Graphical abstract

1 **A methanotroph-based biorefinery: potential scenarios for generating multiple products from**
2 **a single fermentation**

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14

15 **Abstract.** Methane, a carbon source for methanotrophic bacteria, is the principal component of
16 natural gas and is produced during anaerobic digestion of organic matter (biogas). Methanotrophs
17 are a viable source of single cell protein (feed supplement) and can produce various products, since
18 they accumulate osmolytes (e.g. ectoine, sucrose), phospholipids (potential biofuels) and
19 biopolymers (polyhydroxybutyrate, glycogen), among others. Other cell components, such as
20 surface layers, metal chelating proteins (methanobactin), enzymes (methane monooxygenase) or
21 heterologous proteins hold promise as future products. Here, scenarios are presented where ectoine,
22 polyhydroxybutyrate or protein G are synthesised as the primary product, in conjunction with a
23 variety of ancillary products that could enhance process viability. Single or dual-stage processes and
24 volumetric requirements for bioreactors are discussed, in terms of an annual biomass output of 1000
25 tonnes.year⁻¹. Product yields are discussed in relation to methane and oxygen consumption and
26 organic waste generation.

27 **1 Introduction**

28 **1.1 Sustainable methane from organic waste**

29 Methane is the second most abundant greenhouse gas after carbon dioxide. Methane has a global
30 warming potential up to 86 times greater (measured over 20 years) than CO₂, and is responsible for
31 0.48 W.m⁻² of atmospheric radiative forcing (Howarth et al., 2011; IPCC, 2013; Shindell et al.,
32 2009). Anaerobic microbial catabolism of organic substrates generates significant amounts of global
33 methane. Typically, the substrate is converted into a soluble compound (hydrolysis) that is
34 metabolised to generate organic acids (acidogenesis and acetogenesis). Acetic acid is transformed
35 into biogas (methanogenesis) - a gas mixture composed primarily of methane and carbon dioxide
36 (Abbasi et al., 2012). Biogas production and capture is an established and sustainable strategy for
37 treating organic waste. The scrubbed methane is used for generating electricity in gas turbines or
38 steam boilers, piped into homes for domestic heating and cooking, or compressed for use as a
39 vehicle fuel (Nwaoha & Wood, 2014).

40 The rapid expansion of the global market's access to methane, in the forms of natural gas and
41 methane-rich biogas, has improved accessibility and lowered the commodity price. In recent times,
42 there has been growing interest in the biological conversion of methane to fine and commodity
43 chemicals due to economic drivers and the mind shift towards sustainable alternatives for the
44 production of these chemicals. Methane represents an enormous resource and opportunity for
45 conversion into higher value products, especially if it can be sourced from stranded natural gas
46 deposits that are not economical to process for conventional heating, or from sustainable point
47 sources, such as high rate digesters or capped landfills. With natural gas alone, the World Bank
48 estimates 92 Mt.yr⁻¹ is wasted by flaring or venting (WorldBank, 2015). There are large sources of
49 renewable methane that are either already captured, or present the opportunity for capture.

50 Opportunities for methane capture includes landfills (38 Mt.yr⁻¹), wastewater treatment (21 Mt.yr⁻¹)
51 and manure management (11 Mt.yr⁻¹) (Abbasi et al., 2012). The rate and yield of methane
52 production is dependent on the recalcitrance of the organic material, water availability and culture

53 conditions such as pH, temperature and oxygen ingress. Food wastes are readily digestible and can
54 have high yields of methane, while wastes such as hardwoods are recalcitrant and yield relatively
55 low amounts of methane on a carbon basis (see Table 1 for comparative yields of methane).
56 Typically, only large landfills are capable of generating sufficient methane to justify the capital and
57 operational expenditure required for the infrastructure and equipment to capture methane and
58 conventional conversion to electrical energy. It is increasingly popular in developed countries to
59 divert organic waste from landfills to high-rate anaerobic digesters, which may use various urban
60 organic wastes (e.g. food, garden waste), agricultural crop residues (e.g. bagasse, corn stover) or
61 even specifically cultivated biomass. However, the return on capital for any such investment is
62 closely tied to the selling price of the final output of the project. In light of recent lower global
63 energy prices, projects which utilise methane for electricity production or as a vehicle fuel, are
64 generally not economical unless subsidised. Therefore, new technologies are needed to convert
65 methane into higher value products to justify the conversion of smaller scale or stranded methane
66 sources. One such technology is the biological conversion of methane to higher-value products by
67 methanotrophic organisms.

68 **1.2 Phylogeny and Metabolism of Methanotrophic Organisms**

69 Methane serves as a carbon and energy source for a group of aerobic bacteria known as
70 methanotrophs. The first step in aerobic methane metabolism is the oxidation of methane to
71 methanol. The enzyme responsible - methane monooxygenase - occurs in two forms, a soluble
72 (sMMO) enzyme, or membrane-associated particulate (pMMO) enzyme. Methanol is further
73 oxidised to formaldehyde, formate and CO₂, or assimilated as cellular components
74 Methanol is subsequently oxidised to formaldehyde which can be assimilated into cellular
75 metabolites, or further oxidized to formate and CO₂ to provide energy to the cell in the form of
76 reducing equivalents (Kalyuzhnaya et al., 2015). The aerobic methane-oxidisers span different
77 bacterial phyla, including Gammaproteobacteria (Methylococcaceae, commonly referred to as Type
78 I and Type X), Alphaproteobacteria (Methylocystaceae, commonly referred to as Type II,

79 Beijerinckia and unclassified Rhizobiales) and Verrucomicrobia (*Methylacidiphilales*,
 80 “*Methyloacida*”, and “*Methylacidimicrobium*”). Interestingly, members of the division NC10
 81 (Candidatus *Methylophilus* spp), which are generally anaerobic, are able to oxidize methane
 82 aerobically or anaerobically due to the similarity of their MMO enzymes (Ettwig et al., 2010).
 83 The metabolic pathways for methane oxidation, carbon assimilation and product formation have
 84 been reviewed recently (Kalyuzhnaya et al., 2015; Koller et al., 2013), while the metabolic and
 85 physiological comparisons of different microbial groups for industrial strain selections have been
 86 summarised in Kalyuzhnaya (2016). Briefly, gammaproteobacterial methanotrophs are
 87 metabolically versatile, as they may assimilate methane via the ribulose monophosphate (RuMP),
 88 Embden–Meyerhof–Parnas (EMP), Entner-Doudoroff (EDD) and phosphoketolase (PK) pathways,
 89 the partial serine cycle or the Calvin-Benson-Bassham (CBB) cycle. The members of the
 90 Gammaproteobacteria have the highest carbon conversion efficiencies (up to 56%). The
 91 alphaproteobacterial methanotrophs can assimilate C₁-carbon via the serine cycle linked to
 92 ethylmalonyl-CoA pathway (EMCP) or glyoxylate shunt and the CBB cycle. Both,
 93 Verrucomicrobia and *Candidatus Methylophilus* spp assimilate C₁-carbon via the CBB cycle.
 94 Importantly, these pathways all provide a natural route for the formation of carbon-carbon bonds
 95 from a methane substrate, allowing the highly specific conversion of methane to a wide range of
 96 biological products. While these types of reactions are extremely difficult to achieve using
 97 thermochemical means, methanotrophic organisms provide a natural method of achieving efficient
 98 conversion of methane to a variety of products.

