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Bioengineering

Review

Biotechnological conversion of methane to methanol: evaluation of progress and potential

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Abstract: Sources of methane are numerous, and vary greatly in their use and sustainable credentials. A Jekyll and Hyde character, it is a valuable energy source present as geological deposits of natural gas, however it is also potent greenhouse gas, released during many waste management processes. Gas-to-liquid technologies are being investigated as a means to exploit and monetise non-traditional and unutilised methane sources. The product identified as having the greatest potential is methanol due to it being a robust, commercially mature conversion process from methane and its beneficial fuel characteristics. Commercial methane to methanol conversion requires high temperatures and pressures, in an energy intensive and costly process. In contrast methanotrophic bacteria perform the desired transformation under ambient conditions, using methane monooxygenase (MMO) enzymes. Despite the great potential of these bacteria a number of biotechnical difficulties are hindering progress towards an industrially suitable process. We have identified five major challenges that exist as barriers to a viable conversion process that, to our knowledge, have not previously been examined as distinct process challenges. Although biotechnological applications of methanotrophic bacteria have been reviewed in part, no review has comprehensively covered progress and challenges for a methane to methanol process from an industrial perspective. All published examples to date of methanotroph catalysed conversion of methane to methanol are collated, and standardised to allow direct comparison. The focus will be on conversion of methane to methanol by whole-cell, wild type, methanotroph cultures, and the potential for their application in an industrially relevant process. A

recent shift in the research community focus from a mainly biological angle to an overall engineering approach, offers potential to exploit methanotrophs in an industrially relevant biotechnological gas-to-liquid process. Current innovations and future opportunities are discussed.

Keywords: methanotrophs; methane monooxygenase; gas-to-liquid; methane partial oxidation; biocatalysis; methanol synthesis

Abbreviations: MMO: Methane monooxygenase; GTL: Gas-to-liquid; CNG: Compressed natural gas; LNG: Liquified natural gas; MTBE: Methyl tert-butyl ether; DME: Dimethyl ether; MTO: Methanol-to-olefin; GHG: Greenhouse gas; DNA: Deoxyribose nucleic acid; RNA: Ribonucleic acid; MMO: Methane monooxygenase; sMMO: Soluble methane monooxygenase; pMMO: Particulate methane monooxygenase; MDH: Methanol dehydrogenase; FADH: Formaldehyde dehydrogenase; FDH: Formate dehydrogenase; RuMP: Ribulose monophosphate; NADH: Nicotinamide adenine dinucleotide; PQQ: Pyrroloquinoline quinine; GMO: Genetically modified organism; AMO: Ammonia monooxygenase; EDTA: Ethylenediaminetetraacetic acid; GC-MS: Gas chromatography-mass spectrometry; ISPR: In situ product removal; CytC: Cytochrome c; PHB: Polyhydroxybutyrate; TCE: Trichloroethylene; rRNA: Ribosomal ribonucleic acid; OD: Optical density; DEAE: Diethylaminoethanol; MBR: Membrane bioreactor

1. Introduction

Methane is simultaneously a valuable energy resource, significant global waste product and a potent green-house gas (GHG). The volume of waste methane released from anthropogenic sources is increasing, in addition to natural gas sources becoming increasingly remote and diffuse [1,2]. Gas-to-liquid (GTL) technologies are being developed to exploit and monetise a range of underutilised methane resources through chemical conversion to liquid hydrocarbon products that are more readily stored and transported. Methane to methanol conversion is receiving increased research interest due to the drive towards sustainable technologies and renewable fuels.

Compared to methane, methanol can be easily used as a feedstock for further chemical conversion, is suitable for use in the current transportation fuel infrastructure, has a greater energy density, and burns with fewer toxic by-products [3]. Methanol is produced commercially from methane via syngas, however the two-step process requires high temperatures (about 900 °C) and pressures (3 MPa) and as such is energy intensive [4]. Despite the costs associated with the process, chemical conversion has been successfully commercialised, however the high volumes of methane necessary to make large-scale processes economically viable are not applicable for marginal fields and waste methane sources.

In contrast to chemical routes, the oxidation of methane to methanol is performed biologically by methane monooxygenase (MMO) enzymes in a single step at ambient temperature and pressure. Unique to methanotrophic bacteria, MMO enzymes catalyse the initial oxidation of methane to methanol, ultimately allowing the use of methane as a sole carbon and energy source [5]. The

biological conversion offers methane to methanol conversion in an energy efficient and environmentally benign manner. In addition, biotechnological processes are well suited to small-scale operations, appropriate for remote and diffuse methane sources, and require low capital investment. The potential to exploit methanotrophs for the partial oxidation of methane to methanol has been explored, however progress towards an industrially relevant biocatalytical process to date has been minimal due to a range of issues.

Herein, we have identified five major challenges that exist as barriers to a viable conversion process that, to our knowledge, have not previously been examined as distinct process challenges. This review will comprehensively analyse recent progress in these areas from an industrial perspective, in addition to providing tabulated and standardised data for all published examples to date of the whole cell bioconversion of methane to methanol using methanotrophic bacteria. In summary, the potential to exploit methanotrophs in a biotechnological GTL process is vast, however implementation is hindered by the factors identified. Progress in this will be facilitated by the recent shift in the research community from biologically focused research to take a holistic, engineering approach, and further work is required at the interface of these disciplines. Current innovations and future opportunities are discussed.

2. Setting the scene: the energy and environmental context

It is now widely accepted that a significant deviation from the unsustainable global energy situation is necessary. The scientific consensus is that the Earth's climate is being affected by human activities [6], attributed to the release of GHGs, with atmospheric concentrations at unprecedented levels [6]. Combustion of fossil fuels for energy production is responsible for the majority of GHG emissions, whilst also being available as finite resources, and so alternative fuels that are both sustainably sourced and produce lower emissions are of interest.

As such, methane has received increased attention from the scientific community. A Jekyll and Hyde character, sources of methane can be divided into anthropogenic and natural, while there is also a distinction between those that are traditionally utilised commercially and those that result in atmospheric and biogenic accumulation. The resource most frequently exploited is geological deposits of fossil formed natural gas, used predominantly for energy generation. As a product of the anaerobic decay of biomass, however, it can be considered either a renewable carbon source or a potent GHG, depending on its final use or treatment.

2.1. Methane sources and uses

The main component of natural and shale gases, methane is considered a next-generation carbon feedstock due to the vast global reserves [7], with geological deposits of the fossil formed gas frequently exploited for energy generation. In addition, it is the main constituent of biogas, produced by the microbial digestion of biomass under anaerobic conditions, and as such is a product of many waste management processes and agricultural activity including enteric fermentation and rice cultivation [1]. However methane is an abundant and potent GHG. The greatest source to the atmosphere is as a result of anaerobic decay of biomass, with anthropogenic contribution through

industrial waste production on the increase [8]. Also the energy sector is responsible for significant methane emissions released during fossil fuel exploration, extraction and transportation.

Anthropogenic methane emissions in 2010 were estimated to be 481 billion m^3 methane, equivalent in global warming potential to 6867 Mt carbon dioxide [1], with atmospheric methane concentrations at unprecedented levels, having increased from 715 ppb to 1774 ppb over the past 300 years [6]. This, combined with a global warming potential 25 times greater than carbon dioxide, have resulted in methane being the second most significant greenhouse gas after carbon dioxide, contributing more than one-third of current anthropogenic warming [1]. Methane has a much shorter global atmospheric lifetime (12 years) compared with carbon dioxide (5–200 years) [6], so it would be possible to rapidly reduce atmospheric concentrations through a reduction in emissions. Methane mitigation strategies offer both the potential to curb atmospheric accumulation and the associated climate impact in addition to providing a valuable industrial fuel source and chemical feedstock.

Global natural gas production in 2013 was estimated to be 3369.9 billion m^3, with proven reserves of 185.7 trillion m^3 [9]. Of this it is estimated that between 30% and 80% is "stranded gas" [10], defined as natural gas that is wasted or unused because the gas field may be too small or remote for production to be economically feasible [2]. An additional environmental concern is the considerable amount of associated gas that is flared or vented during oil production encouraging the implementation of restrictions on such processes. In 2008, 139 billion m^3 of natural gas was flared globally; equal to 4% of global natural gas production, and resulted in the release of more than 278 Mt of carbon dioxide [11].

Combining the volume of anthropogenic waste methane emissions, stranded and associated gas demonstrates the huge amount of unutilised global methane sources and the waste of a valuable resource.

2.2. Gas-to-liquid technologies

One difficulty in the use of methane is that it occurs as a gas under ambient conditions (boiling point –164 °C) and so storage and transportation are costly, further compounded for diffuse and remote non-traditional sources. Conversion to compressed natural gas (CNG) or liquefied natural gas (LNG) are energy intensive and require large capital investment, in addition to being hazardous due to their high pressure (21–25 MPa) and low temperature (–164 °C) [2]. This, and the potential to monetise unutilised methane sources has initiated interest in GTL technologies to chemically convert methane to liquid hydrocarbon products that are more readily transported. The most widely deployed, commercially demonstrated GTL technologies utilise the Fischer-Tropsch conversion process to produce diesel, naphtha and waxes [10], although other conversion technologies are being investigated to generate products including methanol, dimethyl ether (DME) and olefins. GTL processes offer market diversification and an opportunity to harness remote natural gas resources, although high costs, price risks, reliability and technical difficulties have hindered implementation [4]. A number of factors impact the suitability and success of such technologies including scale, capital cost and potential markets for products [4]. Large-scale projects offer economies of scale but typically require high capital investment and a constant high volume input of methane, not often available at remote sources. Ultimately, the commercial viability of a plant is determined by natural

gas and product prices. Volatility and uncertainty in these markets make justifying the large capital investment problematic. Interest in small-scale, modular GTL units has increased for reasons contrasting large-scale projects, such as the suitability for use at low volume gas sources and reduced capital investment. It is anticipated that unit cost and reliability will have the greatest impact on the uptake and success of such technologies [4].

