂·2H₂O 0.011g; MgSO₄·7H₂O 0.010g; Na₂SO₄ 0.002g; CaCl₂ 0.067g; 1000 mL distilled water, pH 5-7 | Mild acidiphiles from ombrotrophic wetlands | [102] |
| Medium S | NH₄NO₃ 0.5g; Na₂HPO₄·2H₂O 0.5g; KH₂PO₄ 0.1g; FeSO₄·7H₂O 0.005g; NaCl 66.0g; Na₂SO₄ 10.45g; MgSO₄·7H₂O 0.010g; Na₂HPO₄ 0.010g; NaH₂PO₄ 0.28g; NaH₂PO₄·2H₂O 0.067g; NaCl 0.28g; MgCl₂ 10.09g; CaCl₂ 2.21g; 1000 mL distilled water, pH 7.5 | Neutrophilic halophiles | [103] |
| Mjnet medium | 30 g NaN₃, 0.14 g K₂HPO₄, 0.8 g CaCl₂, 3.4 g MgSO₄·7H₂O, 4.18 g MgCl₂·6H₂O, 0.33 g KCl, 0.25 g NH₄Cl, 0.25 g NaNO₃, 0.5 mg MgCl₂·6H₂O, 0.5 mg Na₃PO₄·2H₂O, 0.1 mg Na₂WO₄, 20 mg Fe(NH₄)₂(SO₄)₁₂·6H₂O, 1000 mL distilled water, 1 ml vitamin solution, 6 mL NaHCO₃, 1 μM CaCl₂, pH 3-7 | Hydrothermophilic methanotrophs | [104] |

Identification of optimal growth conditions, cultivation media and/or choosing resilient strains can induce superior methanotrophy and curb undesirable bottlenecks. The notion of robust methanotrophy refers to enhanced valorisation of methane (methane-oxidation capacity), which can refer to ‘innate robustness’ through natural selection or ‘induced robustness’ through biological alternations made in genetic, metabolic and synthetic pathways of methanotrophs. Moreover, it is quintessential to develop efficient tools and strategies to culture and catalogue diverse novel methanotrophs, for harnessing and/or stimulating their metabolic dynamics of CH₄ consumption. Traditional and novel techniques for isolation, cultivation and purification techniques, metabolic evolution methodologies, and their perspectives are comprehensively described in this review. Moreover, adaptive evolution techniques coupled with atmospheric and room-temperature plasma mutagenesis (ARTP) and microbial microdropet culture system (MMC) are also reviewed as advanced systems to isolate robust methanotrophic strains by inducing tolerance to inhibitors or desired products within limited time frames.

Large-scale isolation of methanotrophs through enrichment technique can be done in well-equipped bioreactors. A previously uncultured mixture of ‘aerobic methanotrophs and associated denitrifying methanotrophs’ was isolated through 53-week enrichment of marine water-sample in nitrate rich medium sparged (10 ml/min) with CH₄·CO₂ (95:5 v/v) at 25 °C, 250 rpm in dark using 5.2 L bioreactor. In another study, sourced from volcanic mud, anaerobic methanotrophic archaea (Methylophaga sp., Methylobacter sp.) and sulphate reducing bacteria (Dehaloproteobacteria) were 286-days enriched and isolated in a SR-AOM bioreactor supplied with sulphate and pressurized methane (8 MPa). Enrichment of freshwater lake sediment samples using a membrane biofilm bioreactor operated for 13 months (10–25 °C) led to isolation of Candidatus Methyloarabidobacter oxyfera featuring methanotrophy and coupled denitrification.

Apart from aiding methanotrophic growth, enrichment can also be done to induce, co-production of ectoines, co-metabolism of trichloroethene along with methane, and improving temperature tolerance (from 25 °C to 30/37 °C) for accumulation of
### Table 2

Comparisons of enrichment isolation techniques of various methanotrophic strains, their sources and purity testing methods.

| Isolation technique | Strains | Sources | Purity testing |
|---------------------|---------|---------|----------------|
| Pre-enrichment using NMS medium | Methylotetracoccus oryzae | Acidic peat bog sample: Russia | Transmission electron microscopy, paludis, Phase-contrast, transmission electron microscopy |
| Enrichment culturing using low-salt methanotrophic medium | BRS-K6, GFS-K6, AK-K6 | Waterlogged rice field soil, methane seep pond and AK-K6 sediments: Bangladesh. Warm spring sediments: Armenia | Plating upon NMS agar plates, phase contrast microscopy, water instead of distilled water and sodium nitrate |
| Enrichment technique using NMS medium | Methylocaldum strain S8T | Marine sediment from hydrothermal vent, Japan | Plating upon NMS agar plates, phase contrast microscopy, 300 nM CeCl3 |
| Enrichment technique using MJmet medium | Methylocapsa gorgona | Marine mud sample, Japan | Streaking repeatedly for single colony formation upon gellan gum plates at least six times, electron microscopy |
| Enrichment technique using NMS medium | Methylomarinum vadi | S285 Acidic peat soil, Russia | Iteratively surface plating upon the agar medium M2 |
| Enrichment technique using NMS medium | Methylomonas | Methylosinus species, Peatland plant fragments | Surface plating upon the NMS agar medium, 100 μg/mL of 300 nM CeCl3 |