99

100 **2 Methanotroph products**

101 There are many potential products that could be generated by the diverse bacteria capable of
 102 methane-oxidation (Strong et al., 2015; Trotsenko et al., 2005). These include single-cell protein,
 103 biopolymers, surface layers, lipids, , methanol, organic acids, ectoine, vitamin B12, sucrose
 104 (Khmelenina et al., 2015), or copper-binding proteins such as methanobactin (Balasubramanian &

105 Rosenzweig, 2008). Genetically engineered methanotrophs can overproduce naturally-occurring
106 metabolites or non-native compounds, including such molecules as carotenoids, isoprene, 1,4
107 butanediol, farnesene, or lactic acid (Henard et al., 2016; Saville et al., 2014). Some of their native
108 enzymes (e.g. glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, malate
109 dehydrogenase, alcohol oxidase and catalase) have high catalytic efficiencies (Trotsenko et al.,
110 2005) and, therefore, are potential products when purified or expressed in commercial production
111 platforms. Methanotrophs may also be used for atmospheric methane mitigation, and the broad
112 substrate range of their methane-oxidising enzymes enables bioremediation of contaminated soils,
113 wastewater treatment, and chemical transformation - such as the conversion of propene to propylene
114 oxide (Strong et al., 2015). The potential products pertinent to this article are briefly discussed in
115 this section.

116

117 **2.1 Single-cell protein.**

118 Single cell protein (SCP) production is an intensively researched field from both an academic and
119 industrial view (Bothe et al., 2002; Helm et al., 2006; Overland et al., 2010). Single cell protein
120 from methane-oxidising bacteria is the most advanced and market-ready product. Products based on
121 SCP, such as FeedKind™ protein (Calysta Inc.: Silverman (2015)), are currently being targeted for
122 a variety of applications in the aqua/mariculture and livestock feed industries. The protein is derived
123 from a mixed culture dominated by *Methylococcus capsulatus* Bath, and is approved in the
124 European Union for use in protein nutritional feeds for salmon and livestock (e.g. pigs, poultry and
125 cattle).

126

127 **2.2 Biopolymers**

128 Biopolymer accumulation within microbes occurs as a result of imbalanced growth, i.e. a nutrient
129 limitation in the presence of sufficient or excess carbon. Polyhydroxyalkanoate biopolymers – such
130 as poly- β -hydroxybutyrate (PHB) - are widely recognised as potential replacements for

131 conventional oil-derived polymers. Many methylotrophs and methanotrophs have been assessed for
132 polyhydroxyalkoanate production and these are tabled in reviews by Khosravi-Darani et al. (2013)
133 and Rittmann (2008). Alphaproteobacteria such as *Methylocystis*, *Methylobacterium* and
134 *Methylosinus* are able to accumulate polyhydroxyalkoanates, with molecular weights of up to
135 3.1 MDa (Helm et al., 2008) and yields of up to 82 Wt% (with citrate as a co-substrate: Zuniga et al.
136 (2013). Biosynthesis in Alphaproteobacteria is interconnected with the core metabolic pathways
137 (the serine cycle and the ethylmalonyl-CoA pathway), which limits application of metabolic
138 engineering for further improvements. Biopolymer yield and quality may be affected by pH,
139 temperature and the available concentrations of methane, oxygen, carbon dioxide, macronutrients
140 (nitrogen, phosphorus, sulfur, potassium, magnesium sodium) and trace metals such as copper, iron,
141 zinc, manganese cobalt (Karthikeyan et al., 2015; Pieja et al., 2011). The structure may be enhanced
142 by the presence of co-substrates such as hydroxy-acid precursors, or the addition of citrate or
143 propionate, to generate poly(3-hydroxybutyrate-co-3-hydroxyvalerate) - PHBV - which is more
144 elastic and tougher than PHB (Strong et al., 2016). Using methane to generate bulk polymers is
145 being investigated by a number of US, Russian and Indian companies because of the potential to
146 lower production costs for PHB compared to other carbon feedstocks (Choi & Lee, 1997; Khosravi-
147 Darani et al., 2013). NewLight Technologies Inc. has developed a commercial process for the
148 production of PHB-based products using a proprietary methanotroph (<http://newlight.com>).

149

150 **2.3 Ectoine**

151 Ectoine is a cyclic imino acid produced intra-cellularly by bacteria to survive in a salt-rich
152 environment. It is an efficient stabiliser of enzymes, nucleic acids, and DNA-protein complexes. It
153 is commercially produced using the halophilic Gammaproteobacterium *Halomonas elongata* DSM
154 2581^T (Schwibbert et al., 2011). It is a valuable product that retails at approximately US\$1000/kg
155 and can be used as a moisturiser in cosmetics. Global consumption is at a scale of <15 000
156 tonnes.year⁻¹. The ectoine biosynthesis pathway in *Methylophilum alcaliphilum* 20Z is similar

157 to that used by other halotolerant heterotrophic bacteria and involves three specific enzymes
158 (diaminobutyric acid aminotransferase, diaminobutyric acid acetyltransferase, and ectoine synthase)
159 regulated by a MarR-like transcriptional regulator (Mustakhimov et al., 2010). Ectoine is released
160 into the surrounding medium when the bacteria are exposed to a lower salt concentration. This
161 allows bacterial cells to be reused multiple times for accumulation and excretion by altering the
162 osmotic potential of the surrounding medium (Sauer & Galinski, 1998). Moderately halotolerant
163 methanotrophs and methylotrophs are able to accumulate up to 12 % of their dry mass as ectoine
164 (Khmelenina et al., 1999; Trotsenko et al., 2005).

165

166 **2.4 Lipids**

167 Most methanotrophs contain abundant intracellular cytoplasmic membranes (ICM) that house
168 pMMO. These ICM are arranged as either stacked discs (Gammaproteobacteria and
169 Verrucomicrobia) or follow the contour of the outer cytomembrane (Alphaproteobacteria). There
170 are two major classes of phospholipids in methanotrophs: phosphatidyl-glycerol and phosphatidyl-
171 ethanolamine (phosphatidyl methyl ethanolamine and phosphatidyl dimethyl ethanolamine). With a
172 few exceptions, the phospholipids in gammaproteobacterial methanotrophs are predominantly
173 C16:1 fatty acids, while those in alphaproteobacterial methanotrophs are predominantly C18:1 fatty
174 acids (Bowman et al., 1991; Fang et al., 2000). Methanotroph biomass contains up to 20 % lipids
175 (Conrado & Gonzalez, 2014). An increase to approximately 35 % lipid content could justify
176 methane to biodiesel conversion (Khmelenina et al., 2015). Several projects under the ARPA-e
177 programme in the US are focussed on improving lipid production in methanotrophs using synthetic
178 biology. The list of targeted products includes short to large chain alkanes, fatty acid ethyl esters
179 (FAEE), fatty acid methyl esters (FAME) and free fatty acids derived from methane (or methanol).
180 Although phospholipids suit diesel production, the high hetero-atom content and presence of sugars
181 or PHBs in the extract negatively impact downstream catalysis (Fei et al., 2014). An alternative
182 higher-value application is to include these lipids as part of a health supplement to lower the ratio of

183 LDL to HDL cholesterol and increase docosahexanoic acid levels (immuno-protectant) in the
184 plasma (Müller et al., 2004; Müller et al., 2005).

185

186 **2.5 Surface layers**

187 Bacterial cell surface layers are regular para-crystalline structures that cover the entire surface of a
188 cell and consist of a single layer of identical proteins or glycoproteins. It has been speculated that
189 the surface layer serves as a protective external barrier in salt-rich environments. Regularly
190 arranged glycoprotein surface layers of hexagonal and linear symmetry have been observed on the
191 outer cell walls of two halo-tolerant *Methylobacter* species (Khmelenina et al., 1999). However,
192 many neutrophilic and non-halophilic methanotrophs form these structures. It is likely that proteins
193 associated with surface layers facilitate copper ion transport to pMMO in methanotrophs, and
194 provide an additional mechanism to maintain copper homeostasis in the cells (Anthony, 2004;
195 Culpepper & Rosenzweig, 2014; Johnson et al., 2014).

196 These glycoproteins are potentially of industrial interest because they intrinsically self-assemble
197 and recrystallise to form porous semi-permeable membranes. These characteristics, and subsequent
198 functionalisation of surfaces, has led to new types of ultrafiltration membranes, affinity structures,
199 enzyme membranes, micro-carriers, biosensors, diagnostic devices, biocompatible surfaces and
200 vaccines, as well as targeting, delivery, and encapsulation systems (Egelseer et al., 2009; Rahalkar,
201 2006; Sleytr & Sara, 1997).

