POZNAN UNIVERSITY OF TECHNOLOGY

Faculty of Environmental Engineering and Energy
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Conversion of methane into selected polyhydroxyalkanoates with the use of methanotrophic microorganisms

PhD dissertation

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POZNAN 2024

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Summary

Environmental pollution and climate change caused by increased greenhouse gases (GHGs) emissions are the major concerns connected with the rapid growth of civilization. The prevalent use of fossil-based plastics, which resist degradation and whose production relies on non-renewable resources, highlights the need for biobased, biodegradable alternatives such as polyhydroxyalkanoates (PHA) produced by bacteria. PHAs can be produced from C1 GHG by methanotrophic bacteria utilising methane (CH_4) as a low-cost and abundant carbon source. Under nutrient-limited conditions with CH_4 as the sole carbon source, methanotrophs accumulate poly-3-hydroxybutyrate (PHB) as a carbon and energy storage. When specific compounds are added as a secondary carbon source during the accumulation, PHA copolymers with enhanced properties such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) can be produced. The efficiency of PHA production and the monomer composition of the final product depends on the bacterial strain used and the optimisation of key process conditions, such as pH, carbon availability, and medium composition. This thesis focused on the conversion of CH_4 into selected PHAs with the use of methanotrophic bacteria. Its primary aim is to determine if a co-feeding strategy of precisely timed pulses of CH_4 and secondary carbon source will result in the production of PHAs with defined composition in mixed methanotrophic microbial communities.

A comprehensive study on the biotechnological potential of methanotrophic bacteria for CH4 conversion to valuable bioproducts was summarised in **Chapter 2**. This study identified the production of PHA, methanol, ectoine, and microbial protein as the most promising technologies for CH_4 utilization bioprocesses, based on recent trends in CH_4 bioconversion. The use of enriched mixed cultures was found to be a viable alternative to monocultures and the major challenges of low gas-liquid mass transfer on the bioprocess applicability was pointed out. Conducted literature analysis showcased the limited availability of data on the PHA copolymer production by mixed methanotrophic cultures which was addressed in this thesis.

To investigate the PHA production from CH_4 by mixed culture, at first, the PHB production potential of mixed methanotrophic cultures enriched from various environmental sources was compared in **Chapter 3**. In addition, the effect of the nitrogen source during the enrichment was evaluated, showing that nitrate promotes culture growth and PHB productivity more than ammonium. Among the enriched cultures the ones abundant in

Methylocystis sp. exhibit the highest PHB accumulation (up to 27% PHB in dry cell weight (DCW)). Furthermore, the PHBV accumulation capacity of enriched cultures was studied with valeric acid as cosubstrate and under different CH_4:O_2 ratios in the headspace. At a 1:2 CH_4:O_2 ratio (10% CH_4) an efficient CH_4 oxidation was supported, and the cultures showed the highest biomass growth and PHA productivity. The possible participation of PHB-producing bacteria other than *Methylocystis* sp. was observed, and mixed culture enriched from waste-activated sludge and cultured at 10% CH_4 (AS10 culture), with PHA accumulation of around 27% PHA in DCW and 39 mol% of 3-hydroxyvalerate (3HV) fraction, was designated as the most promising for application in CH_4 to biopolymer conversion technologies.

In **Chapter 4**, the use of different alcohols and organic acids as cosubstrate on PHA accumulation was evaluated. The addition of odd-carbon alcohols and acids results in the synthesis of PHBV with differed 3HV fraction, at the same time the use of C3-C6 fatty acids as cosubstrates increases the PHA accumulation. Valeric acid as the most optimal cosubstrate for PHBV accumulation was applied to examine the time-based co-feeding strategy for PHA production under a feast-famine regime in a bioreactor. The AS10 culture could produce and maintain PHA for most of the one-month process duration, however, the manipulation of the PHA composition through the changes in the cosubstrate feeding time proved to be unfeasible. Rather valeric acid utilisation for 3HV was dependent on the CH_4 supply conditions with a higher 3HV fraction of 60 mol% accumulated at lower gas flow (0.1 standard litre per minute (slpm)) and 40 mol% at 0.2 slpm. The process operation without pH control caused a shift in the microbial composition due to the pH increase, impacting the PHA accumulation capacity. The maximum biomass and PHA concentration of 2.25 g/L and 0.5 g/L, respectively was achieved under pH controlled process.

As gas supply to the reactor was observed to affect cosubstrate utilization and the incorporation of the 3HV monomer, a higher CH_4 supply to the reactor was provided to study the effect of cosubstrate concentration on PHBV accumulation in a fed-batch system. In **Chapter 5**, pure and mixed methanotrophic cultures were compared for PHBV production under two cosubstrate feeding rates. The results showed that a mixed culture enriched in *Methylocystis hirsuta* was as effective for PHBV production as a pure *M. hirsuta* culture. At low valerate feeding, both cultures produced similar amounts of PHA (up to 0.67-0.71 g/L) with the mixed culture having a higher 3HV fraction (27 mol%) compared to the pure culture (20 mol%). A higher valerate feeding led to decreased PHA

production but resulted in a higher 3HV fraction of around 40 mol% for both cultures. Analysis of the extracted polymers (**Chapters 4** and **5**) showed that PHAs with a molecular weight of 4.4-5.0 x 10^5 Da can be obtained from a mixed methanotrophic culture in a bioreactor.

Chapter 6 summarises the main conclusions of this thesis and presents an outlook for future research and considerations for the development of biopolymer production from CH_4 by mixed cultures.

