Chemosphere 355 (2024) 141832

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

The fundamental role of pH in CH4 bioconversion into polyhydroxybutyrate in mixed methanotrophic cultures

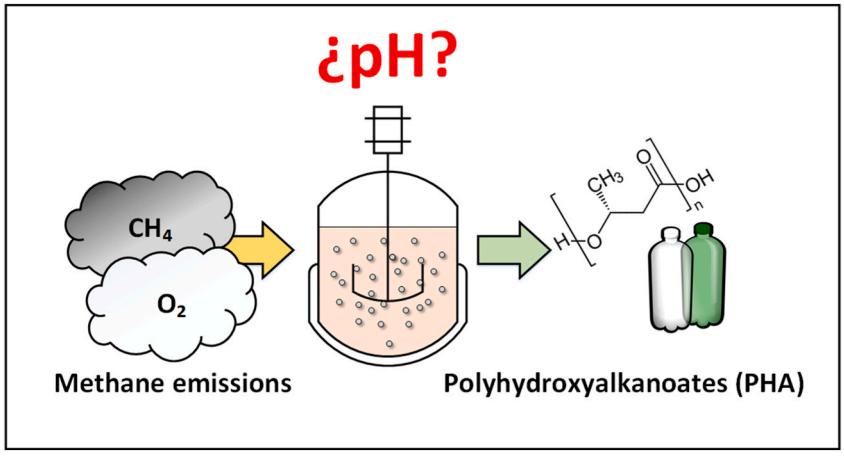
V. Pérez [a,b], R. Lebrero [a,b], R. Muñoz [a,b], R. Pérez [a,b,*]

[a] Institute of Sustainable Processes, Valladolid University, Dr. Mergelina s/n, Spain
[b] Department of Chemical Engineering and Environmental Technology, Valladolid University, Prado de la Magdalena 5, Valladolid, Spain

HIGHLIGHTS

- Low pH (5.5–7.0) is a strong selective pressure towards the growth of *Methylocystis*.
- A higher abundance of *Methylocystis* entails higher PHA accumulation (>40 %w·w^{-1}).
- High pH (8.5) boosts the CH_4 degradation performance (>50 g CH_4·m^{-3}·h^{-1}).
- Very high pH (10) supported a negligible biomass growth and induced cell lysis.
- PHA accumulation started immediately after nitrogen deprivation (<10 mg N·L^{-1}).

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling editor: Ramesh Goel

Keywords:
Bioplastics
Greenhouse gas abatement
Methane
Methanotrophic bacteria
pH
PHA

ABSTRACT

Climate change and plastic pollution are likely the most relevant challenges for the environment in the 21st century. Developing cost-effective technologies for the bioconversion of methane (CH_4) into polyhydroxyalkanoates (PHAs) could simultaneously mitigate CH_4 emissions and boost the commercialization of biodegradable polymers. Despite the fact that the role of temperature, nitrogen deprivation, CH_4:O_2 ratio or micronutrients availability on the PHA accumulation capacity of methanotrophs has been carefully explored, there is still a need for optimization of the CH_4-to-PHA bioconversion process prior to becoming a feasible platform in future biorefineries. In this study, the influence of different cultivation broth pH values (5.5, 7, 8.5 and 10) on bacterial biomass growth, CH_4 bioconversion rate, PHA accumulation capacity and bacterial community structure was investigated in a stirred tank bioreactor under nitrogen deprivation conditions. Higher CH_4 elimination rates were obtained at increasing pH, with a maximum value of 50.4 ± 2.7 g CH_4·m^{-3}·h^{-1} observed at pH 8.5. This was likely mediated by an increased ionic strength in the mineral medium, which enhanced the gas-liquid mass transfer. Interestingly, higher PHB accumulations were observed at decreasing pH, with the highest PHB contents recorded at a pH 5.5 (43.7 ± 3.4 %w·w^{-1}). The strong selective pressure of low pH towards

* Corresponding author. Institute of Sustainable Processes, Valladolid University, Dr. Mergelina s/n, Spain.
E-mail address: rebeca.perez@uva.es (R. Pérez).

https://doi.org/10.1016/j.chemosphere.2024.141832
Received 27 November 2023; Received in revised form 13 March 2024; Accepted 26 March 2024
Available online 1 April 2024

the growth of Type II methanotrophic bacteria could explain this finding. The genus *Methylocystis* increased its abundance from 34 % up to 85 and 90 % at pH 5.5 and 7, respectively. On the contrary, *Methylocystis* was less abundant in the community enriched at pH 8.5 (14 %). The accumulation of intracellular PHB as energy and carbon storage material allowed the maintenance of high CH_4 biodegradation rates during 48 h after complete nitrogen deprivation. The results here obtained demonstrated for the first time a crucial and multifactorial role of pH on the bioconversion performance of CH_4 into PHA.