202

203 **2.6 Methanobactin**

204 Copper plays a key role in the physiology of methanotrophs as the particulate methane mono-
205 oxygenase requires copper for catalytic activity (Bowman, 2006). Methanobactins are high affinity
206 copper-binding compounds synthesised by these bacteria to maintain their copper requirement.
207 Functionally, methanobactin represents an extracellular component of a copper acquisition system
208 (DiSpirito et al., 2016). In addition to particulate methane monooxygenase activity, methanobactin

209 may function in copper uptake, regulation of soluble methane monooxygenase expression and
210 protection against copper toxicity (Balasubramanian & Rosenzweig, 2008). They are analogous to
211 iron siderophores (which solubilise and bind Fe(III)), and are classified as chalkophores as they
212 bind copper. The high affinity of methanobactin for Cu(I) cations, as well its ability to bind other
213 metals such as gold, iron, nickel, zinc, cobalt, cadmium, mercury, and uranium (Heyer et al., 2005;
214 Pieja et al., 2011), could be applied in environmental remediation and metal recovery from mine
215 leachates, or even therapeutics (DiSpirito et al., 2016; Follner et al., 1993). The binding of different
216 metals by methanobactin suggests that methanotrophic activity also may play a role in either the
217 solubilisation or immobilisation of many metals *in situ*.

218

219 **2.7 Novel compounds of pharmaceutical potential**

220 Some methanotrophic cultures produce antibacterial proteins termed bacteriocins (Pashkova et al.,
221 1997; Starostina et al., 1998). The antibacterial protein synthesised by *Methylocystis minimus* was
222 an 8 kD protein (Pashkova et al., 1997), while the *Methylobacter bovis* (now *M. luteus*) protein
223 weighed approximately 70 kD (Starostina et al., 1998). Both proteins were relatively thermo-stable.
224 The gene clusters encoding a putative precursor and the peptidase that generates the bacteriocin
225 were recently identified in the *M. luteus* genome (Hamilton et al., 2015). While further research is
226 still required, the discovery of these antibacterial compounds and the encoding genes for their
227 production illustrates the potential to produce novel antibiotics using methanotrophic cultures.

228

229 **2.7 Products, and potential products from genetically modified methanotrophs**

230 Products from genetically modified (GM) methanotrophs include over-expressed endogenous
231 compounds such as various lipids (FAEE, FAME and free fatty acids) and organic acid (lactic acid),
232 or heterologous compounds (e.g. carotenoids, isoprene, 1,4 butanediol and farnesene). Two recent
233 publications suggest that lactic acid may be over-produced (Henard et al., 2016; Saville et al.,
234 2014). Here, two different methanotrophs (*Methylococcus capsulatus* Bath and a *Methylobacterium*

sp.) were engineered to overexpress lactate dehydrogenase, which can convert pyruvate into lactate - a chemical precursor for polymer synthesis. Calysta has partnered with Natureworks with the intention of commercialising polylactic acid from the methane-derived lactate monomer. Methanotrophs have also been engineered to synthesis isoprene (Calysta; SK Innovation/ San Diego State University), 1,4 butanediol and farnesene (Intrexon). While these are significant achievements, it is difficult to consider these for a bioprocess because the yields and production efficiencies are not readily available, making it implausible to calculate substrate and bioreactor requirements. Of the GM methanotroph products, only data for carotenoid synthesis is available (Sharpe, 2008; Ye et al., 2007).

One alternative product is an exogenous surface protein. Exogenous IgG proteins have been successfully expressed by *Caulobacter crescentus* – which, although not a methanotroph, is an Alphaproteobacteria. It produces a single 98-kDa surface protein (RsaA) in a two-dimensional crystalline array (Doronina et al., 2008). The surface layer protein is up to 12 % of the total cell protein, is tolerant of heterologous peptide insertions, and maintains its surface-attached crystalline character (Zúñiga et al., 2011). Theoretically, exogenous therapeutic or IgG proteins can be synthesised on the outer surface of methanotrophic bacteria as a protein G fusion surface layer protein. *Methylococcus*, *Methylothermus*, and *Methylobaculum* cells are potential candidates as they naturally synthesise surface layers (Khmelenina et al., 2013).

253

254 **2.8 pMMO as a potential catalyst**

The first step of methane catalysis uses molecular oxygen to generate methanol, and may be broadly represented by the equation: $\text{CH}_4 + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ = \text{CH}_3\text{OH} + \text{H}_2\text{O}$ (Shiemke et al., 1995). In addition to oxidising methane, sMMO and pMMO have broad substrate ranges (although the range of sMMO is greater) and can transform C_1 - C_8 n-alkanes to 1- and 2-alcohols, terminal alkenes to 1,2-epoxides (e.g. propene to propene oxide) and diethyl ether to ethanol/ethanal (Colby et al., 1977). Although the particulate form has a smaller substrate range, it is synthesised by nearly all

methanotrophs. pMMO is composed of three subunits, α , β and γ , also known as pmoB, pmoA, and pmoC, respectively, arranged in an $\alpha_3\beta_3\gamma_3$ trimer. The physiological reductant for pMMO has not been identified definitively, but may involve quinones from the quinone pool reduced by a type 2 NADH:quinone oxidoreductase or by methanol dehydrogenase (Culpepper & Rosenzweig, 2012). The most likely physiological electron donor to pMMO is ubiquinol, but the source of electrons to reduce the resultant ubiquinone is not yet known (Kalyuzhnaya et al., 2015). Artificial reductants such as duroquinol and NADH are needed to complete the reaction *in vitro* (Shiemke et al., 1995). Regenerating the reducing agent is a limiting factor for enzymatic catalysis. In living cells these would be regenerated in the metabolic oxidation of formaldehyde to CO₂. Duroquinol has been used as a reducing agent for pMMO, with optimal activity at 45 °C and pH 6.5, oxidising externally-added propene to propylene epoxide at 13.6 nmol.min⁻¹.mg⁻¹ protein (Takeguchi et al., 1998). There are also electrochemical and electro-enzymatic methods that could potentially be integrated to the production process to regenerate NADH directly (Addo et al., 2011). Electrochemical NADH regeneration could potentially pave the way for a solely enzymatic process for transforming methane to methanol, or even propene to propylene oxide, that could be conducted at a high catalyst density, higher temperatures and require less aqueous media than live cell-based catalysis. Methanol and propylene oxide are bulk commodities with vast global demand. Propylene oxide is a polyurethane precursor, while methanol is a feedstock chemical that is more fuel compatible (blending with petroleum), energy dense (15.8 MJ/l; methane: 0.038 MJ/l), and easily transportable (Hwang et al., 2014) compared to methane.

281

282 **3 Gas fermentation and media design**

283 For any microbial process, there are significant challenges in maintaining culture stability,
284 minimizing and controlling contamination, and providing an optimal growth environment. While
285 using methane as the carbon source is highly selective, other bacteria are able to survive in a
286 methanotrophic fermentation by consuming metabolic byproducts such as acetic acid. In the case of

SCP, this was fortuitous as it benefited culture stability (Bothe et al., 2002). Selective conditions such as an alkaline pH and a high salt content (e.g. for alkaliphilic halotolerant methanotrophs) can minimise and control contamination. However, media supporting extreme growth conditions requires specialised hardware components. Although methanotrophs such as *Methylobacterium* are capable of growing in high salt concentrations (up to 116 g/l: Kalyuzhnaya et al. (2008)). Salt is problematic as it may pit or corrode metal reactor vessels, packing material (gas diffusers) and internal pump components. Glass lined vessels are available, as are corrosion resistant pumps, and graphite or polyethylene can be used a packing material for reactors, but these increase capital costs upon scale-up. Barometric testing has to occur more frequently to ensure reactor integrity. To maintain a high pH requires greater base addition to neutralise organic acids produced during fermentation. This is problematic both due to expense, and the accumulation of the counter ion in the media for media recycling. Accumulation of excreted metabolites may limit the number of times media could be recycled. Furthermore, the high pH and salinity of the media may represent a challenge for wastewater treatment and environmental discharge.