Streszczenie

Gwałtowny rozwój cywilizacji w ostatnich dekadach doprowadził do nasilenia się problemów związanych z zanieczyszczeniem środowiska i zmianami klimatycznymi spowodowanymi zwiększoną emisją gazów cieplarnianych. Rozpowszechnione wykorzystanie plastiku, który nie rozkłada się w środowisku naturalnym, wiążę się z problemem kontrolowania jego utylizacji i przedostawania się do środowiska. Dodatkowo znaczna większość produkowanego plastiku wykorzystuje nieodnawialne źródła kopalne co wraz z problemem zanieczyszczenia środowiska podkreśla potrzebę na pozyskiwanie biodegradowalnych alternatyw takich jak polihydroksyalkaniany (PHA) produkowane przez bakterie. PHA mogą być produkowane ze źródeł odpadowych, w tym z gazów cieplarnianych takich jak metan (CH_4) wykorzystując specjalne bakterie metanotroficzne zdolne do utylizacji CH_4 jako głównego źródła węgla. W warunkach ograniczonej dostępności składników odżywczych, podczas gdy źródło węgla jest powszechnie dostępne, metanotrofy akumulują polihydroksymaślan (PHB) jako magazyn węgla i energii. Suplementacja na tym etapie hodowli bakteryjne dodatkowym źródłem węgla może prowadzić do produkcji kopolimerów PHA takich jak poli(3-hydroksymaślan-co-3-hydroksywalerynian) (PHBV), które charakteryzują się lepszymi właściwościami niż PHB. Wydajność produkcji PHA oraz jego skład monomerowy zależy od wykorzystywanego szczepu bakteryjnego oraz optymalizacji kluczowych warunków procesu, takich jak pH, dostępność węgla i skład pożywki. Niniejsza praca doktorska skupia się na badaniu konwersji CH_4 do wybranych PHA z użyciem bakterii metanotroficznych. Głównym celem pracy było sprawdzenie, czy strategia współkarmienia oparta na precyzyjnym dozowaniu CH_4 i dodatkowego źródła węgla będzie skutkowało produkcją PHA o określonym składzie przez mieszane społeczności mikroorganizmów metanotroficznych.

Biotechnologiczny potencjał bakterii metanotroficznych do konwersji CH_4 do cennych bioproduktów został kompleksowo zestawiony i podsumowany w **Rozdziale 2**. Zaobserwowane trendy w biokonwersji CH_4 wskazały produkcję PHA, metanolu, ektoiny i białek pojedynczych komórek jako najbardziej obiecujące technologie biologicznego przetwarzania CH_4. Zauważono, że zastosowanie wzbogaconych kultur mieszanych stanowi realną alternatywę dla monokultur i wskazano główne wyzwanie dla wydajności i komercjalizacji procesu jakim jest niewystarczające przenoszenie masy między fazami. Przeprowadzona analiza literatury wykazała ograniczoną dostępność danych na temat

produkcji kopolimeru PHA przez mieszane kultury metanotroficzne, uzupełnienie tej luki w wiedzy stało się przedmiotem niniejszej pracy doktorskiej.

W celu zbadania produkcji PHA z CH_4 przez mieszaną kulturę pierwszym krokiem, przedstawionym w **Rozdziale 3**, było porównanie potencjału do produkcji PHB przez mieszane kultury metanotroficzne wzbogacone z różnych środowisk. Dodatkowo oceniono wpływ źródła azotu podczas wzbogacania, wykazując, że sole azotanowe bardziej sprzyjają wzrostowi biomasy i wydajności produkcji PHB niż sole amonowe. Spośród wzbogaconych kultur te obfitujące w *Methylocystis* sp. wykazują najwyższą akumulację PHB (do 27% PHB w suchej masie komórek (DCW - dry cel weight)). Ponadto zbadano zdolność wzbogaconych kultur do akumulacji PHBV w różnych stężeniach CH_4 i wykorzystując kwas walerianowy jako kosubstrat. Przy stosunku CH_4:O_2 wynoszącym 1:2 (10% CH_4) wspomagane było efektywne utlenianie CH_4, a kultury wykazywały największy wzrost biomasy i wydajność produkcji PHA. Zaobserwowano możliwą obecność bakterii produkujących PHB innych niż Methylocystis sp., a mieszana kultura wzbogacona z osadu czynnego i hodowana przy 10% CH_4 (kultura AS10), z akumulacją PHA na poziomie około 27% PHA w DCW i frakcją 3-hydroksywalerianu (3HV) wynoszącą 39 mol%, została uznana za najbardziej obiecującą do zastosowania w technologiach konwersji CH_4 do biopolimerów.

W **Rozdziale 4** oceniono zastosowanie różnych alkoholi i kwasów organicznych jako kosubstratu w akumulacji PHA. Zauważono, że dodanie alkoholi i kwasów o nieparzystej liczbie atomów węgla skutkuje syntezą PHBV o różnych frakcjach 3HV, jednocześnie stosowanie kwasów tłuszczowych C3-C6 zwiększa akumulację PHA. Kwas walerianowy, jako najbardziej optymalny kosubstrat do akumulacji PHBV, zastosowano do zbadania opartej na czasie dozowania strategii współkarmienia w bioreaktorze operującym w cyklach feast-famine (okresowego odżywiania i głodzenia hodowli). Kultura AS10 wykazywała produkcję PHA przez większość trwania procesu, jednak manipulacja składem PHA poprzez zmiany czasu podawania kosubstratu okazała się niemożliwa. Wykorzystanie kwasu walerianowego do produkcji 3HV zależało bardziej od warunków dostarczania CH4, z wyższą frakcją 3HV wynoszącą 60 mol% akumulowaną przy niższym przepływie gazu (0,1 standardowego litra na minutę (slpm)) i 40 mol% przy 0,2 slpm. Prowadzenie procesu bez kontroli pH skutkowało zmianą w składzie społeczności mikroorganizmów spowodowanej wzrostem pH, co jednak nie wpłynęło na zdolność do

akumulacji PHA. Maksymalne stężenie biomasy i PHA wynoszące odpowiednio 2,25 g/L i 0,5 g/L osiągnięto w procesie z kontrolą pH.