2.3. Methanol as a sustainable liquid fuel

Conversion to methanol is an attractive option due to the range of applications and growing market; global methanol demand reached 70 Mt in 2015 [12].

Traditionally used as a solvent and feedstock, methanol is utilised in the synthesis of industrially relevant compounds including acetic acid, formaldehyde, methyl tert-butyl ether (MTBE), and dimethyl ether (DME), whilst the methanol-to-olefin (MTO) process can be used to produce ethylene or propylene which can be further processed into a range of hydrocarbon and organic polymeric materials (Figure 1).
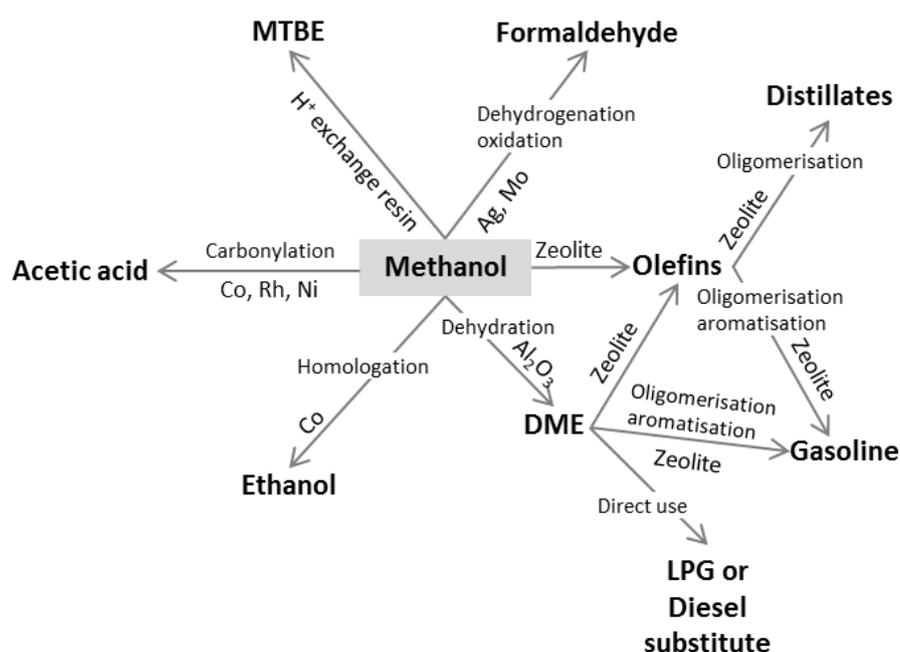

Figure 1. Possible industrial transformations from methanol, including production processes and products [13].

Methanol also offers great potential as an energy carrier or fuel, with an energy density greater than that of methane (15.6 MJ L^{-1} and 36.6×10^{-3} MJ L^{-1} respectively). There are five main fuel applications for methanol: directly as a transportation fuel; blended with petrol; converted to DME to be used as a diesel replacement; in the production of biodiesel via trans-esterification; in fuel cells to generate electricity. The current energy situation has increased interest in methanol in transportation fuel applications due to the potential for sustainable, carbon neutral sources, low cost compared to

other fuels, and clean burning [14], in addition to offering a range of advantages over both methane, and current petrochemical fuels. Methanol exhibits favourable combustion properties, increased engine performance and greater efficiencies over those achieved with gasoline [3]. Another advantage of methanol over petroleum based fuels is that it is considered both safer, with a lower or comparable toxicity, and more environmentally benign in case of uncontrolled release as it is highly biodegradable [15].

Perhaps the greatest advantage of methanol as a fuel is related to the GHG emission reduction potential. Comparison of life cycle carbon intensity analysis shows that emissions from methanol as a fuel are heavily dependent on the feedstock source. Methanol from natural gas has slightly lower carbon emissions compared with those from conventional gasoline fuels. However, sustainably produced bio-methanol is the lowest of results calculated [3]. Combustion of methanol produces fewer toxic by-products—about half as much carbon monoxide and an eighth as much nitrogen oxides (NOx)—compared with gasoline [16]. In terms of point-of-use emissions for transportation fuels, methanol generates lower carbon dioxide emissions per unit energy than conventional petrochemical fuels [17]. It is possible to blend methanol with petrol to increase the octane value and reduce the cost, without the need for any engine modifications [18], however at high levels it is corrosive requiring specific compatible engines. In an interdisciplinary report prepared by MIT [19] on the future role of natural gas as an energy source, conversion to methanol is identified as having the lowest cost and GHG emissions in comparison with alternative liquid fuel products as well as being the only potential transformation that has been produced for a long period at an industrial scale.

2.4. Conversion of methane to methanol

In theory, conversion of methane gas to liquid products offers many advantages, however the current reality is that processes are energetically inefficient and costly. The highly inert nature of saturated hydrocarbons makes chemical transformation challenging, and although high temperatures and pressures can be employed to promote reaction, this often results in loss of selectivity and low yields. As the dissociation energy of the C–H bond in methane (440 kJ mol^{-1}) is greater than methanol (393 kJ mol^{-1}), under oxidising conditions, the product methanol reacts preferentially to methane forming a mixture of products including carbon monoxide, carbon dioxide, formaldehyde, and formic acid [20].

Current commercial methanol production overcomes selectivity issues by utilising a two-step process in which fossil methane is first converted to syngas via steam reforming, followed by the metal-catalysed methanol synthesis step in an overall endothermic process ($\Delta H° = +116$ kJ mol^{-1}) (Figure 2). Initial conversion of methane and water to carbon monoxide and hydrogen is an energy intensive process, operating at temperatures around 900 °C and pressures of 3 MPa [4]. Harsh reaction conditions necessitate costly equipment that account for approximately 60% of the process capital costs [21,22]. In addition, the overall process has a conversion rate of ~25% and selectivity ~70% [21].

Interest in a direct, single step oxidation of methane to methanol is vast, driven by many potential advantages over the conventional two-step process. In contrast to the indirect commercial process, the single step oxidation of methane to methanol is an exothermic reaction ($\Delta H° = -128$ kJ mol^{-1}) (Figure 3), avoiding the energy intensive, inefficient and expensive

syngas formation step. Mild reaction conditions also negate the need for specialist, costly equipment. Despite significant interest and effort in the direct partial oxidation of methane to methanol, many difficulties remain unsolved and as such the selective C–H bond activation required often is described as the "Holy Grail" of Chemistry [23].

$$CH_4 + H_2O \longrightarrow CO + 3\,H_2 \qquad\qquad \Delta_rH° = +\,206\ kJ\ mol^{-1} \qquad (1)$$

$$CO + 2\,H_2 \longrightarrow CH_3OH \qquad\qquad \Delta_rH° = -\,90\ kJ\ mol^{-1} \qquad (2)$$

$$Overall\ \Delta H° = +\,116\ kJ\ mol^{-1}$$

Figure 2. Energies of indirect methanol formation from methane via syngas [24].

$$CH_4 + ½\,O_2 \longrightarrow CO + 3\,H_2 \qquad\qquad \Delta H° = -\,128\ kJ\ mol^{-1} \qquad (3)$$

Figure 3. Energies of direct methanol formation from methane [24].

In summary, methane is a major contributor to the climate change problem that could instead be exploited as a chemical industry feedstock and as a fuel. Conversion to methanol is an attractive proposition but existing processes are difficult to apply to less accessible methane sources. Essentially this is due to difficult chemistry that biology has already evolved mechanisms to exploit. Due to it being an efficient energy store, convenient fuel suitable for use in the existing transport fuel infrastructure and raw-material for synthetic hydrocarbons, arguments exist for the implementation of a "methanol economy" as an alternative to the current fossil-fuel based situation [25].

3. Methanotrophic bacteria

In nature, methanotrophic bacteria are able to perform the controlled partial oxidation of methane to methanol at high conversion and selectivity, allowing the use of methane as a sole carbon and energy source. This unique ability makes methanotrophs a valuable candidate for the bioconversion of methane to methanol. Driven by the prospect of commercial exploitation for biocatalysis and bioremediation, interest in these microorganisms has increased over the last 30 years [5].

3.1. Methanotrophs: a brief introduction

The first methanotroph was isolated by Söhngen in 1906 which he named Bacillus methanicus [26]. Since then the most significant contribution was made by Whittenbury and his colleagues in 1970, in which over 100 methane-utilising bacteria were isolated, characterised and compared [27]. Playing an important role in the global methane cycle, methane-oxidising bacteria are found across a broad range of natural environments, present in nearly all samples taken from soil, swamps, rivers, oceans,

ponds and sewage sludge, reportedly representing up to 8% of the total "heterotrophic" population [28]. As the majority of methane is naturally produced through the anaerobic decay of organic matter, they are found primarily at oxic-anoxic interfaces. The majority of known methanotrophs are aerobic, however, methane oxidation is known to occur in anaerobic environments by coupling oxidation to sulphate [29] and nitrite reduction [30]. As expected from their prevalence within the environment, methanotrophs are found in both mesophilic and extreme environments. Strains have been isolated from temperatures as low as 4 °C [31] and as high as 72 °C [32] and it has been demonstrated that populations of methanotrophs in nature adapt to different temperatures [5].

Two populations of methanotrophs have been identified that exist depending on environmental methane availability [33]. Low affinity methanotrophs are able to utilise methane at high concentrations (>40 ppm), and are observed in soils with high methane exposure, accounting for all isolated methanotroph cultures known to date. High affinity methanotrophs are able to oxidise ambient methane concentrations (~ 2 ppm) and although their existence within soil samples has been verified using molecular techniques, isolation of such bacteria has not yet been possible. Analysis of nucleic acids (DNA and RNA), phospholipids, methane oxidation rates and stable isotope probing (SIP) using ^{13}C labelled methane has provided characterisation information, and confirmed relatively low abundance of these high affinity methanotrophs in soils [34]. In contrast to the relatively high abundance of methanotroph populations present in the environment, these low affinity methanotrophs account for <0.01% of total bacteria biomass in soils, attributed to low atmospheric methane concentrations [34].