1. Introduction

Climate change and plastic pollution are likely the most relevant challenges for the environment in the 21st century (OECD, 2023). Methane (CH_4) constitutes the second most important greenhouse gas (GHG) and holds a global warming potential 80 times higher than carbon dioxide (CO_2) in a 20-years lifespan (Forster et al., 2021). Despite the fact that CH_4 can be used as an efficient energy vector in the form of biogas or natural gas, the high investment and maintenance costs required for electricity and heat recovery in co-generation engines typically result in massive amounts of CH_4 being flared or vented to the atmosphere (Wellinger et al., 2013; World Bank, 2019).On the other hand, the massive usage of non-biodegradable plastics has resulted in a severe accumulation of macro- and micro-plastics in landfills and water bodies, with an expected plastic accumulation of more than 12,000 Mt by 2050 (Geyer et al., 2017).

In this context, the development of cost-effective technologies for the bioconversion of CH_4 into polyhydroxyalkanoates (PHAs) could simultaneously mitigate CH_4 emissions and boost the commercialization of biodegradable and carbon-neutral polymers (Rodríguez et al., 2020b). PHAs, a family of biodegradable polymers that can be produced by multiple microorganisms, exhibit mechanical and thermal properties comparable to those of polypropylene and polyethylene and have gained a great market share with the most recent reports accounting for an annual demand over 80,000 t y^{-1} (European Bioplastics, 2022). Type II methanotrophic bacteria, able to use the CH_4 present in industrial gaseous by-products such as biogas or natural gas as their only carbon and energy source, hold the ability to accumulate PHA under nutrient stress conditions. Typically, nitrogen, phosphorous or magnesium deprivation rank among the most widely studied limiting scenarios (Strong et al., 2015). Despite the fact that the fundamental role of temperature, nitrogen deprivation, CH_4:O_2 ratio or micronutrients availability on the PHA accumulation capacity of methanotrophs has been carefully explored, there is still a need for optimization of the CH_4-to-PHA bioconversion process prior to becoming a cost-effective platform in future biorefineries (Pérez et al., 2019; Rodríguez et al., 2020a).

In this study, the influence of different pH conditions (5.5, 7, 8.5 and 10) on bacterial biomass growth, CH_4 bioconversion rate, PHA accumulation capacity and the evolution of the bacterial community structure under nitrogen deprivation conditions were investigated.

2. Materials and methods

2.1. Inoculum, mineral salt medium and bioreactor set-up

A mixture of *Sphagnum* sp and activated sludge from Valladolid wastewater treatment plant was used as inoculum for the enrichment of microorganisms able to degrade CH_4 and accumulate PHA under nitrogen deprivation. The mineral salt medium supplied for the batch operation was modified from (Mokhtari-Hosseini et al., 2009) as described in (Pérez et al., 2019). A 3 L automated Biostat A bioreactor (Sartorius Stedim, Spain) was operated batchwise with a working volume of 2.5 L until complete nitrogen depletion. The bioreactor was equipped with a thermal jacket that maintained the temperature at 30 °C and with a Rushton turbine operated at a stirring rate of 600 rpm. A mixture of air and biogas with a composition of (CH_4/CO_2/O_2/N_2 9/4/18/69 %v·v^{-1}) was continuously sparged at the bottom of the bioreactor with an empty bed residence time of 60 min pH was continuously monitored with a pH-probe EasyFerm Bio HB MS 225 and adjusted automatically via addition of HCl 1 N and NaOH 1 N. Each condition was tested once and no biological replicates of the experiments were performed.