Ponieważ zaobserwowano, że dopływ gazu do reaktora wpływa na wykorzystanie kosubstratu i frakcję 3HV, w celu zbadania wpływu stężenia kosubstratu na akumulację PHBV w systemie okresowym z zasilaniem (fed-batch) zapewniono wyższe zasilanie CH_4 do reaktora. W **Rozdziale 5** porównano czyste i mieszane kultury metanotroficzne pod kątem produkcji PHBV przy dwóch stosunkach podaży kosubstratu. Mieszana kultura wzbogacona w *Methylocytsis hirsuta* była tak samo efektywna w produkcji PHBV jak czysta kultura *M. hirsuta*. Przy niskiej podaży walerianu, obie kultury produkowały podobne ilości PHA (do 0,67-0,71 g/L), przy czym mieszana kultura miała wyższą frakcję 3HV (27 mol%) w porównaniu do czystej kultury (20 mol%). Wyższe stężenia wprowadzanego walerianu prowadziły do zmniejszenia produkcji PHA, ale skutkowało wyższą frakcją 3HV wynoszącą około 40 mol% dla obu kultur. Analiza otrzymanych polimerów (**Rozdziały 4** i **5**) wykazała, że z mieszanej kultury metanotroficznej w bioreaktorze można uzyskać PHA o masie cząsteczkowej 4,4-5,0 x 10^5 Da.

Rozdział 6 podsumowuje główne wnioski pracy doktorskiej oraz przedstawia perspektywy przyszłych badań i rozważania dotyczące rozwoju produkcji biopolimerów z CH_4 przez mieszane kultury.

Preface

The presented research was performed at Poznan University of Technology, Faculty of Environmental Engineering and Energy, Water Supply and Bioeconomy Division under the supervision of professor Piotr Oleśkowicz-Popiel and Mateusz Łężyk, PhD. The conducted work was under the project "Production of hydroxyalkanoate copolymers in gas fermentation of methane with mixed microbial consortia" financed by the National Science Centre under 2019/35/D/ST8/03530 grant agreement (PI: Mateusz Łężyk, PhD).

Two chapters of this thesis were published in a peer-reviewed journals, and two are currently under preparation and will be submitted in the near future. In all of the published and planned publications I was a primary author.

Chapter 2 was published as Gęsicka, A., Oleskowicz-Popiel, P., Łężyk, M., 2021. Recent trends in methane to bioproduct conversion by methanotrophs. Biotechnol Adv 53, 107861. https://doi.org/10.1016/j.biotechadv.2021.107861 As a primary author I was responsible for data collection, conceptualization, visualization, formal analysis and writing the original manuscript.

Chapter 3 was published as Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M., 2024. Enrichment of mixed methanotrophic cultures producing polyhydroxyalkanoates (PHAs) from various environmental sources. Science of the Total Environment 912. https://doi.org/10.1016/j.scitotenv.2023.168844 As a primary author I conceptualised and conducted the research, collected data, performed formal analysis, visualized presented data and wrote the manuscript.

Chapter 4 is in preparation for publication as Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M., 2024. Sequential feast-famine process for polyhydroxyalkanoates production by mixed methanotrophic culture under different carbon supply and pH control strategies. As a primary author I am responsible for conceptualisation, investigation, formal analysis, visualization of data and writing the manuscript.

Chapter 5 is under preparation for publication as Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, Influence of valerate addition during fed-batch process for PHBV synthesis by pure and mixed methanotrophic cultures. As a primary author I conceptualised and conducted the research, collected data, performed formal analysis, visualized presented data and I am responsible for writing the original manuscript.

Chapter 1

Introduction and thesis outline

1.1. Environmental concerns

The rapid growth of civilization has led to a climate crisis attributed primarily to global warming, caused by the increased greenhouse gases (GHGs) emissions. The reliance on fossil fuels for technological advancement, power generation, and material production alongside the intensification of agriculture and livestock cultivation resulted in a 5 fold increase in GHGs emissions over the last century (Ritchie et al., 2020). Anthropogenic sources of GHGs emissions, predominantly carbon dioxide (CO_2) and methane (CH_4) (Fig. 1.1), have been the main cause of observed climate change and the consequential increase in the global average surface temperature. From the reported data it was shown that the average surface temperature between 1850–1900 and 2011–2020 warmed up by 1.09°C and is estimated to cross 1.5°C in the early 2030s (IPCC, 2023). This surge in surface temperature has resulted in extreme weather events, drought, floods and loss of biodiversity. Application of climate policies could significantly help in mitigating global warming in the long term (Fig. 1.2). Current climate policies are aiming towards reducing GHGs emissions with the goal of achieving climate neutrality by 2050 (European Commission, 2023).

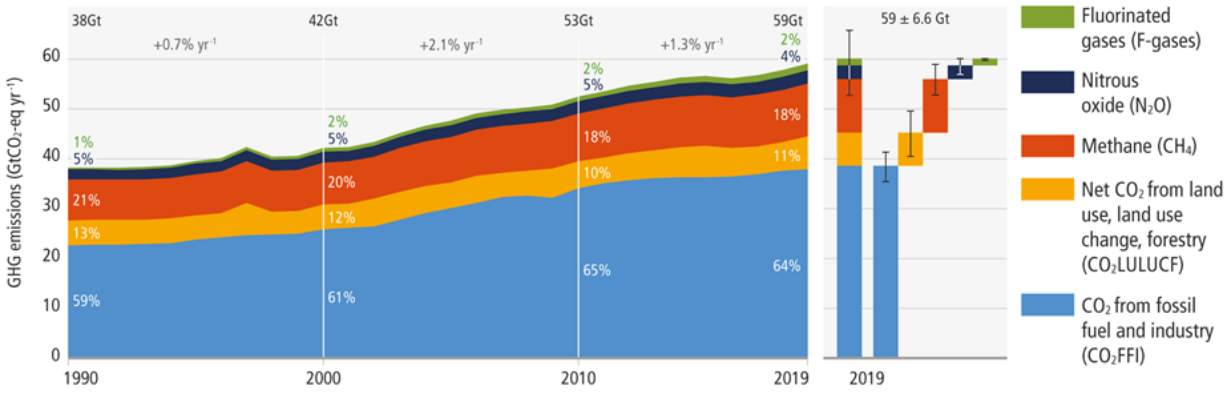


Figure 1.1. Global net anthropogenic GHG emissions 1990-2019 in giga tonnes CO_2 equivalent per year ($GtCO_2$-eq yr^{-1}). Source IPCC (2023b)