Methane monooxygenase (MMO) enzymes catalyse the initial oxidation of methane to methanol, followed by sequential oxidation to formaldehyde by methanol dehydrogenase (MDH), oxidation of formaldehyde to formate by formaldehyde dehydrogenase (FADH), and finally formate to carbon dioxide by formate dehydrogenase (FDH). Formaldehyde is assimilated into biomass by either the ribulose monophosphate (RuMP) pathway, or the serine pathway. Figure 4 illustrates the metabolism of methane by methanotrophs.
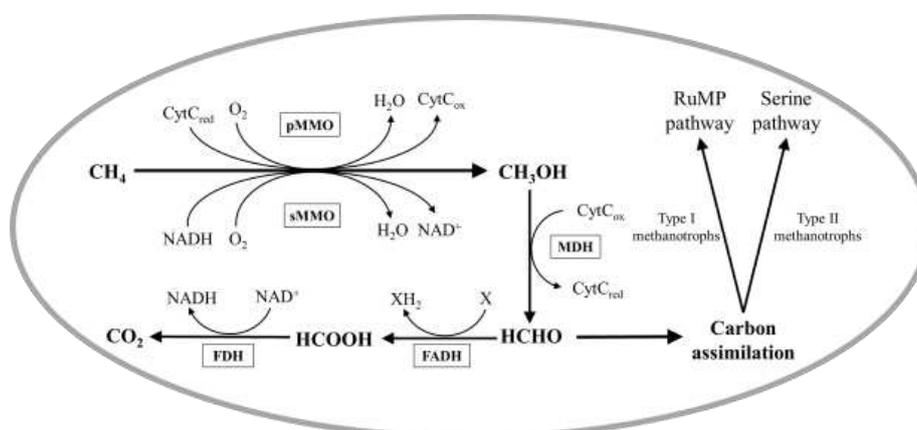

Figure 4. Pathways for the oxidation of methane and assimilation of formaldehyde in methanotrophic bacteria [5,35].

Traditionally, all aerobic methane-oxidising bacteria were of the phylum Proteobacteria, and classified into two major groups: Type I and Type II, based on differences in physiological and morphological traits, with Type X methanotrophs further differentiated from Type I [36,37]. Recent characterisation of several new genera and species, and the subsequent increase in the diversity of known methanotrophs, meant this system was no longer useful to characterise all known species, resulting in an update in the taxonomical description used to classify these organisms.

Methanotroph species are now known in Proteobacteria, Verrucomicrobia and candidate phylum NC10. Proteobacteria methanotrophs are divided into two classes; Alphaproteobacteria and Gammaproteobacteria. Current classification further divides Gammaproteobacteria, of the order Methylococcales, into three families: Methylococcaceae, which is further separated into Type Ia, including a total of 13 genera, and Type Ib including four genera (Methylococcus, Methylocaldum, Methylogaea and Methyloparacoccus); Methylothermaceae, Type Ic, (genera: Methylothermus, Methylohalobius, Methylomarinovum); and Crenotrichaceae which includes a single genus (Crenothrix polyspora) that to date has not been isolated as a pure culture. Methanotrophs of the family Methylococcaceae, of which the majority are Type Ia, utilise the RuMP cycle for carbon assimilation and have intracytoplasmic membranes arranged as a uniform array of bundles of vesicular disks distributed evenly across the cell. Differing from Type Ia methanotrophs in the expression of low levels of the ribulose-1,5-bisphosphate carboxylase enzyme, in addition to the RuMP pathway, genera formally identified as Type X were renamed Type Ib. The methanotrophic Alphaproteobacteria have been divided into two families; Methylocystaceae, Type IIa (genera: Methylocystis, Methylosinus) and Beijerinckiaceae, Type IIb (genera: Methylocella, Methylocapsa, Methyloferula) methanotrophs. Type II methanotrophs of the family Methylocystacea, utilise the serine pathway for formaldehyde assimilation, with the intracytoplasmic membrane arranged as stacks of vesicles in parallel to the cell membrane. Species of the family Beijerinckiaceae, identified as Type IIb, differ from Type IIa methanotrophs in that cells of Methylocella and Methylocapsa do not contain an intracytoplasmic membrane, while in Methyloferula they are found only on one side of the cell. Extremophilic methanotrophs belonging to the phylum Verrucomicrobia, of the genus Methylacidiphilium are sometimes described as Type III. Able to grow across a wide range of temperatures, they are unique in comparison with all other known methanotrophs due to their extremely acidophilic phenotype [38,39]. As with the majority of methanotrophs they possess pMMO but lack the familiar formaldehyde assimilation pathways, instead utilising the Calvin-Benson cycle for carbon fixation [40,41]. Anaerobic methane oxidation, coupled with nitrite reduction, has been observed in bacteria of the candidate phylum NC10, and named Ca. Methylomirabilis oxyfera [30]. See reviews by Knief [42], and Semrau [43] for comprehensive reviews of the current taxonomy of aerobic methanotrophs.

Methylosinus trichosporium OB3b (for "oddball" strain 3b) [44] and Methylococcus capsulatus (Bath) (originally isolated from the hot water baths in Bath, UK) [45], have proven themselves to be the experimental workhorses. The majority of investigation into the effect of environmental growth conditions, metabolic characterisation work, and consideration into commercial applications has been performed using these two strains leading to for full genomic characterisation [44,46], further enhancing molecular and system level research in the species.

3.2. Biochemistry of methane oxidation using methane monooxygenase (MMO)

Unique to methanotrophs is possession of MMO enzymes that catalyse the oxidation of methane to methanol. The initial reaction in the sequential oxidation to carbon dioxide, it is oxygen dependent and the most chemically difficult step. The monooxygenase splits the O–O bond of dioxygen using two reducing equivalents, with one of the oxygen atoms incorporated into methane to form methanol and the other reduced to water [47].

Two types of MMO have been found in methanotrophic bacteria; a soluble cytoplasmic form (sMMO) and a particulate membrane-bound form (pMMO). All methanotrophs, with the exception of members of the genera Methylocella [48] and Methyloferula [49], have the ability to produce pMMO, however Type II methanotrophs are also able to produce sMMO. For those strains able to produce both forms, the environmental growth conditions are responsible for dictating the type of enzyme expressed within the cell, with dependence primarily on the availability of copper. Under conditions of copper excess (>0.85 µmol g^{-1} dry weight of cells) pMMO is produced preferentially, while under conditions of limited copper availability, sMMO is generated, although the two are not mutually exclusive [50]. The dependence on copper availability is attributed to its presence in the active site of the pMMO enzyme [51].

The relative ease of isolation of sMMO has resulted in it being thoroughly studied and fully characterised [47,52]. sMMO is known to be made up of three protein components: a hydroxylase (MMOH), a regulatory protein (MMOB), and a reductase (MMOR). The hydroxylase protein is made up of three polypeptide subunits, arranged as a $\alpha_2\beta_2\gamma_2$ dimer, and contains a di-iron active site where oxygen and methane react using electrons supplied from NADH oxidation at the reductase, facilitated by the regulatory protein [47]. In contrast, significantly less is known about the biocatalysis and structure of pMMO, despite it being more prevalent in nature, due to difficulties in isolation and stability of the membrane bound protein. pMMO is known to be comprised of three subunits: PmoA, PmoB and PmoC, arranged in a trimeric $\alpha_3\beta_3\gamma_3$ complex, with the di-copper active site on the soluble part of the PmoB subunit [53,54]. A number of recent reviews give full structural and mechanistic details of sMMO and pMMO [47,55–57]. Despite their similar function within the cell, sMMO and pMMO are not related structurally or genetically.

Both forms of MMO are able to co-oxidise a range of organic substrates in the presence of methane although they do not support in vivo growth [58,59]. sMMO exhibits broader substrate specificity and is able to catalyse a larger number of biotransformations than pMMO. Preferential oxidation of smaller substrates by the pMMO system led to understanding that access to the active site of pMMO is sterically more restricted than sMMO [60]. The differences between the two forms of MMO have been predicted to lead to specific phenotypic differences. Cells that contain pMMO have greater growth yields, attributed to a reduction in the energetic requirement, and exhibit a higher affinity for methane than those containing sMMO [61,62]. This is related to differences in reducing power utilisation between pMMO and sMMO. Electrons required for the initial oxidation step are provided by NADH in the sMMO catalysed reaction, whereas pMMO utilises reducing equivalents provided by the MDH co-factor pyrroloquinoline-quinone (PQQ) [61,62].

3.3. Utilising methanotrophic bacteria as biocatalysts for the oxidation of methane to methanol

The majority of biological methods to exploit this single step transformation are based on utilising or mimicking powerful MMO enzymes that activate the C–H bond in methane in a highly selective process at ambient temperature and pressure. MMO-catalysed partial oxidation of methane to methanol has a number advantages over thermochemical oxidation routes, including higher selectivity, improved process efficiency and safety, milder reaction conditions and energy savings, all leading to associated economic benefits.

A number of approaches have been investigated to exploit the powerful oxidising ability of methanotrophic bacteria, with varying potential for use in industrial processes. Below is a brief overview of these, including consideration of their suitability for use in an industrially relevant process. As the method with greatest potential, exploiting whole cell methanotroph cultures will be the focus of this review.

3.3.1. Whole cell methanotroph cultures

Whole cell methanotroph cultures have the potential to be a relatively cheap route for the bioconversion of methane to methanol. The generation of biomass is reasonably simple and cost effective, whilst the more involved molecular operations, such as the synthesis of key MMO enzymes and necessary reducing equivalents, are controlled entirely by the bacteria. Whole cells also have the capacity for self-maintenance and replication. Moreover, there are downstream processing benefits because although the biochemical reactions occur within the intracellular space, the methanol accumulates extracellularly, which facilitates product isolation.