2.2. Analytical procedures

Cell growth was measured as Total Suspended Solids (TSS) according to Standard Methods (American Public Health Association (APHA), 2005). Total nitrogen concentration (TN) was determined following sample filtration (0.45 μm) in a TOC-VCSH interconnected to a TNM-1 module (Shimadzu, Japan). Gas concentrations were measured using a Bruker 430 GC-TCD (Palo Alto, USA) endowed with a CP-Molsieve 5A column (15 m × 0.53 μm × 15 μm) for CO_2 measurement and a CP-PoraBOND Q column (25 m × 0.53 μm × 10 μm) for O_2 and CH_4 quantification. Both columns were operated at oven, injector and detector temperatures of 45 °C, 150 °C and 200 °C, respectively. Helium was used as the gas carrier at a flow rate of 13.7 mL min^{-1}. Polyhydroxybutyrate (PHB) and Polyhydroxyvalerate (PHV) concentration were quantified in a GC–MS (GC System 7820A MSD 5977E, Agilent Technologies, Santa Clara, USA) equipped with a DB-wax column (30 m × 250 μm × 0.25 μm) according to (Frutos et al., 2017). The standard solution was prepared with poly [(R)-3-hydroxybutyric acid--co-(R)-3-hydroxyvaleric acid] (molar ratio 88/12, ≥99.99%) purchased from Sigma-Aldrich® (St. Louis, MO, USA). Total genomic DNA was extracted using the Fast DNA Spin kit for soil according to the manufacturer's instructions (Biomedical, USA). The pyrosequencing analysis was carried by the Foundation for the Promotion of Health and Biomedical Research of Valencia Region (FISABIO, Spain) and the statistical analysis was performed according to (Pérez et al., 2019). The data have been deposited with links to BioProject accession number PRJNA1039782 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/). Each aforementioned parameter was analyzed in duplicate (or in triplicate when necessary).

3. Results and discussion

3.1. Influence of the pH on biomass growth and nitrogen consumption

Mild pH conditions showed an immediate biomass growth with a very reduced lag phase, entailing maximum biomass concentration of 1.51 g TSS·L^{-1} after 102 h and 1.16 g TSS·L^{-1} after 93 h, at pH 7 and 8.5, respectively. On the contrary, adaptation of the biomass at pH 5.5 showed a longer lag phase but reached a similar maximum biomass concentration of 1.54 g TSS·L^{-1} after 196 h. The longer lag phase at low pH conditions might be explained by the need for acclimation of the bacterial culture, given that the bacterial inoculum was previously cultivated at pH 6.8. The operation at high pH conditions (pH = 10) probably induced cell lysis and consequently a negligible biomass growth (Fig. 1A). Interestingly, maximum biomass concentrations for pH 5.5, 7 and 8.5 were obtained 80 h, 96 h and 24 h after complete nitrogen depletion (<10 mg N·L^{-1}), respectively (Fig. 1B). These results revealed a prolonged biomass growth in the absence of nitrogen, which might be an indication of a significant accumulation of PHB as intracellular energy and carbon storage compound under severe nutrient

limitation conditions (Damrow et al., 2016). Average biomass productivity values of 0.37, 0.27 and 0.35 g TSS·g CH_4 were obtained before nitrogen depletion for pH 5.5, 7 and 8.5 respectively.

3.2. Influence of the pH on the CH_4 biodegradation kinetics

No relevant difference was observed between the steady state CH_4 elimination capacity at pH 5.5 and 7 (35.1 ± 2.2 and 34.6 ± 1.3 g $CH_4 \cdot m^{-3} \cdot h^{-1}$ respectively). However, a notably higher steady state CH_4 elimination capacity was observed at pH 8.5 (50.4 ± 2.7 g $CH_4 \cdot m^{-3} \cdot h^{-1}$) as a result of the increased ionic strength of the cultivation medium mediated by NaOH addition for pH control (Fig. 1C). The positive effect of ionic strength on the reduction of bubble coalescence and thus on a concomitant improvement of gas-liquid mass transfer has been consistently reported in literature (Zieminski and Whittemore, 1971). The CH_4 elimination capacity at pH 10 was negligible, with maximum values of 4.2 ± 1.7 g $CH_4 \cdot m^{-3} \cdot h^{-1}$ given the cell lysis associated to extreme pH conditions. Similarly, to biomass growth, the CH_4 abatement capacity was maintained approximately 48 h after complete nitrogen depletion, which again suggested a carbon flux utilization in the absence of nitrogen for PHB synthesis as energy and carbon storage material (García-Pérez et al., 2018).