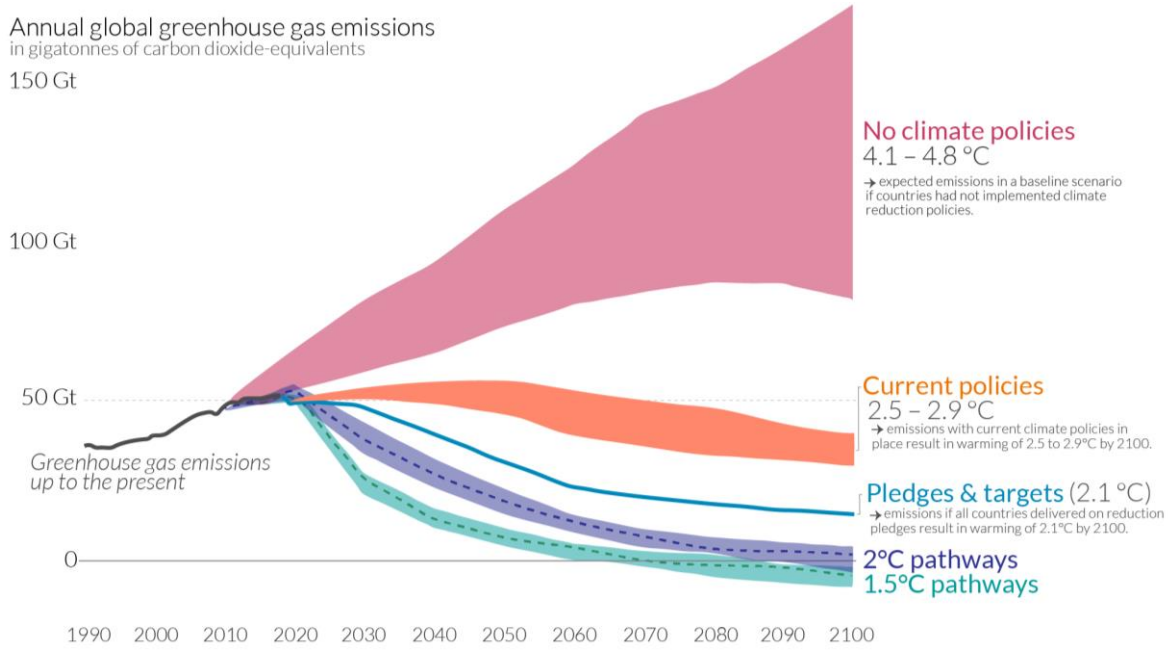


Figure 1.2. Global GHG emissions and warming scenarios. Chart from OurWorldinData.org (Ritchie et al., 2022).

With the development of anaerobic digestion technology, organic wastes can be valorised for the production of biogas (consisting predominantly of CH_4 and CO_2) and biofertilizer (Valentin et al., 2023). The produced biogas can be used for power and heat generation. Additionally, it can be upgraded to biomethane, a "renewable natural gas". This biomethane can be incorporated into existing natural gas networks and utilized in the energy sector as well as for transportation (IEA, 2020). In this sense, carbon capture and sequestration, and the development of biogas and biomethanation plants are some of the key technologies that will support the clean energy transition. Additionally, mitigation of GHGs emissions could involve using CO_2 and CH_4 as carbon sources for the production of platform chemicals and materials within a bioeconomy framework. In the context of global warming, the mitigation of CH_4 is especially important as it is responsible for around 30% of the rise in global temperatures and has a 25 times higher global warming effect as compared to CO_2 (IEA, 2022). As such development of different strategies for restricting CH_4 emissions into the atmosphere is imperative for mitigating climate change (Pratt and Tate, 2018).

Another major concern is the environmental pollution stemming from the challenges in controlling escalating levels of waste. The pollution of terrestrial and marine ecosystems with plastics is particularly alarming in terms of environmental sustainability. The wide range of applications of various conventional fossil fuel-based synthetic polymers has resulted in rapid growth of plastic production since the beginning of the 20th century and

reached over 450 million tonnes in 2019 (OECD, 2022). Conventional plastics are long-lasting in the natural environment and their disposal is realised either by incineration or deposition in landfills. A report on the global analysis of all mass-produced plastics manufactured till 2015 estimated that from all the plastic waste generated only 9% underwent recycling, 12% was incinerated and the rest is being accumulated in landfills (Geyer et al., 2017). The subsequent pollution of terrestrial and marine ecosystems, the concern over the microplastic effect on human health, the depletion of non-renewable fossil fuels resources and the contribution to the GHGs emissions from the plastic production process calls for urgent action. To minimize the negative environmental effects, the sustainable production of biodegradable biopolymers such as polylactide, polysaccharides, aliphatic polyesters, and polyhydroxyalkanoates could substitute the conventional plastics production and consequently decrease the annual release of problematic plastics to the environment (Anjum et al., 2016). However, the lower production efficiency and higher production cost of biopolymers compared to conventional fossil fuel-based plastics make them less competitive in the plastics market. In 2022, less than 1% of global plastics production was bio-based, while 9% came from recycling. Still, 90% of produced plastics are fossil-based (Plastics Europe, 2023). This data indicates the necessity for extensive research and development of bio-based polymer production to enhance their economic appeal and to increase their share in the global plastics market.