3. Modern isolation techniques for methanotrophs

Development and adoption of modern isolation and cultivation techniques like ARTP, MMC, high throughput technologies, single cell isotope imaging, mass-spectroscopy and adaptive metabolic evolution could further help to comprehend and exploit bioprocesses and metabolites of the methanotrophic cultivation, thereby increasing the incidence of novel methanotroph isolation [53–56]. The purity and taxonomic identity of a methanotroph can simultaneously be determined by elaborate molecular identification techniques like 16S rRNA sequencing, T-RFLP analysis, pm0A/mmoX PCR cloning, 13CH4 stable-isotope probing and FISH analysis [57–60]. Some uncultured methanotrophs of unrecognized geothermal ecosystems cannot be identified as methane-oxidisers by comparing with 16S rRNA sequences of known methanotrophs. In such cases, enrichment microcosms of methane-oxidizing geothermal samples can be evaluated in the terms of methane oxidizing capacity and activity at extreme temperatures (37–75 °C) and transcriptional activity (detection of mRNA-transcripts). This a novel technique of metatranscriptomics for detecting stress-responsive genes expressed in microcosms for adaptation to environmental changes, and helps us comprehend and decipher metabolic pathways in thermophilic methanotrophy [61]. Metagenomic approaches can also be used to guide novel methanotrophic cultivation methods by unearthing methanotrophic phylogenies and metabolic diversities, which can be quantified by relevant genes/pathways [62].

ARTP is a tool featuring whole cell mutations induced by radiofrequency and ‘room-temperature and atmospheric plasma (helium-
Based discharges to enhance medium/nutrient tolerance promoting cellular growth/metabolism [63–66]. ARTP can be combined with supplementary mutational methods like γ-ray and CRISPR/Cas9 for delimiting industrial drawbacks in methanotrophs like *Methylococcus capsulatus* [67,68]. ARTP technique using pressurized glow discharges induced genomic mutational breeding in *Methanosinus trichosporium* OB3b [69]. Measurements of intracellular copper uptake inducing mutations in *Methylococcus capsulatus* were done through inductively coupled plasma mass spectrometry (ICP-MS). It was also deciphered that when grown in copper-limited conditions the organism secretes ‘MopE’ protein which can bind with both oxidised and reduced copper, based upon ‘X-ray near edge absorption spectroscopy’ and ‘Electron Paramagnetic Resonance’ analyses [70,71].

MMC is an automated platform integrating adaptive evolution with high-throughput microbial cultivation [72,73]. Here microorganisms can be grown in droplets with gaseous exchange facility allowing continuous sub-cultivation and improving robust strain construction under induced stress conditions [72]. Likewise, MMC can be applied for methanotroph growth and cultivation studies also as fluorinated oil-phase micro-droplets can support CH₄ exchange along with O₂ [74]. Methanotrophic strain MeSV2.2 and *Methylolobacterium extroquens* AM1 were found to exhibit good growth in MMC. Adaptive evolution experiments of MeSV2.2 by cultivation for 18 days in MMC resulted in a mutant with improved growth rate and high final cell density was desired [72,75].

Uncultivable methanotrophic gamma-proteobacteria like *Crenothrix* sp., which have been evading isolations previously are now being isolated and catalogued taxonomically applying cumulative technologies like Raman-Microspectroscopy, and Microfluidics [54]. Single-cell isotopic imaging is a novel technique featuring stable isotope probing, and application of single-cell-level imaging tools using Raman spectra of individual cells to yield bands for modelling cellular profiles and metabolites with higher throughputs and lower costs [76]. Metagenomic analysis using coupled techniques of stable-isotope-labelling and single-cell-imaging mass spectrometry could elucidate isolation and identification of *Crenothrix* population (major methane metabolisers of stratified lakes), which could grow potentially with and without oxygen supply due to possession of genes encoding typical gamma-proteobacterial ‘PmoA’ [77]. Mass specotscopy or gas chromatography can be used for the analysis of fatty acids of whole-cell methanotrophs like *Methylomega oryzae*, *Methylcococcus capsulatus* Bath, *Methylparacoccus murrelli*, *Methyllocaldum gracile* [78]. In cold adapted methanotroph *Methylomolllum psychrotolerans*, analysis of fatty acid methyl esters was done using gas chromatography-mass spectrometry, and intact polar lipid analysis of biomass was done by ultra-high pressure liquid chromatography-high resolution mass spectrometry [79].