3.3. Influence of the pH on the PHA accumulation

A decrease in the intracellular PHB accumulation capacity with increasing pH was observed. Maximum PHB contents of 43.7 ± 3.4, 38.5 ± 2.9 and 26.1 ± 5.5 %w·w^{-1} were recorded at pH 5.5, 7 and 8.5, respectively. Interestingly, PHB accumulation started immediately after nitrogen depletion in the mineral medium regardless of the pH conditions tested, which is in agreement with the observed prolonged biomass growth and maintained CH_4 elimination capacity after nitrogen depletion. However, a sudden decrease in the CH_4 elimination capacity occurred shortly after the maximum PHB accumulation, which indicates a rapid decrease of the cell activity in the absence of nitrogen and under high PHA accumulation rates. Average PHB productivity values of 0.46, 0.29 and 0.09 g PHA·g^{-1} CH_4 were obtained after nitrogen depletion for pH 5.5, 7 and 8.5 respectively. PHV content was considered negligible in all the samples analyzed, regardless of the pH conditions.

3.4. Influence of the pH on the bacterial community structure

The initial inoculum contained 34 % of the *Methylocystis* genera (Fig. 2), known for accumulating high concentrations of PHA under nitrogen deprivation conditions (Bordel et al., 2019). Interestingly, the operation at pH 5.5 and 7 entailed a noteworthy growth of the *Methylocystis* relative abundance, with 86 and 90 %, respectively. This indicated that operation at low pH conditions represented a strong selective pressure for the growth of Type II methanotrophic bacteria, which was in well agreement with the higher PHA accumulation rates. On the contrary, operation at pH 8.5 showed a lower relative abundance of the *Methylocystis* genera with only 14 %. However, the low relative

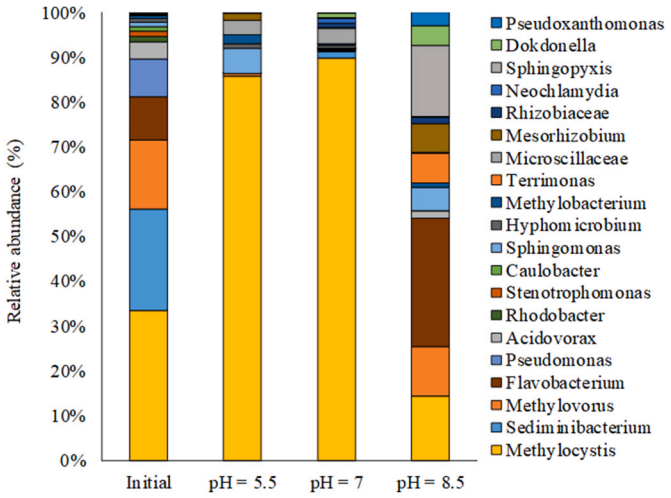


Fig. 2. Influence of pH on the community composition at a genus level of the biomass enriched under nitrogen limitation at pH of 5.5, 7 and 8.5 with respect to the initial community. The abundance is presented in terms of percentage in total effective bacterial sequences in a sample, classified using RDP Classifier.