Additionally, methane-fed aerobic fermentation has unique challenges compared to sugar-based fermentation due to the poor solubility of both methane and oxygen in aqueous solutions. This represents a significant challenge for an efficient large-scale operation. Also, due to the highly reduced nature of methane, a relatively larger amount of oxygen is required relative to sugar-based fermentation. Supply of oxygen represents a challenge regarding gas-liquid mass transfer, and the potential danger when of combining it with a flammable gas – methane has a lower flammable limit of 4.4 % (by volume in air). These challenges have been addressed to some extent by the design of specialised reactors. A variety of reactor types has been developed for conventional aerobic fermentation, ranging from continuous stirred tank reactors (CSTRs) to bubble lift and airlift reactors. CSTRs are common bench-scale reactors, but the capital cost for scale up, energy requirements, and poor gas mass transfer render them unfeasible for large-scale gas fermentation. Airlift and bubble lift reactors are also hampered by poor gas mass transfer properties, where a

313 pressurised headspace is often used to enhance transfer. For the commercial FeedKind™ protein
314 production process, a specialised loop reactor with counter current gas delivery, a large surface area
315 for liquid-headspace gas interface, a specialised static mixer design, and pressurised reactors (up to
316 5 bar) was developed, that can strongly improve gas transfer into the bulk medium. Automated
317 control systems and minimized head space prevent the formation of explosive gas mixtures, thereby
318 assuring safe operation while providing tight control of input gas feeds and nutrients. This reactor
319 design has been validated at large scale (300 m³), demonstrating its suitability for commercial
320 production. However, further improvements are possible and additional research into reactor design
321 is warranted.

322

323 **4 Scenarios for generating multiple products from a single methanotroph**

324 The production of three flagship compounds (ectoine, PHB and protein G), and additional
325 compounds, is presented in the following section. All processes are based on a sequential batch
326 production. For consistency of comparison, 50 % of the reactor broth is replaced with fresh growth
327 media (or recycled media with reconstituted nutrients) for a harvest period based on the strain's
328 doubling time. An additional half an hour per cycle is added for broth and media transfer. It was
329 assumed that the facility would be operate for 24 hours a day, 7 days a week, 340 days annually,
330 and generate 1000 tonnes of biomass (dry mass) per year. Hydraulic volume was assumed to be two
331 thirds of the total reactor volume (i.e. 33 % of the reactor volume was headspace and gas holdup).
332 Methane and oxygen consumption were based on the specific methane consumption (per gram dry
333 weight) of 18.45 mmol.g⁻¹.hr⁻¹ and an oxygen consumption of 23.55 mmol.g⁻¹.h⁻¹, recently
334 reported for *Methylobaculum buryatense* 5GB1 by Gilman et al. (2015), except for the PHB
335 accumulation stage. Lipid content was assumed as 10 % (based on values of up to 10.6 % described
336 in Gilman et al. (2015)). The following applies to individual scenarios, and yield recovery
337 efficiencies are presented in Table 2.

- 338 • *Ectoine – Single stage reactor*. A slightly slower growth rate of 0.20 hour^{-1} was assumed if
 339 growing in a more saline environment (compared to 0.23 hour^{-1} observed by Gilman et al.
 340 (2015)), equating to a doubling time of 3.5 hours and a complete harvest cycle of 4 hours.
- 341 • *Ectoine - Two stage reactor*. The first stage is identical to the single stage reactor, but a second
 342 reactor with a significantly lower NaCl content is used to induce ectoine excretion. Stationary
 343 phase cells were assumed to have half the substrate requirement of growing cells, and a 3.5 hour
 344 retention time allowed for for 9 % (g.g^{-1} dry mass) ectoine excretion in Reactor 2 - based on
 345 15.5 % (g.g^{-1} dry mass) excretion by *Halomonas elongata* (Sauer & Galinski, 1998).
- 346 • *PHB - Two stage reactor*. Biomass is generated in the first stage, and PHB accumulates under a
 347 nutrient deficiency (e.g. phosphorous) in the second stage. The growth rate in the first stage
 348 (0.16 hour^{-1}), the biomass dry weight (50 g.l^{-1}) and PHB content (50 %) is based on data from
 349 Helm et al. (2008). The methane consumption, cell density, final PHB content and accumulation
 350 period for the second stage were based on a 24 hour accumulation period, 51 % PHB content and
 351 product yield of 0.45 g/g CH_4 reported in Helm et al. (2008), while the molar methane to oxygen
 352 ratio (1:1.28) was based on the ratio reported by Gilman et al. (2015).
- 353 • *Protein G synthesis by a GM methanotroph*. In this theoretical scenario, protein G was
 354 synthesised externally as part of a surface layer protein for a methanotroph with a growth rate of
 355 0.37 hour^{-1} (doubling time: 1.87 hours), based on the growth of *Methylococcus capsulatus* (Bath)
 356 reported by Joergensen and Degn (1987), and the ability to harvest at 50 g.l^{-1} (as per PHB
 357 production: Helm et al. (2008)). This scenario was to illustrate the effect of growth rate and cell
 358 harvest density on substrate consumption and reactor volume requirements.

359

360 **4.1 Generating ectoine and other products using a halotolerant *Methylobacterium* or** 361 ***Methylobacter* species**

362 Ectoine production by an alkaliphilic halotolerant methanotroph, with the potential to also harvest
 363 lipids, pMMO, surface layers and methanobactin, is considered in a single and a two-stage process

(Figure 1A). The production of these compounds in *Methylobacterium* or *Methylobacterium* species is verified in literature, except methanobactin – which is assumed. Alkaliphilic cultures have well-developed metabolic models and a range of reliable genetic tools, which makes them an attractive system for biotechnological engineering (Ojala et al., 2010; Kalyuzhnaya et al., 2015; de la Torre et al., 2015).

Ectoine synthesis would require 1328 tonnes of methane and 4352 tonnes of oxygen annually, for a single stage reactor where biomass increased from 12.5 to 25 g/l over 3.5 hour period. An additional 448 tonnes of methane and 567 tonnes of oxygen would be required for the secondary ‘milking’ reactor, but this would provide a purer ectoine concentrate than a cells extract. The single stage process would require a reactor with hydraulic capacity of 39.2 m³ - equating to a total reactor volume of 58.8 m³. The addition of second reactor for harvesting ectoine excreted from the live culture would increase the required hydraulic capacity to 58.8 m³, and total reactor volume to 88.2 m³.

In the single stage process biomass can be concentrated, stored and processed in a batch-wise manner (potentially yielding 102 tonnes ectoine per 1000 tonnes biomass). This is worth considering as ectoine-rich cells may be valuable enough for decentralised production and centralised processing. In the two-stage process ectoine accumulation and excretion are partitioned between reactors with differing osmotic potential, generating 90 tonnes of excreted ectoine annually. Crude ectoine in the aqueous solution would require onsite purification and concentration. This system may be advantageous as biomass can be recycled for multiple bouts of ectoine accumulation and excretion. Using *Halomonas elongata*, ectoine was repeatedly harvested (nine times) when recycling biomass through accumulation and excretion phases, with an average ectoine yield of 15.5 % of the dry mass (Sauer & Galinski, 1998). While adding to gas consumption and the reactor footprint, a reactor for excretion yields a relatively contaminant-free ectoine in comparison to a cell extract. Cell recycling could potentially offset gas usage - as methane could be directed towards product synthesis rather than biomass synthesis. Although SCP is a potential

product, the relatively high residual ectoine content poses an unknown health risk for a feed supplement. Its effect on animal health has not been assessed and it may prove unsuitable. If so, harvesting the ectoine, and individual components such as the external proteins (surface layers: 70 tonnes), internal proteins (methanobactin: 5 tonnes) and lipids (80 tonnes) would be a feasible alternative. Lipid synthesis in methanotrophs does vary according to osmotic potential of the medium. At higher NaCl concentration phosphatidylglycerol content increases, while phosphatidylethanolamine content decreases. *M. alcaliphilus* 20Z grown at pH 9.0 in the presence of NaCl had a higher content of acid phospholipids and unbranched fatty acids than cells grown at pH 7.0 without NaCl (Khmelenina et al., 1997).