High throughput technologies like Omics for molecular analysis of DNA/RNA, proteins and metabolites of methanotrophs can help of comprehend geochemical and communal functions/links of methanotrophy [80,81]. High-throughput cultivation technique applies the dilution-to-extinction-culturing to partition cells singly into tubes/micro-wells with low nutrient media [50,82]. Due to the immense taxonomic and physiological diversity of methanotrophs isolation discoveries expanding their nomenclatural groups, their identification or cataloguing can be done by the application of high-throughput metagenomic screening/sequencing [83–85]. High-throughput sequencing of 16sRNA gene amplicon regions has been used for identifying differences between methanotrophic family clusters of *Methylococaceae* and *Methylocystaceae* isolated from diverse ecosystems by multiplex sequencing [86]. Similarly methanotrophic biodiversity of two soil habitats was distinguished and reported using high-throughput sequencing of their pmoA genes [87]. Global methanotrophic diversity of thermoacidophilic verrucomicrobial species of common ancestral origins are caused by allopatic evolution caused by geographical habitat distances [51].

Adaptive laboratory evolution techniques are methods which promote the growth of methanotrophs by inducing gradual adaptive changes at laboratory level thereby metabolically engineering microbial cells to suit industrial conditions [88,89]. Metabolic adaption is not just phenotypic but genetically stable in methanotrophs as tested in *Methylomicrobium album* B8 [88]. They are simpler than stereotypic genetic engineering experiments, and use metabolic responses like nutrient limitation and/or induced stress factors to incorporate evolutionary changes [90]. As putrescine is inhibitive for methanotroph, adaptive evolution was conducted to induce tolerance of *Methylomicrobium album alcaliphilum* 20Z to putrescine dihydrochloride (400 mM) [91]. In lactate-tolerant *Methylocaldum* sp. DH-1, efficient u-lactate production from CH₄ was induced by adaptive laboratory evolution [92]. Evidently, adaptive laboratory evolution has also been reported for isolating mutants of *Methyllobacterium extroquens* AM1 exhibiting stress tolerance to 1-butanol with augmented survival/growth rates, and was confirmed by metabolomics analysis and whole genome sequencing [93]. Adaptive laboratory evolution can also be carried out to induce higher methane degradation rates [56], by incubating the methanotrophic strains of interest at gradually increasing methane contents.

4. Perspectives

Isolation of novel methanotrophs with biotechnological potentials and devising strategies which could address the same, is the need of essence due to their unique metabolisms to devour harmful greenhouse gases like methane. Increasing methane content supply gradually was proven to be a novel technique to amplify innate methanotrophic metabolism and concomitantly free them from unwanted associated heterotrophic methanotrophs/methylotrophs that feed upon methane derivatives like acetate, formate, methanol, succinate or organic acids [94]. Co-occupant satellite species that associate with methanotrophs form functional communities together. Experimental community dynamics reveal that manipulated synthetic environments could be designed more realistically to represent natural processes in laboratory, by studying their proteins, metabolites, transcripts and mutative manipulations [95]. It was within the context of this review to demonstrate that simple isolation methods could be coupled with adaptive evolution techniques to isolate robust high-methane tolerant pure methanotrophic isolates within limited time periods [56]. To summarize, bringing methanotrophs from their natural habitats and nurturing them in our laboratory conditions requires perseverant skill and knowledge of novel reproducible techniques which can be adopted at economic feasibility. Harnessing methanotroph biotechnology for the greater good and welfare of the human society could include pollution control contributing to balanced biogeochemical cycles through bioremediation of xenobiotically contaminated soil/aquatic environments. Application of diverse methanotrophic biofilters for bioconversion of methane-rich abundances like biogas, natural gas, landfill gas and gaseous exhausts from animal husbandry gas, into simpler marketable and transportable commodities, contributes to mitigating greenhouse gas effects [40,96]. This review paper serves an introductory guide to a researcher wanting to isolate and purify robust methanotrophs in a mediocre laboratory environment with minimal scientific equipment.

CRediT authorship contribution statement

Haritha Meruvu: Conceptualization, Resources, Writing - original draft. Hui Wu: Investigation. Ziyue Jiao: Validation, Resources. Liyan Wang: Resources. Qiang Fei: Conceptualization, Supervision, Writing - review & editing.

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