Although currently the preferred option, whole cell biocatalysts do pose a number of challenges. Being closely specialised to a particular niche constrains their deployment in dissimilar biotechnological process operating conditions. High cell density culture also has proven difficult, which has been attributed to gas-liquid transfer limitations [63,64]. The complex nature of cellular metabolism in the case of methanotrophic bacteria presents the risk of over oxidation to formaldehyde, in addition to complications associated with interrupting the natural biochemical pathways of the cell. An added level of process difficulty exists as it is necessary to design a biphasic growth process for both cell growth (enzyme manufacturing phase) and bioconversion (methanol production phase).

3.3.2. MMO enzyme isolates

Using methanotrophic bacteria for the desired transformation is ultimately a way to exploit the powerful MMO enzyme. Substantial research efforts mean MMO enzymes are fully characterised with a high level of understanding of their biochemistry. An alternative strategy uses enzyme isolates from cell cultures. By avoiding various complex cellular interactions, and therefore performing only the desired reaction, the particular benefit of the isolate strategy is that it avoids over oxidation of methanol through normal cellular metabolism. The bacteria still perform the difficult MMO production and, in the absence of other cellular components, process interactions are simplified and

cellular toxicity is not an issue. Using cell-free preparations of the MMO enzyme however poses difficulties in isolation and purification attributed to instability of the purified enzyme [57]. Complications associated with working with an integral membrane bound protein hinder the use of pMMO, although cytoplasmic sMMO is more readily isolated. Typically stabilisation is achieved by enzyme immobilisation on or in artificial matrices. Even so, Evolution did not optimise Nature's catalysts for technical process conditions and so stability, activity and lifetime become process issues [65]. Additionally, cofactor dependency and the necessary supply of exogenous reducing equivalents favours the use of whole cells. The energy requirements for the system for both biomass production and bioconversion are equivalent to using a whole-cell culture, without the advantage of cell maintenance.

3.3.3. Genetically modified organisms

It is possible to combine the advantages of whole-cell systems with optimised reaction processes. By using recombinant microorganisms containing artificial synthetic pathways, methanotrophs offer the potential for specific biotransformations (beyond just methane oxidation to methanol) and improved product yields. Progress here is currently hindered by the inability to express functional MMO proteins in Escherichia coli [66–68]. Away from scientific ability and innovation, the production and use of GMOs (genetically modified organisms) poses significant ethical consideration [69,70]. In the case of methanotrophs, Calysta have patented a process for the biological oxidation of hydrocarbons using a genetically engineered form of Methylosinus trichosporium OB3b, although a lack of published data to verify the system exists [71].

3.3.4. Synthetic MMO analogues

The thorough characterisation and understanding of the biochemistry of MMO enzymes suggests the design of synthetic "biomimetic" catalysts with the potential to offer the advantages of using enzyme isolates combined with the stability of a thermochemical process. Biologically-inspired organometallic compounds might be designed so as to maximise selectivity, yield, reaction rate and conversion efficiency, whilst increasing tolerance to process conditions compared with purified enzymes, avoiding issues relating to instability of isolated membrane bound proteins, and being less susceptible to product inhibition [72]. However, taken together these objectives constitute a challenging optimisation problem that likely necessitates a similarly complicated molecular machine to MMO enzymes, which will challenge chemical synthesis. This must be contrasted with the ease and efficiency with which methanotrophs produce powerful MMO enzymes, especially given that our rapidly increasing ability to design proteins [73] with improved properties should overcome many of the limitations of MMO enzymes. Despite efforts, synthesis of a chemically active MMO analogue has not yet been achieved [72].

3.3.5. Ammonia-oxidising bacteria

An alternative option utilises ammonia-oxidising bacteria containing the ammonia monooxygenase (AMO) enzyme, a pMMO homologue. Similar in both structure and function to pMMO, under typical cellular conditions, AMO catalyses the oxidation of ammonia (NH_3) to hydroxylamine (NH_2OH), followed by the hydroxylamine oxidoreductase catalysed oxidation to nitrate (NO_2^-). The metabolism of ammonia generates reducing equivalents for the cell, whilst carbon dioxide is used as a carbon source [74]. Being similar in structure to pMMO, the low substrate specificity of AMO also allows it to oxidise methane to methanol [75,76].

Despite the potential of ammonia oxidising bacteria, a number of challenges exist before commercial implementation will be possible, including slow reaction rates, high costs and technical immaturity.

4. Challenges and potential strategies associated with the methanotroph catalysed conversion of methane to methanol

The major challenges faced in developing an industrially relevant biological partial methane oxidation process are described below, in addition to approaches investigated to overcome these and optimise reaction conditions. The success of these can be measured in terms of greater biomass concentration, improved methanol yields and enhanced enzyme activity.

4.1. Challenge I: gas-liquid mass transfer limitations

As in the majority of fermentation processes, biomass and cellular product generation will be limited by the availability of metabolic gases, especially at high cell densities. This problem is intensified by the sparingly soluble nature of gases such as oxygen and methane.

It has been demonstrated that the low rates of gas-liquid mass transfer of methane in aqueous culture is a growth limiting factor [63,64] which is responsible, in part, for slow growth and difficulty in high biomass production. As MMO is a growth associated enzyme, high cell density cultivation can result in an increase in MMO containing biomass [77], which is desirable for the proposed biotransformation.

A number of process factors can be addressed to optimise the solubility of gaseous substrates in methanotroph culture including reactor design, gas delivery method and temperature. As with all gases, the solubility of methane decreases with increasing temperature [78], and so to maximise dissolved methane and availability for the methanotroph culture, a low temperature is desired. This parameter is restricted, however, by the optimum temperature for culture growth and maintenance, as well as for MMO reaction and stability. The impact of temperature is further detailed in Section 4.6.2.

4.1.1. Optimised reactor design to maximise mass transfer

Current investigation has focused on employing membrane reactors for the methanotroph catalysed methane oxidation. The delivery of gaseous substrates through two porous membranes

allows the separate feed of methane and air to the reactor, reducing risks involved in using potentially explosive mixtures of gases (methane in air is explosive between 5% v/v and 15% v/v). The gases are delivered to the methanotroph culture through membrane contactors that offer a large surface area and avoid bubble formation, both of which optimise gas-liquid mass transfer.

Duan et al. first demonstrated methane bioconversion in a dense silicon tube stirred membrane reactor. Through improved methane delivery to the liquid phase, methanotroph culture was possible at high cell densities up to 17.3 dry cell g/L, producing 0.95 g/L methanol after 40 h [79]. Pen et al. since designed and demonstrated methanotroph biocatalysis in a novel recirculating macroporous membrane bioreactor (MBR). The mass transfer achieved was twice that observed in a batch reactor in similar conditions, producing 120 mg/L methanol after 24 h [80]. Calysta's commercial FeedKind® protein process is performed in a patented loop reactor optimised for gas-liquid mass transfer. Rapid liquid flow is used to drive substrate gases downwards against gravity, faster than they rise, leading to in situ pressurisation of the gases and consequently increased gas dissolution [81].

4.1.2. Paraffin oil as a "Methane Vector"

In a novel approach to increase the mass transfer of methane from the gas phase to the liquid medium, Han et al. found that adding water-immiscible organic compounds in which methane has a higher solubility, showed significant improvement on cell density. With the addition of 5% (v/v) paraffin oil in the NMS medium, cell density of M. trichosporium OB3b reached 14 g/L (dry weight), around seven times higher than the control after 240 hours culture [63]. Higher concentrations of paraffin did not improve cell growth, suggesting that methane transfer is not the only limiting factor, and also attributed to the fact that cell growth was observed in the oil phase which could act as a barrier to metal ions and nutrient substrates. Although paraffin in the liquid medium has been shown to enhance methanotroph growth, the effect on methanol synthesis has not been investigated.

4.2. Challenge II: over oxidation of methane beyond methanol

As part of the natural biochemical pathway in methanotrophs, methanol is further metabolised through formaldehyde and formate to carbon dioxide, so it is necessary to stop the reaction at the methanol oxidation level. One approach to enhance the production of methanol is to suppress over oxidation by inhibition of the MDH enzyme as shown in Figure 5. A number of compounds have been identified as MDH inhibitors with varying degrees of success in enhancing methanol production as some have been observed to also reduce MMO activity. The efficiency of methanol conversion is known to be impacted by the nature and concentration of such inhibitors.
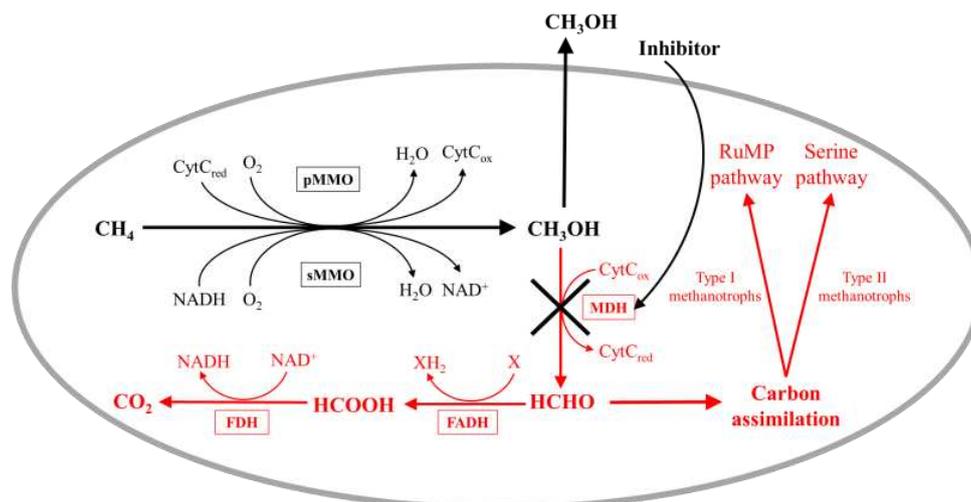
Figure 5. The MDH inhibited pathway of methane oxidation to methanol in methanotrophic bacteria. Sections in red do not occur if 100% of produced methanol is extracted from the cell.

