engineering proceedings

MDPI

Conference proceedings

Analysis of Methanotrophs Population from Various Sources for Production of High-Value Products

Lisa Stephanie H. Dizon[1,2], Robert S. Bertrand[1,2], William E. Holmes[1], Rafael A. Hernandez[1,2], Dhan Lord A. Fortela[1,2], Andre Chistoserdov[1,4], Mark E. Zappi[1,2] and Emmanuel D. Revellame[1,2,3,*]

[1] The Energy Institute of Louisiana, University of Louisiana at Lafayette, Lafayette LA 70504, U.S.A
[2] Department of Chemical Engineering, University of Louisiana at Lafayette, Lafayette LA 70504, U.S.A
[3] Department of Engineering Technology, University of Louisiana at Lafayette, Lafayette LA 70504, U.S.A
* Correspondence: erevellame@louisiana.edu

Abstract: Methanotrophs are bacteria that can consume methane as their sole carbon and energy source to produce a wide variety of high-value products such as lipids, biopolymers, ectoine, and single cell proteins (SCPs). Collected samples from various sources were subjected to DNA extraction followed by 16S rRNA analysis to determine the identity and relative abundance of their microbial population. Several species of methanotrophs were detected in the consortia including Type I (*Methylobacter*), Type X (*Methylocaldum*), Type II (*Methylocystis*, *Methylosinus, and Beijerinckia*), and Type III (*Verrucomicrobium*). This paper expounds the effects of environmental/cultivation conditions on the growth and population of different types of methanotrophs. The results could be used to systematically identify source(s) of natural consortium that can be enriched and developed to produce specific target product(s) under a given cultivation conditions/limitations.

Keywords: methane bioconversion, activated sludge; phospholipids; PHB; ectoine

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Appl. Sci.* **2022**, *12*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

In recent years, the increase in greenhouse gas (GHG) emissions, causing global warming, has been a pressing issue due to its evident harmful environmental effects. Methane (CH_4) is considered as the second most prominent GHG produced next to carbon dioxide (CO_2) and has a global warming potential of 27-30 over 100 years [1]. Methane is the key component of natural gas which is typically used for power, fuel, and heat. The advancement in shale gas production resulted in the instability of natural gas prices in the past decade [e.g., $8.86 per million British thermal unit (MMBtu) in 2008, $2.05/MMBtu in 2020, and $7.88/MMBtu in September 2022 [2]]. As a result, CH_4 is vented and flared into the atmosphere mainly due to unprofitability, operational safety, and costly connection to the pipeline [3]. In 2021 alone, the US flared about 8,763.83 million cubic meter (MCM) of natural gas with 23.37 million metric ton carbon dioxide equivalent (MMT CO_2e) emission and with an equivalent economic value of 1.01 billion dollars [4], causing significant negative environmental impacts and lost revenues. The growing concerns toward climate change mitigation led to the continuous quest for economically viable technologies to reduce these GHG. Hence, there is an opportunity to develop processes to economically convert CH_4 to high-value products. One such process is the utilization of CH_4 as substrate for microbial bioconversion instead of expensive sugar-based feedstocks [5-7].

Methanotrophs are gaining interests because of their ability to utilize CH_4 as their sole carbon and energy source [8]. They play a vital role in carbon cycling since they can convert CH_4 into a wide variety of valuable bioproducts such as lipids, biopolymers, ectoine, and single cell proteins (SCPs) [9-12]. These lipids can be used to produce renewable diesel/green energy or as feedstock for oleochemical manufacturing. Biopolymers such as

polyhydroxyalkanoates (PHAs) are biodegradable, non-toxic, and thermoplastic molecules which can be applied in various energy and environmental applications as well as a potential replacement of conventional plastics [12-14]. On the other hand, ectoine is broadly employed in cosmetics industry, dermatology, and it is also an effective stabilizer for nucleic acids, enzymes, and DNA-protein complexes applied in pharmaceutical industries [12, 14-16] while SCPs can be used as an alternative protein source that has the advantage of being independent of agricultural products (e.g. soybean) as a staring material [7, 14].