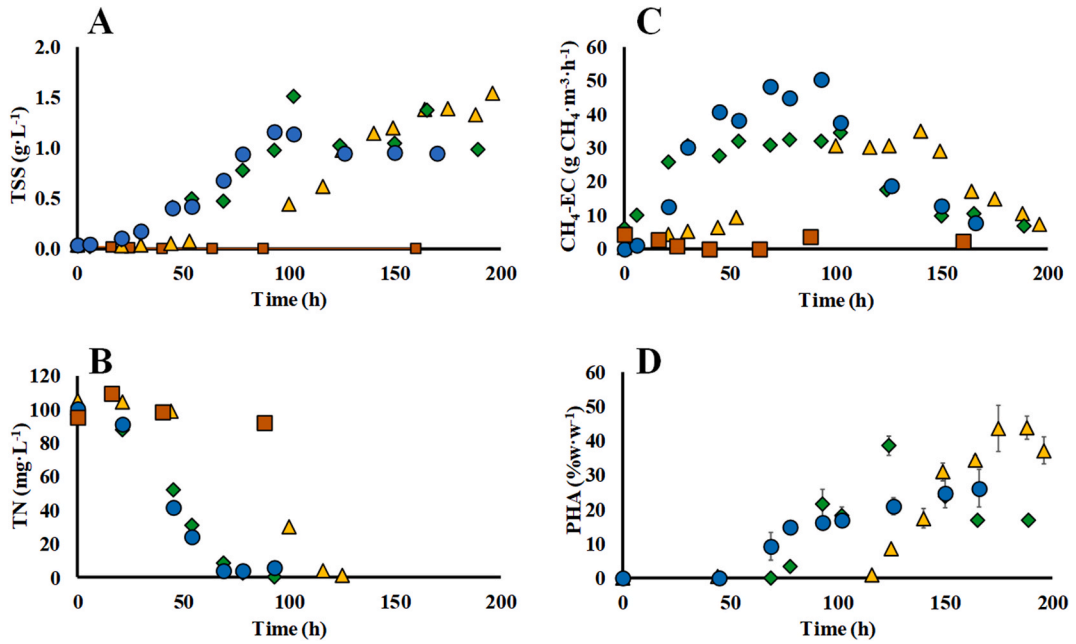


Fig. 1. Influence of pH on: (A) bacterial biomass growth, (B) total nitrogen concentration, (C) CH_4 elimination capacity and (D) intracellular PHA content of the communities enriched at pH 5.5 (yellow triangles), pH 7 (green diamonds), pH 8.5 (blue circles) and pH 10 (brown squares). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

abundance of *Methylocystis* did not match with the high PHA accumulation obtained. Therefore, it could be hypothesized that PHA-accumulating bacteria like *Dokdonella*, *Acidovorax* or *Pseudomonas* could be responsible for PHA accumulation, as they have been observed in previous studies dedicated to PHA production with mixed cultures (Dai et al., 2015). Finally, the genera *Flavobacterium,* described as dominant in PHA-accumulating SBRs (Correa-Galeote et al., 2022; Woraittinuna and Suwannasilp Boonchayaanant, 2017) showed an abundance of 23 %.

4. Conclusions

The pH of the cultivation broth was identified as a key parameter governing the simultaneous CH_4 biodegradation and PHA accumulation. Interestingly, a high pH of 8.5 required a longer acclimation time and supported slightly lower PHA accumulation rates at the expense of a higher CH_4 degradation performance mediated by an increase in the ionic strength of the culture media. On the contrary, the implementation of low pH conditions (5.5–7.0) represented a strong selective pressure towards the growth of *Methylocystis* and entailed a higher PHA accumulation. The accumulation of intracellular PHB as energy and carbon storage material supported an efficient CH_4 biodegradation even 48 h after nitrogen depletion.

CRediT authorship contribution statement

V. Pérez: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **R. Lebrero:** Writing – review & editing, Supervision, Methodology, Conceptualization. **R. Muñoz:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **R. Pérez:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This research was funded by the Spanish Ministry of Economy and Competitiveness, the European FEDER program and the European Commission (CTM2015-73228-JIN). The financial support from the Regional Government of Castilla y León (JCyL) and the FEDER program (CL-EI-2021-07 and UIC315) are also gratefully acknowledged.

References

American Public Health Association (APHA), 2005. American Water Works Association, Water Environment Environment Federation Standard Methods for the Examination of Water and Wastewater, Twenty-First (Standard Methods).

Bordel, S., Rodríguez, E., Muñoz, R., 2019. Genome sequence of Methylocystis hirsuta CSC1, a polyhydroxyalkanoate producing methanotroph. Microbiol. 8, 1–5. https://doi.org/10.1002/mbo3.771.

Correa-Galeote, D., Argiz, L., Val Del Rio, A., Mosquera-Corral, A., Juarez-Jimenez, B., Gonzalez-Lopez, J., Rodelas, B., 2022. Dynamics of PHA-accumulating bacterial communities fed with lipid-rich liquid effluents from fish-canning industries. Polymers 14, 1–22. https://doi.org/10.3390/polym14071396.

Dai, J., Gliniewicz, K., Settles, M.L., Coats, E.R., McDonald, A.G., 2015. Influence of organic loading rate and solid retention time on polyhydroxybutyrate production from hybrid poplar hydrolysates using mixed microbial cultures. Bioresour. Technol. 175, 23–33. https://doi.org/10.1016/j.biortech.2014.10.049.