4.2.1. Cyclopropane-derived inhibitors

Cyclopropane-derived compounds have been found to act as irreversible inhibitors of MDH [82]. The ring opening reaction of the cyclopropane functionality with pyrroloquinoline quinone (PQQ), the coenzyme of MDH, results in deactivation of the MDH [82,83].

Treatment of cell suspensions of M. trichosporium OB3b with cyclopropanol show extracellular methanol accumulation under a methane atmosphere [83–85]. At a cyclopropanol concentration of 6.18 µM, MDH activity in M. trichosporium OB3b has been shown to decrease by 79%, although at this level, simultaneous reduction in pMMO activity of 12% was observed [85]. After 100 hours, M. trichosporium OB3b produced 152 mmol/g (dry cell) methanol which is 51 times higher than produced under "conventional conditions" although direct comparisons of this data is not possible due to the lack of a control, and the distinct differences between "conventional" and "optimum" conditions employed, including temperature and cell density.

4.2.2. High salt concentrations

A range of inorganic compounds have been investigated offering MDH inhibition with varying effects on MMO activity. These are considered advantageous over inhibitors such as cyclopropanol as they are cheaper, more chemically stable and exhibit reversible nature. It is believed that electrostatic interactions between MDH and cytochrome c_L, the primary electron acceptor, can be disrupted by high salt concentrations in the culture medium, thus deactivating the enzyme [86,87].

Initially observed in Methylosinus trichosporium OB3b cell-free extracts [88], and Methanomonas methanooxidans microbial culture [89], the addition of phosphate resulted in methanol accumulation attributed to MDH inhibition. Complete inhibition of MDH has been observed in cell-free extracts of M. trichosporium at 150 mM phosphate [88], with 120 mM

phosphate concentration offering maximum inhibition in whole-cell suspensions of M. trichosporium [90]. At a concentration of 80 mM phosphate ions, 80% of the MDH activity was inhibited, however this was found to simultaneously reduce MMO activity by 16%, and FDH by 20% [90]. Extracellular accumulation of methanol has been maximised using a phosphate concentration of 400 mM, generating 0.96 g/L methanol using a relatively high cell density of 17.3 g dry cell L^{-1}, after 43 hours [79]. Using a mixed microbial culture, Han et al. observed maximum methanol production, and highest methane-to-methanol conversion ratio, at a phosphate concentration of 40 mM [91]. Inhibition with phosphate was found to be fully reversible, with enzyme activity completely restored after washing of the cells with low concentration phosphate buffer [90]. The inhibition mode of phosphate on MDH was found to be uncompetitive, suggesting phosphate binds to a site on the enzyme or enzyme-substrate complex other than the active site [90].

A sodium chloride concentration of 300 mM has been shown to inhibit 100% of MHD activity in M. trichosporium OB3b, while also reducing pMMO activity by 50% [92]. Under an optimal concentration of 200 mM sodium chloride, 7 mM methanol accumulated after 36 hours, compared with no methanol accumulation under the same conditions in an absence of salt [92]. In a methanotroph based consortia, maximum methanol accumulation of 0.5 mmol was observed with an optimum 100 mM sodium chloride concentration, in addition to a conversion ratio of almost 80% [91].

Electron microscopy has shown that at concentrations above 100 mM, sodium chloride disrupts cell structure and, significantly, the intracytoplasmic membrane where pMMO is found [93]. Ethylenediaminetetraacetic acid (EDTA) also inhibits MDH activity, through chelation of metals present in the enzyme, and is not known to impact cell morphology [87]. Kim et al. used a combination of sodium chloride and EDTA, thus allowing reduced sodium chloride concentrations but maintaining MDH inhibition. The study demonstrated that the combination of 1 mM EDTA and 100 mM sodium chloride was optimal for methanol production [93]. At higher concentrations of EDTA, methanol production was reduced, attributed to inhibition of MMO. The efficiency of lone EDTA as an MHD inhibitor was much lower compared with other inhibitors. An optimal concentration of 50 µM in a mixed methanotroph consortia produced methanol at a conversion rate of just 43% [91].

Ammonium chloride is also known as an MDH inhibitor, inducing maximum methane-to-methanol conversion of 80%, and optimal methanol accumulation at a concentration of 40 mM in a mixed microbial culture [91].

4.2.3. Carbon dioxide

Xin et al. investigated the effect of various concentrations of carbon dioxide on the production of methanol using M. trichosporium IMV 3011 [35,94]. They demonstrated that the addition of carbon dioxide to methanotroph cultures under a methane/oxygen atmosphere resulted in the extracellular accumulation of methanol. It was found that 40% v/v carbon dioxide resulted in an optimum accumulation of 14 µmol/L methanol in a sealed flask after 24 h compared with a control where no methanol was detected [35]. At concentrations above 40% v/v methanol synthesis was lower, attributed to greater inhibition of methanol oxidation causing NADH limitation within the cells. Using an appropriate carbon dioxide concentration in the gas feedstock is believed to offer

partial inactivation of MDH, allowing simultaneous accumulation of methanol and NADH recycling through complete oxidation of methane to carbon dioxide. Although reducing the maximum theoretical efficiency to 50%, the advantage of this method is that an external source of reducing equivalents is not needed and instead the natural NADH regenerating cycle can be exploited.

4.3. Challenge III: product inhibition

As with ethanol fermentation, the oxidation of methane to methanol by methanotrophic bacteria is hindered by product inhibition [35,83]. Methanol was first shown to be toxic to most methanotroph strains at concentrations as low as 0.01% v/v [27], supported by the work of Adegbola where methanol was found to completely inhibit growth at 40 g/L [95]. It has since been demonstrated that the pMMO enzyme in M. trichosporium OB3b is directly inhibited at levels as low as 10 mM methanol, confirmed by the complete inhibition of propene epoxidation [83].

It has been hypothesised that under stress conditions, methanotrophs may excrete various other products in addition to methanol, that could have negative effects on the bacterial oxidation ability. An inability to identify unknown compounds by GC-MS and the ultimate loss of oxidation ability after successive media renewals, suggest this is not the mechanism by which biocatalyst activity is lost [96].

4.3.1. In situ product removal (ISPR)

One method to overcome this issue is immediate removal of the methanol product using in situ product removal (ISPR). Maintaining the methanol concentration below inhibitory levels encourages methane oxidation, while also maximising product recovery by preventing over oxidation. ISPR necessitates consideration of reactor design and operation but has the added benefit of reducing downstream processing and associated costs [97].

A number of published examples of continuous and semi-continuous methanol biosynthesis utilise membrane reactors in which methanol is removed in the reaction media as a means to maximise product yield. In a semi-continuous process utilising an ultrafiltration cell, a suspension of M. trichosporium OB3b was investigated for methanol production. After incubation for 90 minutes the reaction mixture was filtered, separating product methanol from the cell suspension. This procedure was repeated five times producing a total of 36.1 µmol methanol compared to 19.6 µmol after 6 h in a batch reactor under the same conditions [83]. Xin et al. utilised a membrane reactor with a reaction volume of 40 mL and a continuous buffer feed to remove produced methanol from the cell suspension. The reactor was run for 198 h without loss of productivity and generated a total ~23 µmol methanol at a rate of 0.13 µmol/h [35] . This was in comparison with a batch reaction under the same conditions in which a calculated total ~3.7 µmol (18.8 µmol/L quoted) methanol was produced, as a consequence of product inhibition. Continued productivity can be attributed to methanol removal, in addition to allowing a portion of the methanol produced to oxidise to carbon dioxide, generating NADH and maintaining MMO activity.

Pen et al. performed successive reaction medium renewals over a 22 h methanol production process, demonstrating increased methane oxidation activity compared to a process without media

renewal. A plateau in methanol concentration was observed at 22 h for both the reaction with and without media change, and the total methanol quantity produced was also comparable: 16.5 mg for the consistent reaction media and 18.0 mg after 3 medium renewals. The apparent lack of improvement on methanol production was attributed to a limit to bacterial oxidation capacity [96].

4.4. Challenge IV: maintaining catalytic activity and methanotroph viability

One issue in using whole cell cultures for catalysis is the need to maintain the physiological activity, catalytic activity and viability of the microbes.

During the MMO catalysed oxidation of methane, two electrons are used to split the O–O bond in molecular oxygen, supplied by the cell in the form of NADH or cytochrome c (CytC) depending on whether sMMO or pMMO are utilised. Under standard cell conditions, reducing equivalents are regenerated from NAD+ and $CytC_{ox}$ during oxidation of methanol via formaldehyde and formate to carbon dioxide. However, interruption of metabolic pathways by MDH inhibition and extraction of methanol results in the sequential oxidation of methanol to carbon dioxide not being possible, thus preventing regeneration of reducing equivalents. Eventually exhaustion of the energy source results in loss of MMO activity and cell viability (Figure 5).

An important point in considering the suitability of an electron source for an industrial process, it that it is low cost and sustainable.

4.4.1. Formate addition

The addition of external metabolic electron donors to the reaction media overcomes this issue, allowing continued production of methanol. Formate is a preferred choice as a downstream metabolite of the process of interest, employed in the majority of studies [79,80,83,85,92,93,96,98,99]. Formate added to the reaction mixture is oxidised by FDH in the cell, generating an electron and carbon dioxide. Mehta et al. [98] were first to demonstrate the restoration of methanol synthesis by formate addition through regeneration of $NADH_2$. The rate of methanol synthesis in an MDH inhibited methanotroph culture was observed to fall off after 6 h, attributed to depletion of reducing equivalents, and the addition of 40 mM sodium formate to the reaction mixture restored biocatalytic activity to the previous level. Takeguchi et al. reported methanol accumulation in MHD inhibited M. trichosporium OB3b increased with increasing sodium formate concentration in the reaction media up to a maximum 14.3 mmol/L [85].