The two-stage process presents an opportunity to harvest intact dead cells that house the pMMO and associated cell machinery - with an internal content of ectoine that could stabilise the pMMO. This would represent a major fraction of the biomass (>80 %) and render the process nearly free of organic waste disposal. In contrast, the single-stage scenario could conceivably generate up to half the biomass as organic waste if the protein within the cells could not be harvested.

The specific growth conditions used in this process (a high pH, and high salt content) are highly selective and would ensure a culture relatively free of contaminant bacteria and predatory eukaryotes. Literature supports these culture conditions, where *M. buryatense* 5B grew optimally at pH 9.5 with 7.5 g/l NaCl (Eshinimaev et al., 2002) and *M. alcaliphilus* 20Z cells oxidized and assimilated methane at the highest rate at pH 9.0 in the presence of 41 g/l NaCl (Khmelenina et al., 1997). However, culture at pilot and commercial scale is still an unknown. While high salinity and pH offers some advantages, such as preventing fungal and microbial contamination, these conditions represent a number of technical hurdles (Section 3). However, if these operating conditions are unsustainable, it is likely that halotolerant alkaliphilic methanotrophs can be improved via the adaptive evolution to grow in a less extreme environment. Currently, several novel strains of *M. alcaliphilus* 20Z are capable of growth at a lower pH, low salinity and/or in the presence of high concentrations of organic acids or alcohols (Kalyuzhnaya, unpublished).

416

417 **4.2 Generating PHB and other products using *Methylocystis* or *Methylosinus* species**

418 Here, PHB production by an alphaproteobacterial methanotroph is considered for a two-stage
419 process. Other potential products considered here are lipids, a 7 kD bacteriocin, and potentially an
420 extruded solid of the remaining biomass after PHB extraction and crystallisation. Biomass is
421 generated in the first stage and PHB is accumulated in the second stage (Figure 1B). The first stage
422 would consume 1328 tonnes of methane and 1700 tonnes of oxygen annually. The second stage
423 would require a further 1111 tonnes of methane and 2844 tonnes of oxygen. Total methane
424 consumption and oxygen consumption would approximate 3180 and 8141 tonnes.year⁻¹
425 respectively. This is the highest gas requirement for any of the production scenarios presented here,
426 and illustrates the disadvantage of a secondary reactor where the cells must be maintained for an
427 extended period of time (24 hours). The first stage in this process only requires a hydraulic capacity
428 of 23.5 m³ (total reactor volume of 35.3 m³). However, the hydraulic retention time in second
429 reactor is five times longer than the harvest cycle in the first stage, and increased the required
430 hydraulic volume by 58.8 m³ (total reactor volume: 123.5 m³).

431 At an 80 % recovery efficiency, there is potential to generate 400 tonnes of high molecular weight
432 (3 kDa) PHB. In a techno-economic analysis of PHB generated using methane as the microbial
433 carbon source, Levett et al. (2016) proposed acetone-based PHB extraction of dried biomass
434 (<6 Wt% water), where PHB was solubilised at 90 °C under pressure (3 bar) for two hours.

435 Insoluble biomass components were removed by centrifugation and the PHB dissolved in the
436 acetone was crystallised/precipitated by cooling to 40 °C and mixing with water (2:1). The
437 insoluble biomass components would retain a fraction of acetone containing solubilised PHB.

438 Typically, this would be recovered with solvent washing, but an alternative option would be
439 extruding the remaining insoluble cell material biomass as a pellet, wafer or rod prior to recovering
440 the remaining acetone. The extruded biomass would effectively be considered a heat-sterilised
441 product nearly completely free of water, and could potentially be used as packaging/filler material

442 or, at worst, burnt if the calorific value and ash content were within acceptable ranges. It would
443 consist predominantly of thermally degraded intracellular components and peptidoglycan
444 (crosslinked *N*-acetylglucosamine and *N*-acetylmuramic acid), and could find higher-value
445 applications if surface active groups were identified that allowed easy functionalisation. If the
446 polymer-bonded insoluble biomass and degraded organic compounds could find an application,
447 such as packaging or pellets for combustion, and additional 180 tonnes.year⁻¹ of waste would
448 become a product.

449 The organic solvent is recovered by distillation, and a concentrated lipid solution would remain.
450 The extraction process would not favour subsequent enzyme or protein recovery, as the drying and
451 thermal degradation would affect the tertiary structure. However, a smaller protein such as a 7kD
452 bacteriocin may survive the thermo-solvent extraction process and partition into the aqueous phase
453 after solvent recovery. As with Scenario 2 for ectoine production, this scenario could produce very
454 little organic waste. The possibility of sacrificing a fraction of the soluble PHB in the solvent phase
455 to produce an extruded material will notably lower the organic waste generated in this scenario. The
456 organic waste would primarily be soluble, degraded compounds remaining in the aqueous phase
457 after crystallisation of the polymer and solvent recovery. An added advantage for this process is the
458 lack of sterility required to produce PHB. Literature indicates that significant quantities can be
459 produced in a stable fashion by these methanotrophs in mixed cultures (Strong et al., 2016). The
460 opportunity to operate under non-sterile conditions at an industrial scale would reduce operating
461 costs.

462

463 **4.3 Generating protein G and other products using a genetically modified *Methylococcus*,** 464 ***Methyломicrobium* or *Methylothermus* species**

465 The third flagship product is the theoretical production of an IgG protein on the outer surface of a
466 GM methanotroph as a protein G fusion surface layer protein (Figure 1C). Based on an increase of
467 biomass from 25 to 50 g/l over a 2.4 hour period, the methanotrophs would consume 1039 tonnes of

methane and 2659 tonnes of oxygen. Compared to the single stage production of ectoine, this process scenario would use 660 tonnes less methane, and 1690 tonnes less oxygen per year, illustrating the direct operational savings from a faster growth rate and a higher working concentration. Additionally, a faster growth rate is advantageous to reactor volume requirements. This process had the smallest reactor footprint: needing a hydraulic capacity of only 11.8 m³ and a total reactor volume of 17.6 m³. Assuming the GMO had a typical total protein content of 70 % obtained with *M. capsulatus* (Bath) and that the fusion protein comprised 10 % of total cell protein, a 90 % recovery efficiency would yield 63 tonnes of protein G. If 90 % of the remaining protein could be recovered as SCP, this would yield 560 tonnes SCP annually and significantly lower the waste from the process. While single cell protein from *M. capsulatus* (Bath) has regulatory approval for use as an animal feed, genetic modification would require reassessing the SCP for use as a feed supplement. An alternative to harvesting the SCP would be to harvest the lipids (80 tonnes annually) and methanobactin (5 tonnes annually), or even the entire cell mass for the pMMO - if the fusion protein could be enzymatically cleaved from the cell surface in a cell concentrate.

483

484 **5 Conclusion**

Bacteria that use methane as their carbon source could be used in a biorefinery fashion to generate multiple products during a single fermentation, including SCP, PHB, ectoine and lipids, or potential future products such as pMMO, methanobactin, bacteriocin and surface layers. Optimal production is an interplay between facility costs, yield, production rate, process efficiency and market value. The process scenarios presented illustrate the effects of growth rate and product accumulation on substrate use and reactor volume required. Studies at pilot scale, or that close the C, N and O mass balances, are vital to better determine financial feasibility.