1.2. Biological methane conversion

In a global CH_4 cycle, CH_4 emissions are partially curbed by the presence of methanotrophic bacteria, a group of methane-oxidizing bacteria that utilise CH_4 as their only carbon and energy source, and work as a natural CH_4 sink (Conrad, 2009). Methanotrophs are ubiquitous and particularly active in ecosystems with high CH_4 emissions, such as landfills, anaerobic digestion sites, rice paddy fields, wetlands, and oil and gas wells, where they can consume CH_4 in a wide range of concentrations in air (He et al., 2023). Aerobic methanotrophs owe their unique ability of CH_4 oxidation at ambient temperature and pressure to the expression of methane monooxygenase, a key enzyme that catalyses the first step of CH_4 oxidation to methanol (Ross and Rosenzweig, 2017). The high diversity of metabolic pathways within different methanotrophic species makes these bacteria attractive not only for onsite CH_4 emissions mitigation but also for the

biodegradation of organic pollutants and the production of valuable compounds with a wide range of applications, as seen in Figure 1.3 (Knief, 2015).

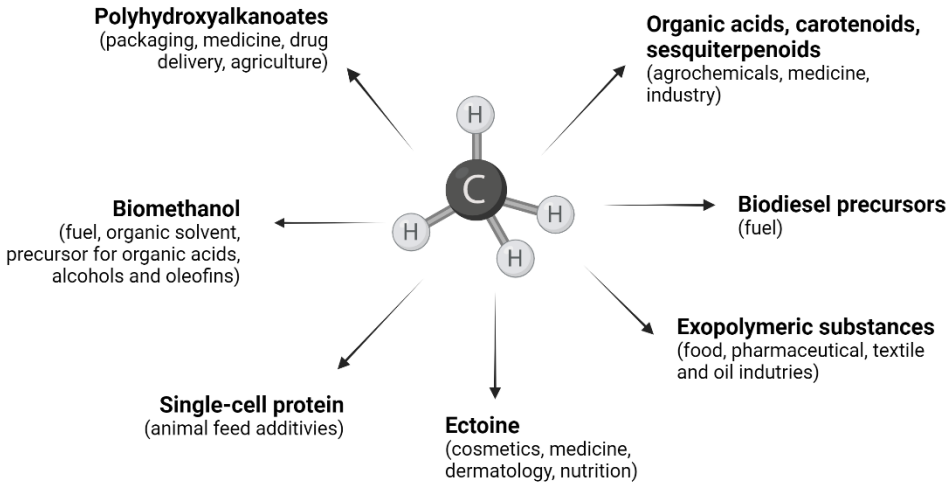


Figure 1.3. Possible bioproducts (and their application) of CH4 conversion by methanotrophs.

The development of gas collection systems used in landfills and from anaerobic digestion allows for the capture and utilisation of CH_4 for heat and power generation or simply flaring, thus limiting its direct emission into the atmosphere. This has made other sources of CH_4-rich gas streams, apart from natural gas or pure CH_4, available as carbon sources for microbial processes (Jawaharraj et al., 2020). Hence the significant biotechnological potential of CH_4 bioconversion technologies has attracted substantial research attention (refer to Table 2.1-2.7), particularly in the areas of biopolymers, single-cell protein, and biofuel production. Among these products, the synthesis of polyhydroxyalkanoates, a type of biopolymer that could replace conventional plastic, has become a desired technology to develop. This offers a means to address environmental plastic pollution and climate change crises simultaneously.

1.3. Polyhydroxyalkanoates from methane

The search for eco-friendly alternatives to fossil fuel-based plastics has put a lot of effort into developing microbial biopolymers such as polyhydroxyalkanoates (PHAs), polylactic acid (PLA), and polycaprolactone (PCL) among others (Behera et al., 2022). Among these, PHAs represent a category of thermoplastic polyesters that are widely acknowledged as biopolymers due to their biodegradability, biocompatibility and sustainable nature (Kumar

et al., 2020). PHAs polymers are especially attractive as their properties are similar to those of polypropylene (PP) and polyethylene (PE). Unlike some other biopolymers such as PLA, PHAs do not necessitate specific conditions for degradation and can break down in various environments including landfills, anaerobic digestion or composting, as well as marine and freshwater environments (Jin et al., 2023). Extensive research on microbial PHA synthesis has yielded over 150 types of PHAs, including homopolymers such as poly-3-hydroxybutyrate (PHB) and copolymers like poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) or more complex heteropolymers (Gao et al., 2022). PHA polymers exhibit a wide range of physical properties such as stiffness, strength, and flexibility that can be tailored for specific applications. The particular properties of these polymers depend on their monomer composition and chemical structure (Kumar, 2020). Furthermore, they can be blended with other polymers or to enhance their physical properties further and expand their potential applications. Research on PHAs has widened its possible uses from traditional packaging materials in diverse fields such as agriculture, biomedicine, and pharmaceuticals, including long-term release of insecticides and herbicides, drug delivery systems and tissue bioengineering (Koller, 2020; Kumar et al., 2020).

PHAs are naturally produced by a wide variety of bacteria as carbon and energy storage under nutrient-limiting conditions from a diverse range of carbon sources (Kumar et al., 2020). Methanotrophs are also a well-known group of PHA producers with a PHB accumulation potential of up to 78% PHB in dry cell weight (Cal et al., 2016). The use of CH_4 as a low-cost alternative for sugar substrates has proven to decrease overall production costs by reducing the expenses on raw materials, underscoring the commercial potential of this bioprocess (Levett et al., 2016). However, despite PHB production being well-studied, the product has several drawbacks due to its high crystallinity, stiffness, and brittleness. On the other hand, when specific compounds are added as a secondary carbon source during the CH_4 conversion process the PHBV with superior properties of low crystallinity, high elongation at the break point, and a lower melting point can be synthesised. Providing odd-carbon cosubstrates such as propionic or valeric acids during the accumulation of PHA supports the synthesis of 3-hydroxyvalerate (3HV) units and their incorporation into the polymer (Myung et al., 2016). Generally, a higher 3HV molar fraction of polymer improves its biodegradation rate, ductility, flexibility, and thermal processivity (Lhamo and Mahanty, 2023). Up-to-date PHBV with varied 3HV fractions were obtained in the studies on the

effect of cosubstrate type and concentration on PHA accumulation (Amabile et al., 2024a). However, there is still much to explore, particularly in the field of PHBV production by mixed methanotrophic cultures. Enriched mixed cultures offer various advantages such as broader metabolic capability and improved process stability by restricting the inhibitory effect of by-products and limiting the risk of contamination. Methanotrophic bacteria often coexist with heterotrophic bacteria that grow on metabolites, such as methanol or organic acids, derived from CH_4. The mutualistic interactions within the culture can significantly stimulate methanotroph growth (Stock et al., 2013), which in turn can enhance PHA production, as it is dependent on the cell biomass. In addition, the reduced operating costs due to operating under non-sterile conditions (Strong et al., 2016b), will make biopolymers produced in a mixed culture system more affordable and competitive with conventional plastics. Figure 1.4 illustrates the schematic concept of this procedure.