Methanotrophs are gram-negative *Proteobacteria* that are ubiquitous in nature commonly found in soil, natural gas fields, wetlands, sewage sludges, and waste treatment facilities [9, 16-18]. They are classified by 16S rRNA gene sequence into taxonomic groups based on their cell morphology, ultra-structure, phylogeny, and metabolic pathways [8, 11, 16] as shown in Figure 1.

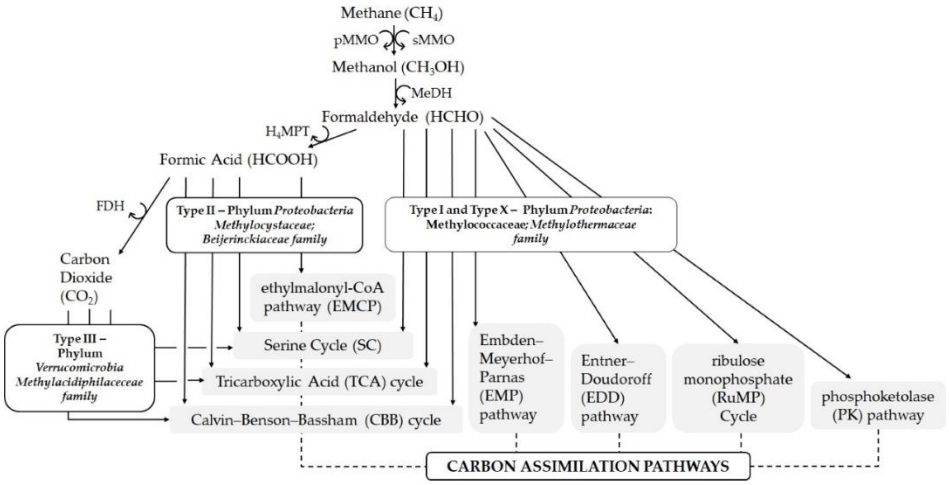

Figure 1. Different types of methanotrophs and their metabolic pathways: pMMO - particulate monooxygenase, sMMO - soluble monooxygenase

Distinct types of methanotrophs react differently to changing environmental conditions. The knowledge on the behavior of methanotrophs under different conditions is critical in choosing the suitable type of methanotrophs for culture enrichment and cultivation optimization tailored for producing a certain bio-product. In this work, natural microbial consortia present in samples collected from various sources (particularly low and high O_2 levels) were tested for the presence of methanotrophs. The results could be used to identify possible source of seed that contains certain type(s) of methanotrophs for further studies.

2. Materials and Methods

Samples were collected from various sites including sediments from three open drainage ditches and sludges from different wastewater treatment plants (WWTPs) located in Lafayette, LA, USA – South WWTP (activated sludge and aerobic digester), East WWTP (activated sludge) and Ambassador Caffery WWTP (activated sludge). The samples were immediately subjected to DNA extraction using DNeasy® Powersoil® Pro Kit (Qiagen, Germany) and the extracts were subjected to full length 16S rRNA gene diversity analysis using bTEFAP® technology (Mr. DNA Molecular Research LP, Shallowater, TX). Sequencing was performed on a MiSeq following the manufacturer's protocols and sequence data were processed using ribosomal and functional gene analysis pipeline. The final zero-radius operational taxonomic units (zOTUs) were classified using BLASTn

against a curated database derived from National Center for Biotechnology Information (NCBI).

3. Results and Discussion

In all the samples, low but detectable levels of methanotrophs were identified including Type I (*Methylobacter*), Type X (*Methylocaldum*), Type II (*Methylocystis*, *Methylosinus*, and *Beijerinckia*), and Type III (*Verrucomicrobium*) (see Table 1).