Damrow, R., Maldener, I., Zilliges, Y., 2016. The multiple functions of common microbial carbon polymers, glycogen and PHB, during stress responses in the non-diazotrophic cyanobacterium synechocystis sp. PCC 6803. Front. Microbiol. 7, 1–10. https://doi.org/10.3389/fmicb.2016.00966.

European Bioplastics, 2022. Bioplastics: Facts and Figures. Berlin.

Forster, P.T., Storelvmo, K., Armour, W., Collins, W., Dufresne, J.L., Frame, D., Lunt, D. J., Mauritsen, T., Palmer, M.D., Watanabe, M., Wild, M., Zhang, H., 2021. Earth's energy budget, climate feedbacks, and climate sensitivity. In: Climate Change 2021: the Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel, pp. 691–698. https://doi.org/10.12006/j.issn.1673-1719.2021.191.

Frutos, O.D., Cortes, I., Cantera, S., Arnaiz, E., Lebrero, R., Muñoz, R., 2017. Nitrous oxide abatement coupled with biopolymer production as a model GHG biorefinery for cost-effective climate change mitigation. Environ. Sci. Technol. 51, 6319–6325. https://doi.org/10.1021/acs.est.7b00643.

García-Pérez, T., López, J.C., Passos, F., Lebrero, R., Revah, S., Muñoz, R., 2018. Simultaneous methane abatement and PHB production by Methylocystis hirsuta in a novel gas-recycling bubble column bioreactor. Chem. Eng. J. 334, 691–697. https://doi.org/10.1016/j.cej.2017.10.106.

Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made - supplementary Information. Sci. Adv. 3, 19–24. https://doi.org/10.1126/sciadv.1700782.

Mokhtari-Hosseini, Z.B., Vasheghani-Farahani, E., Heidarzadeh-Vazifekhoran, A., Shojaosadati, S.A., Karimzadeh, R., Khosravi-Darani, K., 2009. Statistical media optimization for growth and PHB production from methanol by a methylotrophic bacterium. Bioresour. Technol. 100, 2436–2443. https://doi.org/10.1016/j.biortech.2008.11.024.

OECD, 2023. Organisation for economic Co-operation and development. In: Climate Change and Plastics Pollution. Synergies between two crucial environmental challenges, Paris.

Pérez, R., Cantera, S., Bordel, S., García-Encina, P.A., Muñoz, R., 2019. The effect of temperature during culture enrichment on methanotrophic polyhydroxyalkanoate production. Int. Biodeterior. Biodegrad. 140, 144–151. https://doi.org/10.1016/j.ibiod.2019.04.004.

Rodríguez, Y., Firmino, P.I.M., Arnáiz, E., Lebrero, R., Muñoz, R., 2020a. Elucidating the influence of environmental factors on biogas-based polyhydroxybutyrate production by Methylocystis hirsuta CSC1. Sci. Total Environ. 706, 135136 https://doi.org/10.1016/j.scitotenv.2019.135136.

Rodríguez, Y., Pérez, V., López, J.C., Bordel, S., Igor Firmino, P., Lebrero, R., Muñoz, R., 2020b. Coupling biogas with PHA biosynthesis. In: The Handbook of Polyhydroxyalkanoates. https://doi.org/10.1201/9780429296611-16.

Strong, P.J., Xie, S., Clarke, W.P., 2015. Methane as a resource : can the methanotrophs add value. Environ. Sci. Technol. 49, 4001–4018. https://doi.org/10.1021/es504242n.

Wellinger, A., Murphy, J., Baxter, D., 2013. The biogas handbook: science, production and applications, the biogas handbook: science, production and applications. https://doi.org/10.1533/9780857097415.

Woraittinuna, N., Suwannasilp Boonchayaanant, B., 2017. Polyhydroxyalkanoate production from different carbon substrates using sludge from a wastewater treatment plant: microbial communities, polymer compositions, and thermal characteristics. Environ. Prog. Sustain. Energy 36, 1754–1764. https://doi.org/10.1002/ep.12645.

World Bank, 2019. Zero routine flaring by 2030 [WWW Document] URL. https://www.worldbank.org/en/programs/zero-routine-flaring-by-2030/about.

Zieminski, S.A., Whittemore, R.C., 1971. Behavior of gas bubbles in aqueous electrolyte solutions. Chem. Eng. Sci. 26, 509–520. https://doi.org/10.1016/0009-2509(71)83031-2.