Understanding how the process parameters affect the culture growth, microbial community dynamics, PHA accumulation capacity, and monomer composition would allow for controlling the final product properties based on its composition. The development of PHA copolymer production with tuned monomer composition in a cost-effective system run by mixed culture from CH_4 is expected to play a key role in the transition towards a more sustainable and circular economy.

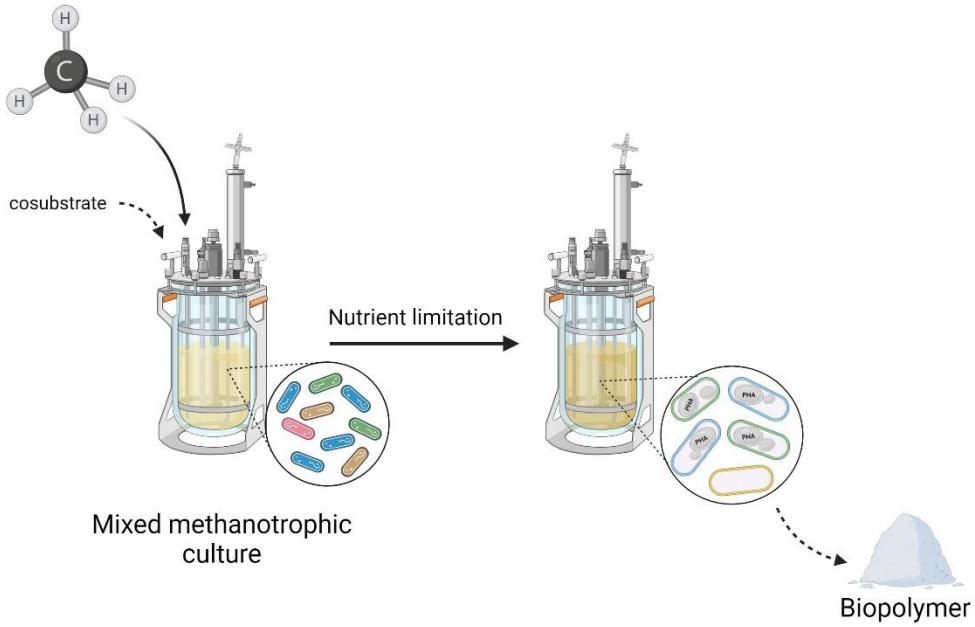


Figure 1.4. The concept of biopolymer production from CH_4 by mixed methanotrophic culture.

1.4. Thesis outline

This thesis investigated the conversion of CH_4 into selected PHAs by mixed methanotrophic culture. The primary research objective was to determine whether a co-feeding strategy of precisely timed pulses of CH_4 and secondary carbon source will result in the production of PHAs with defined composition in mixed methanotrophic microbial communities. Through this research, the aim was to deepen the current understanding of PHAs accumulation under specific process conditions in a complex culture enriched with methanotrophic bacteria.

The particular aims of the thesis were to:

- investigate the conditions that select a stable community of PHB-producing methanotrophs,
- study the microbial composition and process conditions influence on properties of PHAs produced and the process efficiency,
- to determine whether the composition of the desired copolymer can be obtained via alternating the feeding regime between methane and a secondary carbon source,
- to compare the PHBV copolymer production by pure and mixed methanotrophic culture.

In **Chapter 2**, an extensive review of recent advances in CH_4 bioconversion to value-added products is provided. The challenges and limitations of existing technologies were identified and the current research trends in the methanotroph-driven bioprocesses were pointed out. The particular research aims were addressed in **Chapters 3-5**. At first, in **Chapter 3**, the enrichment of PHB-producing methanotrophic cultures from various environments was studied under CH_4 as a carbon source and the effect of nitrogen source on the microbial culture composition and PHB production was evaluated. Furthermore, the PHBV production using methane and valeric acid as a cosubstrate was tested under varied CH_4:O_2 ratios. The mixed culture enriched from waste-activated sludge, which showed the most promise for PHA production, was used in consecutive studies. In **Chapter 4**, the effect of various alcohols and carboxylic acids, as cosubstrates on PHAs accumulation, was evaluated. The most effective cosubstrate was selected to investigate PHBV production in a bioreactor under a feast-famine regime. Various carbon supply and pH control strategies

were then applied to assess the potential for adjusting polymer composition based on operating conditions. In **Chapter 5**, different cosubstrate feeding rates were tested under a fed-batch process to assess the impact of cosubstrate concentration on copolymer production and composition. This study compared PHBV production under the same conditions between pure and mixed methanotrophic cultures to evaluate the potential benefits of using mixed culture for industrial-scale applications. In addition, the molecular weight of the extracted polymer from feast-famine reactors with different pH conditions and from fed-batch reactors under two cosubstrate concentrations are analysed in **Chapters 4** and **5** respectively. Finally, **Chapter 6**, summarises the key findings of the thesis research and their implications. It provides an outlook and recommendations for ongoing developments of CH_4-based PHA copolymer production by mixed methanotrophic cultures.