Intensive studies involving methanotrophs revealed that changing parameters such as CH_4 and O_2 concentrations, nitrogen sources, copper content, pH, and temperature promote the growth and enhance the population of particular types of methanotrophs. In general, Type I methanotrophs prefer low CH_4 and high O_2 concentrations while Type II methanotrophs favor high CH_4 and low O_2 concentrations [7, 19]. This is evident from the results in Table 1, showing that Type I methanotrophs were not detected in samples collected from drainage sediments (DS1, DS2, and DS3). These samples were collected in low O_2 environments (i.e., under <1 foot of stagnant muddy water), and thus, favored Type II methanotrophs. In contrast, samples collected from WWTPs (EWAS, SWAS, SWDS, and AWAS) contain Types I, X and II. These samples were collected from aerobic treatment units (i.e., high O_2 environments) that favors Type I. Nevertheless, localized low O_2 regions within these treatment units might have allowed the proliferations of Type II.

Among the parameters that affect methanotroph growth, only the CH_4 and O_2 levels do not require chemical analyses of the growth environment. In particular, the level of O_2 can be easily speculated as illustrated above. In this work, only the level of O_2 was used as parameter for choosing the source of consortia. Nevertheless, whenever chemical assays are feasible, the following can be used in deciding the source of seed consortium. In terms of nitrogen, Type I methanotrophs preferred an environment with high nitrogen content or lower carbon to nitrogen (C/N) ratio while Type II methanotrophs are more common in N-limited (or high C/N ratio) conditions [20]. Type II methanotrophs and some strains of *Methylobacter* (Type I) have the ability to fix atmospheric N_2 because they possess the nitrogenase enzyme. Moreover, studies revealed that methanotrophs grow better on inorganic nitrogen sources (nitrate or ammonia) than atmospheric N_2 [7, 20]. Copper content, on the other hand, greatly influences the growth of methanotrophs that have the particulate monooxygenase (pMMO) since copper regulates the expression of this enzyme [7, 20].

Methanotrophs are not known to produce neutral lipids (e.g., triglycerides, waxes). Membrane lipids in the form of phospholipids are the only class of lipids typically found in these microbes. As such, the amount of phospholipids that can be obtained from methanotrophs is directly proportional to the biomass produced during cultivation. However, the type of phospholipids is dependent on the type of methanotrophs. For example, phosphatidyl dimethyl ethanolamine and phosphatidyl methyl ethanolamine are found in Type I (*Methylobacter*), Type II (*Methylocystis* and *Methylosinus*), and Type X (*Methylocaldum*) methanotrophs [12]. Thus, if these types of phospholipid are the target products, the consortium in EWAS is most suitable seed for cultivation. The most studied species for ectoine production is *Methylomicrobium alcaliphilum* 20Z (Type I), but it can also be synthesized by *Methylosinus sporium* (Type II) and *Methylobacter marinus* 7C (Type I) [14]. Any of the samples collected can be used as seed for ectoine production since *Methylosinus sporium* was detected in all of them. However, the most suitable might be DS1 as it contains the highest concentration of this species (Table 1). Favorable characteristics of methanotrophs that can produce SCP should have a rapid growth rate, easy to cultivate, and with high protein production capacity [16]. *Methylocystis* sp. (Type II) is one of the methanotrophs species that had been used for SCP production [14] at broad pH and temperature ranges. Although any of the samples can be used as seed consortium for SCP

production, DS1 is the best choice if abundance is required. Otherwise, if abundance and species diversity is sought, SWDS should be chosen.