492

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496

497 **6 References**

- 498 1. Abbasi, T., Tauseef, S.M., Abbasi, S.A. 2012. Anaerobic digestion for global warming control
499 and energy generation—An overview. *Renewable and Sustainable Energy Reviews*, **16**(5), 3228-
500 3242.
- 501 2. Addo, P.K., Arechederra, R.L., Waheed, A., Shoemaker, J.D., Sly, W.S., Minteer, S.D. 2011.
502 Methanol Production via Bioelectrocatalytic Reduction of Carbon Dioxide: Role of Carbonic
503 Anhydrase in Improving Electrode Performance. *Electrochemical and Solid-State Letters*, **14**(4),
504 E9-E13.
- 505 3. Anthony, C. 2004. The quinoprotein dehydrogenases for methanol and glucose. *Arch Biochem*
506 *Biophys*, **428**(1), 2-9.
- 507 4. Balasubramanian, R., Rosenzweig, A.C. 2008. Copper methanobactin: a molecule whose time
508 has come. *Current Opinion in Chemical Biology*, **12**(2), 245-249.
- 509 5. Bothe, H., Møller Jensen, K., Mergel, A., Larsen, J., Jørgensen, C., Bothe, H., Jørgensen, L.
510 2002. Heterotrophic bacteria growing in association with *Methylococcus capsulatus* (Bath) in a
511 single cell protein production process. *Applied Microbiology and Biotechnology*, **59**(1), 33-39.
- 512 6. Bowman, J. 2006. The Methanotrophs — The Families Methylococcaceae and
513 Methylocystaceae. in: *The Prokaryotes*, (Eds.) M. Dworkin, S. Falkow, E. Rosenberg, K.-H.
514 Schleifer, E. Stackebrandt, Springer New York, pp. 266-289.
- 515 7. Bowman, J.P., Skerratt, J.H., Nichols, P.D., Sly, L.I. 1991. Phospholipid fatty acid and
516 lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria. *FEMS Microbiology*
517 *Letters*, **85**(1), 15-22.
- 518 8. Choi, J.I., Lee, S.Y. 1997. Process analysis and economic evaluation for poly(3-
519 hydroxybutyrate) production by fermentation. *Bioprocess Engineering*, **17**(6), 335-342.
- 520 9. Colby, J., Stirling, D., Dalton, H. 1977. The soluble methane monooxygenase of *Methylococcus*
521 *capsulatus* (Bath): its ability to oxygenate n-alkanes, n-alkenes, ethers, and acyclic, aromatic and
522 heterocyclic compounds. *Biochemistry Journal*, **165**, 395-402.

- 523 10. Conrado, R.J., Gonzalez, R. 2014. Envisioning the bioconversion of methane to liquid fuels.
524 *Science*, **343**(6171), 621-623.
- 525 11. Culpepper, M.A., Rosenzweig, A.C. 2012. Architecture and active site of particulate methane
526 monooxygenase. *Crit Rev Biochem Mol Biol*, **47**(6), 483-92.
- 527 12. Culpepper, M.A., Rosenzweig, A.C. 2014. Structure and protein-protein interactions of methanol
528 dehydrogenase from *Methylococcus capsulatus* (Bath). *Biochemistry*, **53**(39), 6211-9.
- 529 13. DiSpirito, A.A., Semrau, J.D., Murrell, J.C., Gallagher, W.H., Dennison, C., Vuilleumier, S.
530 2016. Methanobactin and the Link between Copper and Bacterial Methane Oxidation. *Microbiol*
531 *Mol Biol Rev*, **80**(2), 387-409.
- 532 14. Doronina, N.V., Ezhov, V.A., Trotsenko, Y.A. 2008. Growth of *Methylosinus trichosporium*
533 OB3b on methane and poly-b-hydroxybutyrate biosynthesis. *Applied Biochemistry and*
534 *Microbiology*, **44**(2), 182-185.
- 535 15. Egelseer, E.M., Ilk, N., Pum, D., Messner, P., Schäffer, C., Schuster, B., Sleytr, U.B., Flickinger,
536 M.C. 2009. S-Layers, Microbial, Biotechnological Applications. in: *Encyclopedia of Industrial*
537 *Biotechnology*, John Wiley & Sons, Inc.
- 538 16. Eshinimaev, B.T., Khmelenina, V.N., Sakharovskii, V.G., Suzina, N.E., Trotsenko, Y.A. 2002.
539 Physiological, Biochemical, and Cytological Characteristics of a Haloalkalitolerant
540 Methanotroph Grown on Methanol. *Microbiology*, **71**(5), 512-518.
- 541 17. Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M.,
542 Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H.J.C.T., van Alen,
543 T., Luesken, F., Wu, M.L., van de Pas-Schoonen, K.T., Op den Camp, H.J.M., Janssen-Megens,
544 E.M., Francoijs, K.-J., Stunnenberg, H., Weissenbach, J., Jetten, M.S.M., Strous, M. 2010.
545 Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature*, **464**(7288), 543-548.
- 546 18. Fang, J., Barcelona, M.J., Semrau, J.D. 2000. Characterization of methanotrophic bacteria on the
547 basis of intact phospholipid profiles. *FEMS Microbiol Lett*, **189**(1), 67-72.

- 548 19. Fei, Q., Guarnieri, M.T., Tao, L., Laurens, L.M.L., Dowe, N., Pienkos, P.T. 2014. Bioconversion
549 of natural gas to liquid fuel: Opportunities and challenges. *Biotechnology Advances*, **32**(3), 596-
550 614.
- 551 20. Follner, C.G., Babel, W., Valentin, H.E., Steinbuchel, A. 1993. Expression of
552 polyhydroxyalkanoic-acid-biosynthesis genes in methylotrophic bacteria relying on the ribulose
553 monophosphate pathway. *Applied Microbiology and Biotechnology*, **40**(2-3), 284-291.
- 554 21. Gilman, A., Laurens, L.M., Puri, A.W., Chu, F., Pienkos, P.T., Lidstrom, M.E. 2015. Bioreactor
555 performance parameters for an industrially-promising methanotroph *Methylobacterium*
556 *buryatense* 5GB1. *Microb Cell Fact*, **14**(1), 182.
- 557 22. Hamilton, R., Kits, K.D., Ramonovskaya, V.A., Rozova, O.N., Yurimoto, H., Iguchi, H.,
558 Khmelenina, V.N., Sakai, Y., Dunfield, P.F., Klotz, M.G., Knief, C., Op den Camp, H.J.M.,
559 Jetten, M.S.M., Bringel, F., Vuilleumier, S., Svenning, M.M., Shapiro, N., Woyke, T.,
560 Trotsenko, Y.A., Stein, L.Y., Kalyuzhnaya, M.G. 2015. Draft Genomes of
561 Gammaproteobacterial Methanotrophs Isolated from Terrestrial Ecosystems. *Genome*
562 *Announcements*, **3**(3), e00515-15.
- 563 23. Helm, J., Wendlandt, K.D., Jechorek, M., Stottmeister, U. 2008. Potassium deficiency results in
564 accumulation of ultra-high molecular weight poly-beta-hydroxybutyrate in a methane-utilizing
565 mixed culture. *J Appl Microbiol*, **105**(4), 1054-61.
- 566 24. Helm, J., Wendlandt, K.D., Rogge, G., Kappelmeyer, U. 2006. Characterizing a stable methane-
567 utilizing mixed culture used in the synthesis of a high-quality biopolymer in an open system.
568 *Journal of applied microbiology*, **101**(2), 387-395.
- 569 25. Henard, C., Smith, H., Dowe, N., Kalyuzhnaya, M., Pienkos, P., Guarnieri, M. 2016. Methane
570 biocatalysis to lactate by an obligate methanotrophic bacterium. *Scientific Reports*, **In press**.
- 571 26. Heyer, J., Berger, U., Hardt, M., Dunfield, P.F. 2005. *Methylohalobius crimeensis* gen. nov., sp.
572 nov., a moderately halophilic, methanotrophic bacterium isolated from hypersaline lakes of
573 Crimea. *International Journal of Systematic and Evolutionary Microbiology*, **55**(5), 1817-1826.