Chapter 2

Recent trends in methane to bioproduct conversion by methanotrophs

This chapter is published as:

Gęsicka, A., Oleskowicz-Popiel, P., Łężyk, M., 2021. Recent trends in methane to bioproduct conversion by methanotrophs. Biotechnology Advances 53, 107861. https://doi.org/10.1016/j.biotechadv.2021.107861

Abstract

Methane is an abundant and low-cost gas with high global warming potential and its use as a feedstock can help mitigate climate change. Variety of valuable products can be produced from methane by methanotrophs in gas fermentation processes. By using methane as a sole carbon source, methanotrophic bacteria can produce bioplastics, biofuels, feed additives, ectoine and variety of other high-value chemical compounds. A lot of studies have been conducted through the years for natural methanotrophs and engineered strains as well as methanotrophic consortia. These have focused on increasing yields of native products as well as proof of concept for the synthesis of new range of chemicals by metabolic engineering. This review shows trends in the research on key methanotrophic bioproducts since 2015. Despite certain limitations of the known production strategies that makes commercialization of methane-based products challenging there is currently much attention placed on the promising further development.

2.1. Introduction

The development of microbial gas fermentation as a technology that offers a solution for environmental pollution by greenhouse gases (GHGs) and for a better economy of biotechnological processes is gaining much attention. The use of GHGs as a carbon source for the production of chemicals, fuels and other components not only helps mitigate climate change by lowering harmful gases emissions but also improves the process economy by replacing costly sugar-based carbon sources at the same time resolving the issue of food security. The basis of this technology is the application of microorganisms that can grow on gaseous C_1 substrates such as CO, CO_2 or CH_4 and possess natural or engineered pathways for their conversion into more valuable products.

Particularly methane, which is one of the most prevailing greenhouse gases, with global warming potential much higher than CO_2 (Abbasi et al., 2012) is an attractive alternative as a feedstock for biotechnological processes. Bioconversion of methane, while helping mitigate CH_4 emissions, offers a broad range of possibly synthesized products such as bioplastics, chemicals and animal feed additives. Direct utilization of CH_4 is made possible by using methanotrophic bacteria as cell factories. Methanotrophs are a group of ubiquitous bacteria possessing a unique ability to oxidize methane and utilize it as a sole carbon and energy source (Hanson and Hanson, 1996). They have been greatly studied for possible biotechnological applications and for uncovering the metabolic mechanism behind these processes. Through the years many reviews engaging in methane bioconversion by methanotrophs were published. Various authors elaborated on this subject from different perspectives and for a wide range of products and their applications, both naturally produced or achieved through a synthetic biology (A. D. Nguyen and Lee, 2021; Pieja et al., 2017; Sahoo et al., 2021; Strong et al., 2015).

In this review, recent advances and trends in methanotrophic bioproducts studies since 2015 have been compiled and overviewed. General characterization of methanotrophic bacteria and their metabolic pathways for CH_4 oxidation and target bioproducts synthesis are elaborated. Characterization of the most often studied products as well as still remaining challenges and limitations of existing technologies of methane bioconversion are also presented.

2.2. Methanotrophs and methane metabolism

Methanotrophic bacteria are a part of a larger methylotrophic group that associates microorganisms able to utilize C_1 substrates such as methane, methanol and other methylated compounds and assimilate created formaldehyde as a source of cellular carbon (Hanson and Hanson, 1996). The ability to use methane as a sole carbon and energy source and directly oxidize it and convert it in the cell into other products is a unique characteristic of methanotrophs that differentiate them from other methylotrophs. First reports on methanotrophs began to appear at the beginning of the 20th century but the true breakthrough in the studies in this area appeared in 1970 when Whittenbury et al. (1970) isolated more than 100 Gram-negative methane-utilizing bacteria (MOB). Since then many new studies on methanotrophs have been conducted, with growing interest every year. These MOB can be isolated from a wide range of habitats, typically from ecosystems in which methane is released, such as oilfields or coil deposits, landfill covers, sewage sludge, freshwater and marine sediments, wetlands, aquifers, wastewater treatment and biogas plants, but also from extreme environments in terms of temperature, pH or salinity (Knief, 2015; Ross and Rosenzweig, 2017; Strong et al., 2015). Representatives of methanotrophs can be found in three phyla: Proteobacteria (the largest faction), Verrucomicrobia and newly separated candidate phylum NC10 (Hakobyan and Liesack, 2020). MOB that belongs to Proteobacteria are found in two groups: gammaproteobacteria and alphaproteobacteria. Gammaproteobacteria include a wide range of MOB, such as *Methylomonas*, *Methylococcus*, *Methylomicrobium*, *Methylosarcina*, *Methylocaldum*, *Methylobacter* genera and many others in comparison to alphaproteobacteria that includes only four: *Methylosinus*, *Methylocystis*, *Methylocella* and *Methylocapsa* (Kalyuzhnaya et al., 2015). Despite a high diversity of methanotrophic bacteria, the general metabolic pathway for CH_4 oxidation into CO_2 through formaldehyde as an intermediate remains the same for all representatives of this microbial group (Semrau et al., 2010).

Methane metabolism in methanotrophs cells (Figure 2.1) begins from CH_4 oxidation into methanol in the reaction catalyzed by methane monooxygenase (MMO) which presence and activity is a defining characteristic of methanotrophic bacteria (Hanson and Hanson, 1996). The use of MMO enzyme complex enables activation of a strong C-H bond in CH_4 and its oxidation into methanol using oxygen at ambient temperature and pressure (Ross and Rosenzweig, 2017). Methanotrophs can express two types of MMO; located in the

cytoplasm soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO) bound to the membrane (Dalton, 2005). Bacteria can express either one of those two forms or both with pMMO being more prevalent. Although sMMO has wider substrate specificity its expression is inhibited by high copper concentration and only a few methanotrophs produce MMO in this form (Hakemian and Rosenzweig, 2007). In bacteria that produce both sMMO and pMMO concentration of copper becomes a key factor in regulating the expression of genes for these enzymes and their enzymatic activity (Semrau et al., 2010). In comparison, pMMO due to its membrane association, appears to have greater access to methane than sMMO, which on the other hand is thought to be able to oxidize CH_4 more quickly (Ge et al., 2014).