Poly(3-hydroxybutyrate) or PHB, which is a member of the PHA family, is another potential high-value product from methanotrophs. PHBs are accumulated in all Type II methanotrophs as a survival mechanism under nutrients starvation [16, 20]. The results (Table 1) suggest that any of the samples could be used as seed for PHB production. The final choice comes down to whether abundance or diversity or both is required by the cultivation. For PHB or for any of the target products, the ultimate choice for which seed to use will also depend on the cultivation conditions. Generally, the growth conditions that lead to PHB accumulation include: (i) low N level (ammonia or nitrate), (ii) copper deficiency and (iii) fed-batch cultivation [16]. Additionally, AlSayed, et al. [20] reported that most PHB accumulation studies were conducted at temperature from 20 to 40°C and pH of 6 - 7. Some report suggests that increasing the medium acidity also increased PHB accumulation in Type II methanotrophs [20]. PHB accumulation in *Methylocystis* sp. GB25 DSM 7674 was successfully enhanced under N-limited condition during fed-batch cultivation [16]. In any case, the cultivation temperature and pH should be considered noting that some species are more tolerant to drastic conditions than others (Table 2) and might necessitate species diversity over abundance. Additionally, the composition of feed gas should also be considered. As indicated in Figure 1, Types II and III methanotrophs can simultaneously consume CO_2 and CH_4 and should preferably be used for cultivation involving biogases (a mixture mainly composed of CH_4 and CO_2).

4. Conclusions

In this work, samples from several locations, including sediments from three open drainage ditches and sludges from different WWTPs, were collected to identify and quantitate different types of methanotrophs. Based on the 16S rRNA analysis, the samples from each location were composed of diverse types of methanotrophs including Type I (*Methylobacter*), Type X (*Methylocaldum*), Type II (*Methylocystis*, *Methylosinus*, and *Beijerinckia*), and Type III (*Verrucomicrobium*). Although different parameters could affect growth and proliferation of methanotrophs, this work focused mainly on O_2 levels. As anticipated, samples collected from locations with low O_2 levels (i.e., drainage ditches) contained non-detectable levels of Type I methanotrophs. The results of this work emphasized the importance of environmental condition on the choice of the natural source of methanotrophic consortium. It should be noted, however, that other parameters might still need to be considered, along with target product(s) and cultivation conditions, to identify the most suitable natural consortium source for further studies.

Author Contributions: Conceptualization, E.D.R. and L.S.H.D.; methodology, E.D.R. and L.S.H.D.; software, E.D.R. and L.S.H.D. ; validation, E.D.R. and L.S.H.D.; formal analysis, E.D.R. and L.S.H.D.; investigation, E.D.R., L.S.H.D. and R.S.B.; resources, E.D.R. and W.E.H.; analytics, W.E.H..; writing—original draft preparation, E.D.R. and L.S.H.D.; writing—review and editing, E.D.R., R.A.H., A.C., D.L.A.F., M.E.Z., and L.S.H.D.; visualization, E.D.R. and L.S.H.D.; supervision, E.D.R., R.A.H., A.C., D.L.A.F., M.E.Z.; project administration, E.D.R.; funding acquisition, E.D.R.. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by NASA EPSCoR (Grant No. 80NSSC18M0062) and Louisiana Board of Regents (ITRS) (Grant No. LEQSF(2019-22)-RD-B-06).

Data Availability Statement: Not applicable.

Acknowledgments: This research was conducted with the support of the staff of the Energy Institute of Louisiana (EIL) at the University of Louisiana at Lafayette. The authors also acknowledge the financial supports from NASA EPSCoR and Louisiana Board of Regents (ITRS).

Table 1. Methanotrophs composition from various sources.

Type	Species	Abundance						
		DS1[a]	DS2[b]	DS3[b]	EWAS[c]	SWAS[d]	SWDS[e]	AWAS[f]
Type I	*Methylobacter* spp.	-	-	-	0.0042	0.0215	0.0231	0.0082
TypeX	*Methylocaldum* spp.	0.0086	0.0832	0.2370	0.0084	-	-	0.0123
γ-Proteobacteria	*Methylocaldum* sp.	-	-	-	0.1256	0.0086	0.0185	0.1809
Type II α-Proteobacteria	*Methylocystis* spp.	-	-	-	0.0126	0.0258	0.0507	-
	Methylocystis aldrichii	0.2395	0.0832	0.0421	0.0586	0.0129	0.0554	-
	Methylocystis echinoides	-	0.0059	0.0158	0.0419	0.0215	0.0369	0.0041
	Methylosinus trichosporium	0.1796	0.1605	0.0474	0.1298	-	0.0046	0.0041
	Methylosinus sporium	0.0941	0.0416	0.0316	0.0209	0.0043	0.0185	0.0452
	Beijerinckia spp.	0.0941	0.0535	0.0632	0.0670	0.4782	0.3829	0.0946
Type III *Verrucomicrobium*	*Verrucomicrobium* spp.	1.2061	0.4755	0.3160	0.0712	0.0646	0.4613	0.2591