In a study to establish the optimal reaction conditions for methanol synthesis, varying sodium formate concentration was investigated [92]. In agreement with Takeguchi et al., methanol synthesis increased with sodium formate addition, although an optimum concentration of 20 mM formate was determined above which there was no increase in methanol accumulation. In conflict however, Duan et al. found that under the reaction conditions employed, increased sodium formate concentrations between 10 and 80 mM resulted in almost equivalent maximum methanol production, and the accumulation rate decreased with increasing formate concentration [79]. This lead to the suggestion that to maximise rate of methanol synthesis, formate should be added to the media at low concentrations throughout reaction.

The effect on methanol synthesis of supplementing the microbial culture with formate throughout reaction was investigated by Pen et al.. In a culture where maximum methanol synthesis had been achieved, and oxidation activity dropped off, the addition of 20 mM sodium formate did not restart bacterial activity. This suggests that once lost, methane oxidation activity is irreversible. When formate was added whilst the bacteria were still active, an adverse effect was observed. The methanol production rate immediately dropped off compared with a culture without formate addition, and a lower total methanol concentration was achieved (50 mg/L compared with 120 mg/L) [96].

It is believed that sodium formate can be used by the cell in the serine pathway for carbon fixation. This process would compete with NADH regeneration, with the two processes in equilibrium, and could explain the noted trend in increased methanol production with formate addition [96].

4.4.2. Use of cellular regeneration pathways

An alternative method to ensure the sustained activity of MMO is to use the cells' natural regeneration mechanism through the complete oxidation of methane to carbon dioxide [35,94]. By supplying methane to the cell and not extracting methanol, reducing equivalents are generated that can be utilised during the MMO catalysed oxidation of methane. Through alternating between methanol production and regeneration cycles, semi continuous methanol biosynthesis can be maintained. Although use of the methane feedstock in this way reduces the overall process yield, it is necessary to maintain the viability of the cell and provides a relatively cheap and simple solution.

This principle has been demonstrated successfully by Xin et al. for the biosynthesis of methanol from carbon dioxide with M. trichosporium IMV 3011 [94,100]. The hydrogenase enzymes responsible for the oxidation of methanol to carbon dioxide, via formaldehyde and formate, are able to catalyse the reverse reactions, although progressing against the natural biochemical pathway is an energy intensive process requiring an electron for each sequential reduction. In batch experiments after continuous reaction for 48 hours, almost 100% of the methanol synthesis ability of the cells was lost, however by alternate reaction for 24 hours and regeneration for 12 hours with methane and air (1:10, v/v) there was no notable loss in methanol synthesis after 9 cycles [94]. It was proposed, due to the rapid resumption of cellular viability and reduction ability, that regeneration of reducing equivalents was responsible rather than growth of additional cells that would be considerably slower.

4.4.3. Using poly-β-hydroxybutyrate cellular energy store

In addition to NADH as a direct source of reducing equivalents, the ability of the cell to store energy in the form of poly-β-hydroxybutyrate (PHB) has been considered. PHB is a lipid, produced and accumulated as an intracellular carbon and energy storage molecule by a variety of microorganisms in response to stress conditions, and undergoes metabolism releasing reducing equivalents when standard energy sources are not available [100]. PHB accumulation has been observed in methanotrophic bacteria [27,28,101], and is known to be synthesised by the RuMP and serine pathways in response to nitrogen, phosphate and oxygen limitation [100]. PHB production is believed to be mainly non-growth associated, with maximum accumulation occurring during late

growth and early stationary phases [102]. The main factor that determines the extent of PHB production is availability of the synthetic precursor, acetyl-CoA. Type II methanotrophs employing the serine pathway are the most effective producers able to accumulate up to 80% PHB by dry weight [103,104].

Thomson et al. noted the metabolism of PHB in the presence of C_2 compounds, attributed in part to the satisfaction of energy requirements in utilising non-growth associated substrates [105]. Additionally, the capacity for M. trichosporium OB3B to degrade trichloroethylene (TCE) in an sMMO catalysed reaction was enhanced 160% in cells containing 10% PHB compared with 2% PHB [106]. Similarly a positive correlation has been noted between PHB content and TCE oxidation ability in a mixed methanotroph culture, supported by the observed increase oxidation rate on addition of the PHB monomer, β-hydroxybutyrate [107]. It is believed that the finite supply of reducing equivalents within the cell is supplemented by the metabolism of stored PHB.

4.4.4. Microbial electrosynthesis

Microbial electrosynthesis is the process by which electrons can be transferred from an electrode to living cells to provide energy for biocatalytic synthetic processes. A relatively new concept, the direct supply of electrons from an electrode to microbes was initially investigated by Gregory et al. for the anaerobic respiration of Geobacteraceae [108]. The idea has been further developed to utilise an applied current in an electrochemical cell to drive microbial metabolism for the production of a range of fuels and chemicals [109,110]. In a novel approach, it is proposed that microbial electrosynthesis could be used to supply reducing equivalents directly to the methanotroph culture in place of formate.

To date only a small number of bacterial species have been found to accept electrons directly from an electrode, with no electrotrophic methanotrophs identified. Should such species be identified and isolated, and using renewably sourced electrical power, it may be possible to provide a cheap and sustainable source of electrons for methane oxidation.

4.4.5. Cell integrity

It has been demonstrated, that the addition of high concentrations of sodium chloride as an MDH inhibitor has a negative impaction on cell integrity and methane oxidation ability by disrupting the structure of intracytoplasmic membranes [93]. This discovery prompted investigation into alternative MDH inhibitors that do not negatively impact cell morphology.

Pen et al. employed flow cytometry to investigate the integrity of the bacterial culture before and after a methanol production experiment. At the beginning of the experiment, 81% of cells were viable, with damaged cells constituting 5% of the bacterial population, which dropped to 41% viable and 38% dead after 48 h methanol production [96]. Loss in methane oxidation ability of the cells is believed to be related to loss in cell membrane integrity. Despite the presence of 41% viable cells, the oxidation activity loss was measured as 97%, which indicated that the loss of biocatalytic activity of the cells was due to both cell death and an alternative mechanism [96].

Pen et al. noted that the proportion of viable cells halved and the bacterial concentration was reduced by 23% after 48 h of methanol synthesis. The reduction in optical density (OD) measurements was attributed to cell lysis and absorption of bacteria onto the bioreactor [96]. Cell lysis would result in the release of MMO enzymes from the cell into the reaction media. However, the low stability and activity of MMO isolates suggest the catalytic contribution from these to be minimal.

The effect of fresh biocatalyst addition during the methane oxidation process has also been investigated and is surprisingly shown to have a negative impact on methanol production. After 22 h, at the point of loss in methanol oxidation activity, addition of fresh methanotroph culture to double the total biocatalyst concentration, showed a sudden and strong decrease in the methanol concentration, with 70% of the produced methanol lost after a further 24 h [96]. Biocatalyst addition at a stage whilst the bacteria were still viable and activity high, also resulted in methanol consumption by the bacteria, even in the presence of MDH inhibitors. The explanation for this previously unreported phenomenon is that the accumulated methanol gives rise to configuration changes on the PQQ group, restoring the ability for electron transfer from PQQ to cytochrome C_L, ultimately overriding the sodium chloride MDH inhibition and restoring methanol oxidation [96].

4.5. Challenge V: toxicity of source methane impurities

The oxidation of methane by methanotrophs is well known to be sensitive to impurities in the methane feedstock, attributed to the low substrate specificity of the oxidation enzymes in methanotrophs including MMO, MDH and FDH [27] (the names of which do not describe the specificity for such substrates but their metabolic function within the cell). Non-growth hydrocarbons are co-oxidised by the bacteria, producing toxic metabolites that have the potential to disrupt metabolic pathways through both competitive inhibition of catalysts and accumulation of toxic products, which ultimately results in cell death [84,105]. Ethane is particularly problematic as it is generally the most abundant organic compound in natural gas after methane, and is oxidised through ethanol and acetaldehyde to acetate followed by build-up in the cells.

Although free from higher hydrocarbons, methane biogas - the product of industrial anaerobic digestion-contains a mixture of gases depending on the feedstock composition. Primarily methane (30%–70%) and carbon dioxide (25%–50%), it also contains trace impurities including hydrogen sulphide (<2000 ppm), ammonia (<100 ppm) and organic chlorine and silicon compounds [111]. These impurities pose process difficulties if biogas sources of methane are utilised.

The removal of impurities in the methane feedstock can be achieved by the implementation of gas purification processes. Options are vast depending on the methane source and problematic contaminants. Generally however a number of stages are required resulting in a complex, energy intensive and expensive process [112].

4.5.1. Microbial consortium

Interestingly, methanotroph cultures are abundant in natural gas environments such as around petroleum seeps and vents where longer chain gaseous alkanes including ethane, propane and butane

are present at inhibitory concentrations [113–115]. It is well known that natural methanotrophic populations exist in symbiotic communities with a range of organisms, including a variety of alkane oxidising organisms able to utilise both C_1 and $\geq C_2$ substrates [116]. It is believed that within such situations methanotrophs are able to selectively utilise methane whilst potentially toxic $\geq C_2$ alkanes are removed by other bacterial species. Of the methanotrophic strains identified, only those of the genus Methylocella are able to grow on substrates containing C–C bonds [48,117,118], in contrast to numerous bacteria that are capable of growth on linear alkanes C_2–C_9 but not methane [113].

Han et al. were the first to investigate methanol production using a mixed culture consortium from a natural environment [91]. Isolated from a land fill site, 16S rRNA gene analysis identified the key species present as Methylosinus sporium NCIMB 11126, M. trichosporium OB3b and M. capsulatus (Bath). Maximum methanol accumulation was demonstrated, with sodium chloride MDH inhibition, at a production rate of 9 µmol/mg h. No change to microbial community structure was observed over the 24 h time course experiment, suggesting stability of the methanotroph community and methane oxidation process.