27. Howarth, R., Santoro, R., Ingraffea, A. 2011. Methane and the greenhouse-gas footprint of natural gas from shale formations. *Climatic Change*, **106**(4), 679-690.
28. Hwang, I.Y., Lee, S.H., Choi, Y.S., Park, S.J., Na, J.G., Chang, I.S., Kim, C., Kim, H.C., Kim, Y.H., Lee, J.W., Lee, E.Y. 2014. Biocatalytic conversion of methane to methanol as a key step for development of methane-based biorefineries. *J Microbiol Biotechnol*, **24**(12), 1597-605.
29. IPCC. 2013. IPCC Fifth Assessment Report (AR4). Climate Change 2013: The Physical Science Basis. in: *Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, (Eds.) T.F. Stocker, D. Qin, Cambridge University Press. Cambridge, United Kingdom and New York, NY, USA, pp. 93-129.
30. Joergensen, L., Degn, H. 1987. Growth rate and methane affinity of a turbidostatic and oxystatic continuous culture of *Methylococcus capsulatus* (Bath). *Biotechnology Letters*, **9**(1), 71-76.
31. Johnson, K.A., Ve, T., Larsen, Ø., Pedersen, R.B., Lillehaug, J.R., Jensen, H.B., Helland, R., Karlsen, O.A. 2014. CorA Is a Copper Repressible Surface-Associated Copper(I)-Binding Protein Produced in *Methylobacterium album* BG8. *PLoS ONE*, **9**(2), e87750.
32. Kalyuzhnaya, M.G. 2016. Chapter 13 - Methane Biocatalysis: Selecting the Right Microbe. Edited by Trinh, Carrie A. Eckert Cong T. in: *Biotechnology for Biofuel Production and Optimization*, Elsevier. Amsterdam, pp. 353-383.
33. Kalyuzhnaya, M.G., Khmelenina, V., Eshinimaev, B., Sorokin, D., Fuse, H., Lidstrom, M., Trotsenko, Y. 2008. Classification of halo(alkali)philic and halo(alkali)tolerant methanotrophs provisionally assigned to the genera *Methylobacterium* and *Methylobacter* and emended description of the genus *Methylobacterium*. *Int J Syst Evol Microbiol*, **58**(Pt 3), 591-6.
34. Kalyuzhnaya, M.G., Puri, A.W., Lidstrom, M.E. 2015. Metabolic engineering in methanotrophic bacteria. *Metab Eng*, **29**, 142-52.
35. Karthikeyan, O., Karthigeyan, C., Cirés, S., Heimann, K. 2015. Review of sustainable methane mitigation and bio-polymer production. *Critical Reviews in Environmental Science and Technology*, **45**(15), 1579-1610.

- 600 36. Khmelenina, V.N., Kalyuzhnaya, M.G., Sakharovsky, V.G., Suzina, N.E., Trotsenko, Y.A.,
601 Gottschalk, G. 1999. Osmoadaptation in halophilic and alkaliphilic methanotrophs. *Archives of*
602 *Microbiology*, **172**(5), 321-329.
- 603 37. Khmelenina, V.N., Kalyuzhnaya, M.G., Trotsenko, Y.A. 1997. Physiological and Biochemical
604 Properties of a Haloalkalitolerant Methanotroph. *Mikrobiologiya*, **66**(3), 447–453.
- 605 38. Khmelenina, V.N., Rozova, N., But, C.Y., Mustakhimov, II, Reshetnikov, A.S., Beschastnyi,
606 A.P., Trotsenko, Y.A. 2015. Biosynthesis of secondary metabolites in methanotrophs:
607 biochemical and genetic aspects (review). *Applied Biochemistry and Microbiology*, **51**(2), 150-
608 158.
- 609 39. Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A. 2013. Surface layers of methanotrophic
610 bacteria. *Microbiology*, **82**(5), 529-541.
- 611 40. Khosravi-Darani, K., Mokhtari, Z.B., Amai, T., Tanaka, K. 2013. Microbial production of
612 poly(hydroxybutyrate) from C-1 carbon sources. *Applied Microbiology and Biotechnology*,
613 **97**(4), 1407-1424.
- 614 41. Koller, M., Salerno, A., Muhr, A., Reiterer, A., Braunegg, G. 2013. Polyhydroxyalkanoates:
615 biodegradable polymers and plastics from renewable resources. *Mater. Tehnol.*, **47**(Copyright
616 (C) 2013 American Chemical Society (ACS). All Rights Reserved.), 5-12.
- 617 42. Levett, Birkett, Davies, Bell, Langford, A., Laycock, B., Lant, P., Pratt, S. 2016. Techno-
618 economic assessment of poly-3-hydroxybutyrate (PHB) production from methane. *Applied*
619 *Biochemistry and Biotechnology.*, **in submission**.
- 620 43. Müller, H., Hellgren, L., Olsen, E., Skrede, A. 2004. Lipids rich in phosphatidylethanolamine
621 from natural gas-utilizing bacteria reduce plasma cholesterol and classes of phospholipids: A
622 comparison with soybean oil. *Lipids*, **39**(9), 833-841.
- 623 44. Müller, H., Skrede, A., Kleppe, G. 2005. Lipids from methanotrophic bacteria for cholesterol
624 reduction, Patent: WO 2005004888 A1.

- 625 45. Mustakhimov, I.I., Reshetnikov, A.S., Glukhov, A.S., Khmelenina, V.N., Kalyuzhnaya, M.G.,
626 Trotsenko, Y.A. 2010. Identification and Characterization of EctR1, a New Transcriptional
627 Regulator of the Ectoine Biosynthesis Genes in the Halotolerant Methanotroph
628 *Methylobacterium alcaliphilum* 20Z. *Journal of Bacteriology*, **192**(2), 410-417.
- 629 46. Nwaoha, C., Wood, D.A. 2014. A review of the utilization and monetization of Nigeria's natural
630 gas resources: Current realities. *Journal of Natural Gas Science and Engineering*, **18**(0), 412-
631 432.
- 632 47. Overland, M., Tauson, A.H., Shearer, K., Skrede, A. 2010. Evaluation of methane-utilising
633 bacteria products as feed ingredients for monogastric animals. *Archives of Animal Nutrition*,
634 **64**(3), 171-189.
- 635 48. Pashkova, N.I., Starostina, N.G., Tsiomenko, A.B. 1997. A secretory protein involved in the
636 antagonistic interactions between methanotrophic bacteria. *Biochemistry (Mosc)*, **62**(4), 386-90.
- 637 49. Pieja, A.J., Rostkowski, K.H., Criddle, C.S. 2011. Distribution and selection of poly-3-
638 hydroxybutyrate production capacity in methanotrophic proteobacteria. *Microbial Ecology*,
639 **62**(3), 564-573.
- 640 50. Rahalkar, M. 2006. Aerobic methanotrophic bacterial communities in sediments of Lake
641 Constance. in: *Fachbereich Biologie*, Vol. PhD, Universitat Konstanz. Germany.
- 642 51. Rittmann, B.E. 2008. Opportunities for renewable bioenergy using microorganisms.
643 *Biotechnology and Bioengineering*, **100**(2), 203-12.
- 644 52. Sauer, T., Galinski, E.A. 1998. Bacterial milking: A novel bioprocess for production of
645 compatible solutes. *Biotechnol Bioeng*, **57**(3), 306-13.
- 646 53. Saville, R.M., Lee, S., Regitsky, D.D., Resnick, S.M., Silverman, J. 2014. Compositions and
647 methods for biological production of lactate from c1 compounds using lactate dehydrogenase
648 transformants, Patent.
- 649 54. Schwibbert, K., Marin-Sanguino, A., Bagyan, I., Heidrich, G., Lentzen, G., Seitz, H., Rampp,
650 M., Schuster, S.C., Klenk, H.P., Pfeiffer, F., Oesterhelt, D., Kunte, H.J. 2011. A blueprint of

651 ectoine metabolism from the genome of the industrial producer *Halomonas elongata* DSM 2581
652 T. *Environ Microbiol*, **13**(8), 1973-94.

653 55. Sharpe, P.L. 2008. Metabolic Engineering of a Methanotroph for the Production of C40
654 Carotenoids for Aquaculture Applications. *The FASEB Journal*, **22**, 413.2.