[a]DS1 – drainage sediment from a cow farm; [b]DS2 and DS3 – sediments from storm drainage; [c]EWAS – east WWTP activated sludge; [d]SWAS – South WWTP activated sludge; [e]SWDS - South WWTP digester sludge; [f]AWAS - Ambassador Caffery WWTP activated sludge.

Table 2. Temperature and pH growth conditions of different types of methanotrophs.

Type	Species	Temperature (°C)	pH	Reference
Type I	*Methylobacter* spp.	20 – 62	6.0 – 8.5	[7]
Type X γ-Proteobacteria	*Methylocaldum* spp.	0 – 40	5.5 – 9.5	[7]
Type II α-Proteobacteria	*Methylocystis* spp.	10 – 40	6.0 – 9.0	[21]
	Methylosinus spp.	10 – 40	5.5 – 9.0	[21]
	Beijerinckia spp.	10 – 35	3.0 – 10.0	[21]
Type III *Verrucomicrobium*	*Verrucomicrobium* spp.	37 – 65	0.8 – 6.0	[7]

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

[1] EPA. "Understanding Global Warming Potentials." U.S. Environmental Protection Agency. https://www.epa.gov/ghgemissions/understanding-global-warming-potentials#:~:text=Methane%20(CH4)%20is%20estimated,less%20time%20than%20CO2. (accessed October 25, 2022).

[2] EIA. "Natural gas: Henry Hub Natural Gas Spot Price." U.S. Energy Information Administration. https://www.eia.gov/dnav/ng/hist/rngwhhdm.htm (accessed August 7, 2022.

[3] DOE, "Natural Gas Flaring and Venting: State and Federal Regulatory Overview, Trends, and Impacts," U.S. Department of Energy, 2019. [Online]. Available: https://www.energy.gov/sites/prod/files/2019/08/f65/Natural%20Gas%20Flaring%20and%20Venting%20Report.pdf

[4] TheWorldBank. "Global Gas Flaring Reduction Partnership (GGFR)." The World Bank. https://www.worldbank.org/en/programs/gasflaringreduction/global-flaring-data (accessed October 25, 2022).

[5] Q. Fei, M. T. Guarnieri, L. Tao, L. M. Laurens, N. Dowe, and P. T. Pienkos, "Bioconversion of natural gas to liquid fuel: opportunities and challenges," *Biotechnol Adv,* vol. 32, no. 3, pp. 596-614, May-Jun 2014, doi: 10.1016/j.biotechadv.2014.03.011.

[6] A. AlSayed, A. Fergala, and A. Eldyasti, "Enhancement of the cultivation process conditions of mixed culture methanotrophic Proteobacteria phylum enriched from waste activated sludge as the first step for value added recovery process," *J Biosci Bioeng,* vol. 127, no. 5, pp. 602-608, May 2019, doi: 10.1016/j.jbiosc.2018.10.018.

[7] J. D. Semrau, A. A. DiSpirito, and S. Yoon, "Methanotrophs and copper," *FEMS Microbiol Rev,* vol. 34, no. 4, pp. 496-531, Jul 2010, doi: 10.1111/j.1574-6976.2010.00212.x.

[8] X. M. Wei, Y. Su, H. T. Zhang, M. Chen, and R. He, "Responses of methanotrophic activity, community and EPS production to CH_4 and O_2 concentrations in waste biocover soils," *Waste Manag,* vol. 42, pp. 118-27, Aug 2015, doi: 10.1016/j.wasman.2015.04.005.

[9] X. Ge, L. Yang, J. P. Sheets, Z. Yu, and Y. Li, "Biological conversion of methane to liquid fuels: status and opportunities," *Biotechnol Adv,* vol. 32, no. 8, pp. 1460-75, Dec 2014, doi: 10.1016/j.biotechadv.2014.09.004.