This has been demonstrated commercially by Norferm AS who have developed an industrial process utilising a synthetic bacterial community including M. capsulatus (Bath) to convert natural gas, to a bacterial biomass product known as BioProtein® [116].

4.6. Challenge VI: optimised biotechnological conditions and consideration of requirements of the biphasic process

The first stage in the biocatalytic process is growth of biomass, during which the bacteria multiply and manufacture the critical MMO enzymes. This is followed by the bioconversion of methane to methanol which can be considered the production phase and the specific reaction of interest. Unsurprisingly the optimum conditions for these two processes differ which presents the option for using a single vessel with compromised conditions, or a two-step process allowing conditions tailored to culture and reaction separately.

4.6.1. Copper concentration

It is well documented that the concentration of copper in the reaction medium is responsible for the expression of sMMO and pMMO, and consequently the bacterial growth rate. For strains able to produce both MMO forms, under conditions of copper excess pMMO is made preferentially, while under conditions of limited copper availability sMMO is present [50]. Excess copper beyond that required to switch from sMMO to pMMO expression increases the activity of pMMO [119], and it has been demonstrated that cells producing pMMO have a faster growth rate and higher catalytic activity with methane [120]. Cells producing pMMO have greater growth yields than those expressing sMMO attributed in part to the reduced energy requirements on the cell [61,62].

In terms of methanol synthesis, it is proposed that the copper concentration has an indirect effect in the form of MMO synthesised and the relative enzymatic activity. Markowska & Michalkiewicz showed a positive correlation between copper content in the media and methanol synthesis by M. trichosporium OB3b up to 1.0 µmol/L, above which productivity decreased [121].

4.6.2. Temperature

It has been observed that the optimum temperature is different for MMO activity and cell growth in methanotrophic bacteria. Mehta et al. found the optimum temperature for methanol accumulation in M. trichosporium OB3b to be 35 °C [90], confirmed by Lee et al. who noted that maximum sMMO activity is observed at 35 °C whereas the rate of cell growth is maximised at 37 °C [122]. Above 40 °C led to a decrease in methanol accumulation [90], attributed to instability in the MMO system. Takeguchi et al., however, observed maximum methanol accumulation in M. trichosporium OB3b by pMMO at 25 °C [85]. Increasing temperature resulted in reduced methanol accumulation attributed to instability of the pMMO enzyme at elevated temperatures.

4.6.3. pH

The effect of pH on methanotroph growth rate and MMO activity is less sensitive compared to the temperature and copper concentration effect. In M. trichosporium OB3b the optimal pH for sMMO activity was shown to be 6.2–6.4 compared with pH 7.0 for cell growth [122]. In M. trichosporium OB3b expressing pMMO optimal methanol synthesis was observed at pH 6.5 [90].

4.6.4. Cell concentration

The cell concentration in the reaction medium influences extracellular methanol accumulation as MMO is a growth associated enzyme. Mehta et al. found maximum methanol production in M. trichosporium OB3b at a cell concentration of 4 mg ml^{-1} [90], in close agreement with the findings of Xin et al. at 3 mg/ml [35]. In contradiction Lee et al. noted an optimum cell concentration of 0.6 mg/ml for methanol synthesis [92]. It is believed that the maximum cell concentration able to be supported is limited by the availability of methane in the reaction mixture, related to gas-liquid mass transfer limitations and as such delivery methods and culture conditions.

The methanol production kinetics, for reaction in a membrane bioreactor, exhibited the same profile at biocatalytic concentrations between 11 u/ml and 150 u/ml, with little correlation between total methanol production and biocatalyst concentration [80]. This behaviour was shown to be related to oxygen limitation due to absorption of bacteria and fouling of bioreactor membranes.

4.6.5. Bacterial strain

To date Methylosinus trichosporium OB3b has been the preferred methanotroph species for investigation into the potential for a biocatalytic partial methane oxidation process, aided by the wealth of characterisation data available. The application of alternative species, in particular extremophilic and extremotolerant methanotrophs, able to either thrive or tolerate living in extreme environments, offers huge potential in terms of maximising methanol yield through process efficiencies.

The use of psychrophilic methanotrophs, capable of growth at low temperatures, would be well suited to tackling gas-liquid mass transfer limitations. The solubility of methane increases with

decreasing temperature, however this is currently constrained in methanol biosynthesis by the optimum temperature of M. trichosporium OB3b which is 30 °C. A number of psychrophilic methanotrophs have been identified and isolated from a range of low temperature ecosystems including Siberian tundra bogs, Antarctic lakes, and bottom sediments and water from Pacific and Atlantic Oceans [123]. The potential of such species in an industrial process is limited as the rates of in vivo methane oxidation and in vitro metabolic activity have been demonstrated to decrease significantly as the temperature is lowered from 30 °C to 5 °C [124].

MMO isolated from thermophilic methanotrophs has the potential to be better suited to industrial process conditions, offering increased stability under high temperature reaction conditions which would promote increased reaction rates. Tolerance and active growth of such species in environments of elevated temperatures suggests development of metabolic systems to survive under these extreme conditions. Isolated and characterised by Bodrossy et al., strain HB was isolated from Japanese and Hungarian hot springs, capable of growth at temperatures up to 70 °C [32]. Representing a new genus, the name *"Methylothermus"* was proposed, however this organism was not extensively characterised and is no longer extant. With investigation prompted by loss of the HB strain, the only truly thermophilic methanotroph currently known is strain MYHT, described as Methylothermus thermalis. Isolated from a Japanese hot spring, and capable of growth at temperatures 37–67 °C (optimum 57–59 °C), it is closely related to the HB strain [125].

The biochemical and molecular mechanisms by which methanotrophs are able to survive in such harsh conditions is still uncertain, although de novo synthesis of ectoine as a stress protectant is known [123]. Further understanding of responses to stress conditions is needed, in addition to bioenergetic and genetic aspects of extremophile adaptation. Advances have been made, and as such we are increasingly able to isolate and culture extremophilic methanotrophs, which furthers the potential for industrial biotechnological applications.

MMO activity is not only dependent on the expression of either soluble or particulate forms, regulated by copper availability, but also the methanotroph species in question. An example of this is the low methanol productivity recorded by Xin et al. attributed to low specific MMO activity of M. trichosporium 3011, which is about one percent compared with M. trichosporium OB3b [35]. It would therefore be reasonable to expect methanotroph species to exist with MMO activities greater than that in OB3b, which would offer the potential for higher methanol production rates.

4.6.6. Citric acid as a Krebs cycle substrate

The effect of various organic chemicals on the growth of M. trichosporium OB3b was studied as a means to improve the cell density by Xing et al. [126]. The addition of vitamins, amino acids and organic acids involved in the Krebs cycle and serine pathway of Type II methanotrophs were anticipated to enhance assimilation of formaldehyde to biomass. Addition of citrate had the most pronounced positive effect, at an optimal concentration of 0.015 mmol/L the cell density was 0.66 g/L (dry weight), more than 3.5 times that of the control, after 4 days cultivation [126]. It is believed the addition of such organic acids alters the metabolic flow of formaldehyde from oxidation to formate, and ultimately carbon dioxide, into the Krebs cycle for cell growth. Although the addition

of citric acid was shown to greatly increase the cell density of the bacterial culture, the effect on methanol synthesis was not investigated.

5. Literature examples to date of the methanotroph facilitated methane oxidation to methanol

Table 1 reviews work published on whole cell bioconversion of methane to methanol utilising wild type methanotrophic bacteria. Sorted by year of publication, the utilised bacterial strains are listed along with reaction conditions and the process yield. Where multiple data for different reaction conditions are published in the same article, the conditions with the highest productivity are listed. If different reaction modes are investigated, these too are listed independently. The volume of methanol produced is recorded as published, in addition to the calculated volume of methanol in mmol/L/h dry cell mass for comparison.

Table 1. Summary of experimental biological conversion on methane to methanol.

Ref.	Bacteria strain	Process mode and reaction vessel	Reaction volume (mL)	Gas Feedstock	Cell density (dry weight basis) (g/L)	Temp. (°C)	pH	Total production period (hours)	MDH inhibition method	Exogenous reducing agent	Quoted amount of methanol produced	Calculated methanol produced (mmol/L/h)
[90]	Methylosinus trichosporium OB3b (NCIB 11131)	Batch	5	CH$_4$:air (1:1 v/v)	3.00×10^{-3}	35	6.5	3	80 mM phosphate	-	2.7 µmol/mg/h	8.1×10^{-3}
[127]	Unidentified isolate 1 (from digester sludge)	Batch	250	100% CH$_4$	0.3	a	a	24	-	-	0.5 g/L	0.65
[127]	Unidentified isolate 2 (from digester sludge)	Batch	250	100% CH$_4$	0.3	a	a	24	-	-	1.0 g/L	1.30
[98]	Methylosinus trichosporium NCIB 11131 (OB3b)	Batch Cells immobilised on DEAE-cellulose	5	CH$_4$:O$_2$ (1:1 v/v)	3.6	35	6.4	12	100 mM phosphate	40 mM sodium formate at 6 h	50 µmol/mg	15
[98]	Methylosinus trichosporium NCIB 11131 (OB3b)	Continuous Stirred membrane reactor with ISPR Cells immobilised on DEAE-cellulose	50	CH$_4$:O$_2$ (1:1 v/v)	2	35	6.4	70	100 mM phosphate	Sodium formate multiple pulsed addition throughout reaction	267 µmol/h	5.34
[99]	Methylosinus trichosporium OB3b	Batch	3.5	CH$_4$:air (1:3 v/v) Positive pressure	0.36×10^{-3} (wet cell wt.)	25	7.0	120	0.234 µmol Cyclopropanol	50 µmol sodium formate	23 mmol/g (wet cell wt.)	6.90×10^{-5}