655 56. Shiemke, A.K., Cook, S.A., Miley, T., Singleton, P. 1995. Detergent solubilization of
656 membrane-bound methane monooxygenase requires plastoquinol analogs as electron donors.
657 *Arch Biochem Biophys*, **321**(2), 421-8.

658 57. Shindell, D.T., Faluvegi, G., Koch, D.M., Schmidt, G.A., Unger, N., Bauer, S.E. 2009. Improved
659 Attribution of Climate Forcing to Emissions. *Science*, **326**(5953), 716-718.

660 58. Silverman, J. 2015. Single Cell Protein: A sustainable approach to meeting the growing protein
661 demand. *Aquafeed*, **7**(2), 10-14.

662 59. Sleytr, U.B., Sara, M. 1997. Bacterial and archaeal S-layer proteins: structure-function
663 relationships and their biotechnological applications. *Trends Biotechnol*, **15**(1), 20-6.

664 60. Starostina, N.G., Pashkova, N.I., Tsiomenko, A.B. 1998. Detection and partial characterization
665 of bacteriocin in the methanotrophic bacterium *Methylobacter bovis*. *Biochemistry (Mosc)*,
666 **63**(10), 1122-5.

667 61. Strong, P., Xie, S., Clarke, W.P. 2015. Methane as a resource: can the methanotrophs add value?
668 *Environmental Science & Technology*, **49**(7), 4001-4018.

669 62. Strong, P.J., Laycock, B., Mahamud, S.N.S., Jensen, P.D., Lant, P.A., Tyson, G., Pratt, S. 2016.
670 The opportunity for high performance biomaterials from methane. *Microorganisms*, **Online**.

671 63. Takeguchi, M., Miyakawa, K., Okura, I. 1998. Properties of the membranes containing the
672 particulate methane monooxygenase from *Methylosinus trichosporium* OB3b. *Biometals*, **11**(3),
673 229-34.

674 64. Trotsenko, I.A., Doronina, N.V., Khmelenina, V.N. 2005. Biotechnological potential of
675 methylotrophic bacteria: a review of current status and future prospects. *Prikl Biokhim*
676 *Mikrobiol*, **41**(5), 495-503.

- 677 65. WorldBank. 2015. Zero Routine Flaring by 2030. [http://www.worldbank.org/en/programs/zero-](http://www.worldbank.org/en/programs/zero-routine-flaring-by-2030)
678 *routine-flaring-by-2030*.
- 679 66. Ye, R.W., Yao, H., Stead, K., Wang, T., Tao, L., Cheng, Q., Sharpe, P.L., Suh, W., Nagel, E.,
680 Arcilla, D., Dragotta, D., Miller, E.S. 2007. Construction of the astaxanthin biosynthetic pathway
681 in a methanotrophic bacterium *Methylomonas* sp. strain 16a. *J Ind Microbiol Biotechnol*, **34**(4),
682 289-99.
- 683 67. Zúñiga, C., Morales, M., Le Borgne, S., Revah, S. 2011. Production of poly- β -hydroxybutyrate
684 (PHB) by *Methylobacterium organophilum* isolated from a methanotrophic consortium in a two-
685 phase partition bioreactor. *Journal of Hazardous Materials*, **190**(1–3), 876-882.
- 686 68. Zuniga, C., Morales, M., Revah, S. 2013. Polyhydroxyalkanoates accumulation by
687 *Methylobacterium organophilum* CZ-2 during methane degradation using citrate or propionate as
688 cosubstrates. *Bioresource technology*, **130**, 686.

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Figure 1: Process flow diagram for a biorefinery primarily based on the production of **A)** ectoine, where 1: single stage and 2: two stage, **B)** PHB and **C)** Protein G from methanotrophic bacteria.

1 **Table 1:** Methane yields from various organic wastes.

Waste type	Waste	Methane Yield (Nm ³ .t ⁻¹ VS) unless otherwise stated	Reference
Municipal solid waste	• Sub-8cm fraction shredded MSW	92 ± 2 Nm ³ . 'as received' t ⁻¹ 211 ± 38	Clarke et al. (2016)
	• Sub-10cm of organic fraction of MSW	200	Chynoweth et al. (1992)
	• Source separated organic waste	140 Nm ³ .dry t ⁻¹	De Baere (2000)
Waste water sludges	• Primary sludge	110-190 Nm ³ .t ⁻¹ TCOD	Elbeshbishy et al. (2012)
	• Activated sludge	136 ± 7	Bougrier et al. (2006)
	• Activated sludge with 170°C thermal pretreatment	242 ± 9	
Food waste	• Reject bananas	398 ± 20	Clarke et al. (2008)
	• Vegetable waste	412	Rajeshwari et al. (2001)
Green waste	• Leaves (Laurel oak)	129	Owens and Chynoweth (1993)
	• Grass (Floritam St. Augustine)	183	

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3 TCOD: total chemical oxygen demand and VS: volatile solids.

4 **Table 2:** Potential product yields, gas consumption and reactor volume requirements for processing scenarios based primarily on ectoine, PHB or protein G
5 synthesis producing 1000 tonnes of methanotroph biomass annually.

	Ectoine (1-Stage)	Ectoine (2-stage)		PHB (2-stage)		Protein G (1-Stage)
Culture parameters		Reactor 1	Reactor 2	Reactor 1	Reactor 2	
Initial dry mass (g .l ⁻¹)	12.5	12.5	50	25	50	25
Harvest dry mass (g .l ⁻¹)	25	25	44	50	50	50
Dilution (hour ⁻¹)	0.2	0.2	-	0.16	-	0.37
Doubling time (hours)	3.47	3.47	-	4.33	-	1.87
Drain and fill time (hours)	0.5	0.5	-	0.5	-	0.5
Time for full cycle (hours)	4.0	4.0	4.0	4.8	24	2.4
Cycles/harvests (day ⁻¹)	6	6	6	5.0	1	10
Biomass generated daily per litre bioreactor (g.day ⁻¹)	75	75	-	125	-	250
Operational days per year	340	340	340	340	340	340
Biomass generated annually per litre (tonnes.year ⁻¹)	0.0255	0.0255	-	0.0425	-	0.085
Reactor volume for 1000 tonnes.year⁻¹ (total reactor volume)						
Daily required hydraulic volume (m ³)	39.2	39.2	19.6 (58.8)	23.5	58.8 (82.4)	11.8
Total reactor volume (m ³)	58.8	58.8	29.4 (88.2)	35.3	88.2 (123.5)	17.6
Annual yield (per 1000 tonnes)						
Primary product	Ectoine 102 tonnes (12% CDW at 85% recovery)	Ectoine 90 tonnes (12% CDW at 70% recovery)		PHB 400 tonnes (50% CDW at 80% recovery)		Protein G 63 tonnes (10% proteins at 90% recovery)
Lipids	80 tonnes (10% CDW at 80% recovery)			80 tonnes (10% CDW at 80% recovery)		80 tonnes (10% CDW at 80% recovery)
Single cell protein	400 tonnes (50% CDW at 90% recovery)					560 tonnes (70% CDW at 90% recovery)
Surface layers	75 tonnes (10% CDW at 75% recovery)					
Methanobactin	5 tonnes					5 tonnes

	(1% CDW at 50% recovery)					(1% CDW at 50% recovery)
pMMO-containing biomass		817 tonnes (86% CDW recovery of remainder)				
Bacteriocin				5 tonnes (1% at 50% recovery)		
Extruded cell material				250 tonnes (100% recovery)		
Gas consumption* (total gas use)						
g CH ₄ per gram biomass	1.70	1.70	1.03	2.07	1.11	1.04
CH ₄ (tonnes.year ⁻¹)	1700	1700	1033 (2733)	2069	1111 (3180)	1039
O ₂ (tonnes.year ⁻¹)	4352	4352	2267 (6996)	5296	2844 (8141)	2659

6 *Based on a specific methane uptake 18.45 mmol gCDW⁻¹ h⁻¹ and 1.28:1 moles O₂:CH₄ (except for PHB R2).
7 CDW: cell dry weight