[10] A. AlSayed, A. Fergala, S. Khattab, and A. Eldyasti, "Kinetics of type I methanotrophs mixed culture enriched from waste activated sludge," *Biochemical Engineering Journal,* vol. 132, pp. 60-67, 2018, doi: 10.1016/j.bej.2018.01.003.

[11] A. Kasprzycka, J. Lalak-Kańczugowska, A. Walkiewicz, P. Bulak, K. Proc, and Ł. Stępień, "Biocatalytic conversion of methane – selected aspects," *Current Opinion in Chemical Engineering,* vol. 26, pp. 28-32, 2019, doi: 10.1016/j.coche.2019.07.006.

[12] K. Jawaharraj, N. Shrestha, G. Chilkoor, S. S. Dhiman, J. Islam, and V. Gadhamshetty, "Valorization of methane from environmental engineering applications: A critical review," *Water Res,* vol. 187, p. 116400, Dec 15 2020, doi: 10.1016/j.watres.2020.116400.

[13] J. M. Pastor *et al.*, "Ectoines in cell stress protection: Uses and biotechnological production," *Biotechnology Advances,* vol. 28, no. 6, pp. 782-801, 2010/11/01/ 2010, doi: https://doi.org/10.1016/j.biotechadv.2010.06.005.

[14] K. K. Sahoo, G. Goswami, and D. Das, "Biotransformation of Methane and Carbon Dioxide Into High-Value Products by Methanotrophs: Current State of Art and Future Prospects," (in English), *Frontiers in Microbiology,* Mini Review vol. 12, 2021-March-10 2021, doi: 10.3389/fmicb.2021.636486.

[15] S. Cantera, R. Lebrero, E. Rodríguez, P. A. García-Encina, and R. Muñoz, "Continuous abatement of methane coupled with ectoine production by *Methylomicrobium alcaliphilum* 20Z in stirred tank reactors: A step further towards greenhouse gas biorefineries," *Journal of Cleaner Production,* vol. 152, pp. 134-141, 2017, doi: 10.1016/j.jclepro.2017.03.123.

[16] S. Guerrero-Cruz, A. Vaksmaa, M. A. Horn, H. Niemann, M. Pijuan, and A. Ho, "Methanotrophs: Discoveries, Environmental Relevance, and a Perspective on Current and Future Applications," (in eng), *Frontiers in microbiology,* vol. 12, pp. 678057-678057, 2021, doi: 10.3389/fmicb.2021.678057.

[17] R. S. Hanson and T. E. Hanson, "Methanotrophic bacteria," *Microbiol Rev,* vol. 60, no. 2, pp. 439-71, Jun 1996. [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/8801441.

[18] O. P. Karthikeyan, K. Chidambarampadmavathy, S. Nadarajan, and K. Heimann, "Influence of nutrients on oxidation of low level methane by mixed methanotrophic consortia," *Environ Sci Pollut Res Int,* vol. 23, no. 5, pp. 4346-57, Mar 2016, doi: 10.1007/s11356-016-6174-7.

[19] J. A. Amaral and R. Knowles, "Growth of methanotrophs in methane and oxygen counter gradients," *FEMS Microbiology Letters,* vol. 126, no. 3, pp. 215-220, 1995, doi: 10.1111/j.1574-6968.1995.tb07421.x.

[20] A. AlSayed, A. Fergala, and A. Eldyasti, "Sustainable biogas mitigation and value-added resources recovery using methanotrophs intergrated into wastewater treatment plants," *Reviews in Environmental Science and Bio/Technology,* vol. 17, no. 2, pp. 351-393, 2018, doi: 10.1007/s11157-018-9464-3.

[21] J. Bowman, "The Methanotrophs — The Families *Methylococcaceae* and *Methylocystaceae*," in *The Prokaryotes*, 2006, ch. Chapter 15, pp. 266-289.