Ref.	Bacteria strain	Process mode and reaction vessel	Reaction volume (mL)	Gas Feedstock	Cell density (dry weight basis) (g/L)	Temp. (°C)	pH	Total production period (hours)	MDH inhibition method	Exogenous reducing agent	Quoted amount of methanol produced	Calculated methanol produced (mmol/L/h)
[85]	Methylosinus trichosporium OB3	Batch	3.5	CH$_4$:air (1:4 v/v) Positive pressure	1.39	30	7.0	~3	251 µM cyclopropanol	14.3 mM sodium formate	3 mmol/g dry cell	1.39
[85]	Methylosinus trichosporium OB3b	Batch	3.5	CH$_4$:air (1:4 v/v) Positive pressure	3.46×10^{-2}	25	7.0	100	67 nM cyclopropanol	14.3 mM sodium formate	152 mmol/g dry cell	5.26×10^{-2}
[83]	Methylosinus trichosporium OB3b	Batch	17.5	CH$_4$:air (1:2.6 v/v)	3.46×10^{-2}	30	7.0	7.5	67 nM cyclopropanol	14.3 mM sodium formate	19.6 µmol	0.15
[83]	Methylosinus trichosporium OB3b	Repeated batch 5 cycles of 1.5 h	17.5	CH$_4$:air (1:2.6 v/v)	3.46×10^{-2}	30	7.0	7.5	67 nM cyclopropanol	14.3 mM sodium formate	36.1 µmol	0.28
[35]	Methylosinus trichosporium IMV 3011	Batch	25	CO$_2$:CH$_4$:O$_2$:N$_2$ (40:20:20:20 v/v)	3	32	7.0	24	CO$_2$	Oxidation of a portion of metabolic methanol for NADH regeneration	14 µmol/L	5.83×10^{-4}
[35]	Methylosinus trichosporium IMV 3011	Batch	25	CO$_2$:CH$_4$:O$_2$:N$_2$ (40:20:20:20 v/v)	3	32	7.0	30	CO$_2$	Oxidation of a portion of metabolic methanol for NADH regeneration	18.8 µmol/L	6.26×10^{-4}
[35]	Methylosinus trichosporium IMV 3011	Continuous Stirred membrane reactor with ISPR	40	CO$_2$:CH$_4$:O$_2$:N$_2$ (40:20:20:20 v/v)	3	32	7.0	198	CO$_2$	Oxidation of a portion of metabolic methanol for NADH regeneration	23 µmol 0.13 µmol/h	2.90×10^{-3}

Ref.	Bacteria strain	Process mode and reaction vessel	Reaction volume (mL)	Gas Feedstock	Cell density (dry weight basis) (g/L)	Temp. (°C)	pH	Total production period (hours)	MDH inhibition method	Exogenous reducing agent	Quoted amount of methanol produced	Calculated methanol produced (mmol/L/h)
[92]	Methylosinus trichosporium OB3b	Batch	5	CH_4:air (1:5 v/v)	0.6	25	7.0	36	200 mM NaCl	20 mM sodium formate	7.7 mmol/L	0.21
[121]	Methylosinus trichosporium OB3b	Batch	500	CH_4:O_2 (1:2 v/v)	$\sim 1.5 \times 10^{12}$ dm^3	30	7	72	-	-	47.6 µmol/L	6.61×10^{-4}
[93]	Methylosinus trichosporium OB3b	Batch	100	CH_4:air (1:3 v/v)	0.6	25	7.0	28	100 mM NaCl 1 mM EDTA	20 mM sodium formate	13.2 mM	0.47
[93]	Methylosinus trichosporium OB3b	Repeated batch 3 cycles of 8 h	100	CH_4:air (1:3 v/v)	0.6	25	7.0	24	100 mM NaCl 1 mM EDTA	20 mM sodium formate	2.17 µmol/h/mg dry cell wt.	1.30×10^{-6}
[93]	Methylosinus trichosporium OB3b	Continuous	1,000	CH_4:air (1:1 v/v)	0.6	25	7.0	24	100 mM NaCl 1 mM EDTA	20 mM sodium formate	13.7 mM	0.57
[79]	Methylosinus trichosporium OB3b	Batch Sealed flask	10	CH_4:O_2 (1:1 v/v)	17.3	30	6.3	40	400 mM phosphate	20 mmol/L sodium formate	1.12 g/L	0.87
[79]	Methylosinus trichosporium OB3b	Continuous Bubble free membrane reactor	300	CH_4:O_2 (1:1 v/v)	17.3	30	6.3	40	400 mM phosphate	20 mmol/L sodium formate	0.95 g/L	0.74
[91]	Mixed methanotroph consortium from landfill soil	Batch	100	Artificial biogas:air (4:6 v/v)	4×10^{-3}	30	a	a	100 mM NaCl	-	1.49 g/g (g CH3OH per g CH4)	

Ref.	Bacteria strain	Process mode and reaction vessel	Reaction volume (mL)	Gas Feedstock	Cell density (dry weight basis) (g/L)	Temp. (°C)	pH	Total production period (hours)	MDH inhibition method	Exogenous reducing agent	Quoted amount of methanol produced	Calculated methanol produced (mmol/L/h)
[91]	Mixed methanotroph consortium from landfill soil	Batch	5,000 reactor, reaction volume not stated	CH₄:air (1:9 v/v)	a	a	a	24	100 mM NaCl	-	9.0 µmol/h mg cell	
[80]	Methylosinus trichosporium OB3b	Batch	50	CH₄:air (1:1 v/v) Vgas/Vliq = 9	70 u/mL ^{b}	30	7.0	24	12.9 mM phosphate 100 mM NaCl 1.0 mM EDTA	20 mM sodium formate	290 mg/L	0.38
[96]	Methylosinus trichosporium OB3b	Fed-batch Membrane bio-reactor (MBR)	150	CH₄:air (1:1 v/v)	11 u/mL ^{b}	25	7.0	48	12.9 mM phosphate 100 mM NaCl 1.0 mM EDTA	20 mM sodium formate	18.8 mg	8.15×10^{-2}

a Complete reaction conditions not given; b Catalytic concentration defined as "the mass of bacteria required to form 1 µg of methanol in the reaction media within 1h in a 50 mL-batch reactor incubated at 30 °C under stirring at 160 rpm and with a volume ratio Vgas/Vliq of 9".

6. Conclusion

Methane is an abundant natural resource used in the generation of heat and electrical power. Most notably, methane is extracted from geological fossil deposits. A significant proportion of these are identified as stranded methane deposits. GTL technologies are being developed as a means to exploit these remote, often diffuse sources. An additional and often undervalued resource is methane produced during anaerobic digestion that results in large volumes released as a waste product from numerous industrial processes. Consequently atmospheric methane concentrations are at unprecedented levels that, as a potent greenhouse gas, are a major cause for concern. This has led to the instigation of various methane abatement schemes.

GTL technologies offer the opportunity to convert methane into a liquid hydrocarbon fuel that is more readily handled and transported than the gas precursor. Of the range of possible products, methanol is considered an attractive option with the potential for a "methanol economy" to fulfil both the energy and hydrocarbon feedstock demands currently satisfied by fossil fuels. The commercial production of methanol from methane is an energy intensive two-step process.

A direct, single-step oxidation would be an attractive option with the potential for energy and cost savings. Methanotrophic bacteria utilise powerful MMO enzymes to perform the desired reaction with a high level of selectivity under mild conditions. It is proposed that the methanotroph catalysed oxidation of methane to methanol is a potential GTL technology. The advantages of such a process are the low energy and cost requirements; suitability for small scale, modular processes thus allowing use of diffuse and remote gas sources; and the contribution towards methane abatement.

Figure 6 summarises the five main challenges faced in developing an industrially relevant biocatalytic methane oxidation process, and the potential strategies to overcome these.

Challenges:	Potential strategies:
1 Gas-liquid mass transfer limitation	- Reactor design - Addition of 'Methane vectors'
2 Over oxidation of methanol	- MDH inhibitors
3 Biocatalyst methanol toxicity	- ISPR
4 Exogenous energy source	- Exploiting cellular regeneration pathways - Microbial electrosynthesis
5 Toxicity of methane impurities	- Using Methanotroph based microbial consortia

Figure 6. Current challenges and potential strategies for the methanotroph biocatalysed conversion of methane to methanol.

Relatively slow growth and the inability to obtain high cell density cultures is attributed to low methane solubility being a growth limiting factor. This can be addressed through process and reactor

design to allow optimised gas-liquid mass transfer. In terms of methanol synthesis, the first problem arises as methanol is not the final product in the oxidation of methane, but a precursor used by the cell to generate electrons and synthesise various essential metabolites. For this reason, it is necessary to inhibit the MDH enzyme, which results in the accumulation of methanol but also causes depletion of cellular reducing equivalents. It is possible to provide exogenous electrons to the biocatalyst from a number of sources, although partial suppression of MDH, rather than complete inhibition, allows a portion of methanol to be fully oxidised and so exploits the cell's natural regeneration pathways. Additionally, methanol is toxic to most methanotroph strains and so must be removed from the reaction medium before growth and methanol synthesis is affected. Implementation of ISPR methods in the initial process design avoids biocatalyst poisoning whilst also maximising methanol production yields. Although the low specificity of the MMO enzymes is one of the factors that give methanotrophs such potential for applications in biotechnological processes, it presents a complication in biocatalysis of methane. The co-oxidation of contaminants in the feedstock methane gas are further metabolised generating toxic by-products that accumulate within the cell, causing death and loss of biocatalytic activity. It is proposed that use of either natural or synthetic microbial consortia will overcome this, mimicking natural conditions where methanotrophs are able to selectively utilise methane whilst potentially toxic compounds are removed by other bacterial species that utilise higher hydrocarbons. As new strains of methanotrophs are constantly being discovered, isolated and characterised, this also offers potential to identify extremophilic species that may address some of the identified challenges. It is believed that continued progress in these areas will ultimately allow the development of a technically feasible and economically viable methanotroph bioconversion of methane to methanol.

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Conflict of interest

All authors declare that there are no conflicts of interest.

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