After the first step of methane conversion, methanol is then oxidized into formaldehyde, which can be further oxidized or assimilated as a substrate for cell carbon fixation pathways. Oxidation of methanol is catalyzed by methanol dehydrogenase (MDH) which for Gram-negative methanotrophic bacteria exists in a particular form in the periplasm and contains pyrroloquinoline quinone (PQQ) as its catalytic cofactor (Keltjens et al., 2014). Created formaldehyde can be then used in three ways i) further oxidized into CO_2 ii) assimilated via ribulose monophosphate (RuMP) cycle or iii) assimilated via serine cycle. Operating of both of mentioned cycles leads to creating pyruvate and then acetyl-CoA as key intermediates for further cell metabolism. In the case of the oxidation into CO_2, formaldehyde is first converted into formate by formaldehyde dehydrogenase (FADH) and then formate dehydrogenase (FDH) oxidizes formate to CO_2 (Hanson and Hanson, 1996). In the RuMP pathway, formaldehyde is used for the synthesis of fructose-6-phosphate and further into ribulose-5-phosphate through sugar-phosphate intermediates using specified enzymes. There also exists another version for operating this cycle in which pyruvate is mainly synthesized via the Embden–Meyerhof–Parnas (EMP) pathway (Kalyuzhnaya et al., 2015). As a result, formaldehyde is either assimilated for biomass growth or oxidized to CO_2 via an incomplete tricarboxylic acid (TCA) cycle (Strong et al., 2016). Bacteria, which assimilate C_1 substrate via the serine pathway, first incorporate formaldehyde into the serine cycle through the H_4-F pathway (Vorholt et al., 2000). In the serine cycle, C_1 substrates are first converted into C_3 and C_4 compounds and then into acetyl-CoA which in nutrient sufficient conditions enter the functional TCA cycle (Khmelenina et al., 2015).

Carbon fixation mechanism through RuMP or serine cycle is one of the major distinctive characteristics that differentiate methanotrophs and constitutes the basis for their

classification. Methanotrophs using the RuMP pathway for carbon fixation belong to type I (gammaproteobacteria) and type II (alphaproteobacteria) consists of methanotrophs operating via serine cycle. A detailed comparison between type I and type II methanotrophs was described by (Karthikeyan et al., 2015a). While type I and II are the main groups of methanotrophs, type X, which combines characteristics of both of these types, was differentiated from type I. Methanotrophs belonging to type X are also able to grow at higher temperatures and possess ribulose-1,5-bisphosphate carboxylase (Hanson and Hanson, 1996). Different types of methanotrophs vary also in the location of intracytoplasmic membranes, the capability of nitrogen fixation and major phospholipids fatty acids (Knief, 2015).

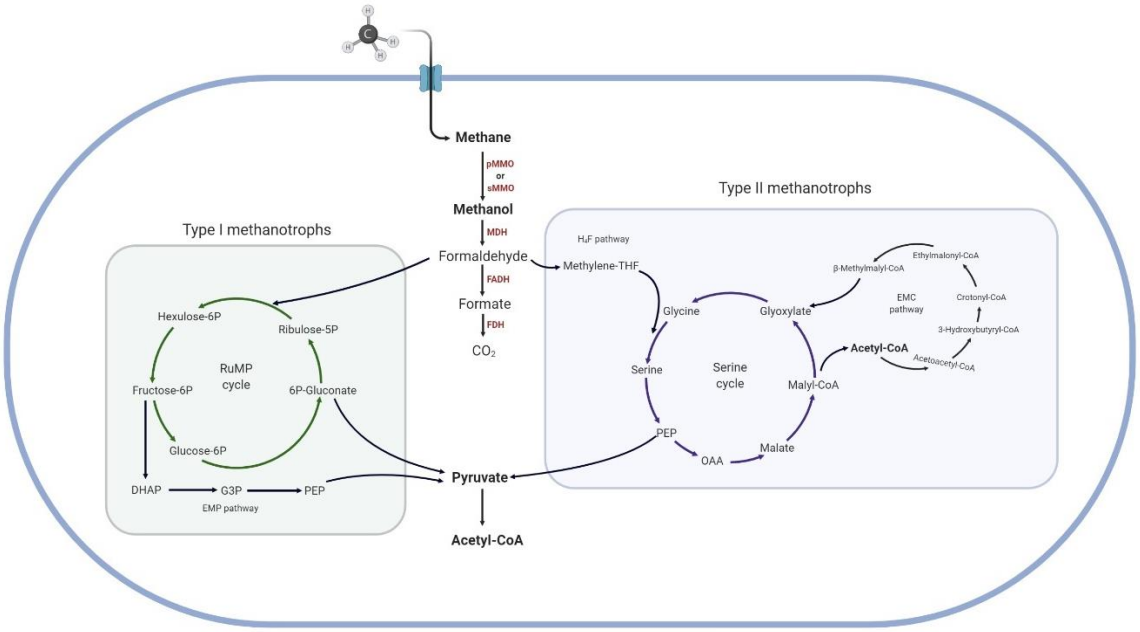


Figure 2.1. Methane assimilation pathways in methanotrophs. Abbreviations: DHAP–dihydroxyacetone phosphate, EMC–ethylmalonyl-coenzyme A, G3P–glyceraldehyde 3-phosphate, methylene-THF-methylene tetrahydrofolate, OAA–oxaloacetate, PEP–phosphoenolpyruvate, RuMP – ribulose monophosphate.

Table 2.1. PHAs production by methanotrophs since 2015, expressed as accumulation of PHAs in the cells (percentage of DCW). Abbreviations: CSTR – continuous stirred-tank reactor, BCB – bubble column bioreactor, DCW – dry cell weight.

STRAIN	METHANE	TEMP.	CULTIVATION TYPE	PROCESS DETAILS	ACCUMULATION	REFERNECE
PHB						
Heterotrophic-methanotrophic consortium (dominated by *Methylocystis* sp.)	10% CH_4	25ºC	Batch	-	8.4% (84.17 mg/g DCW)	(Karthikeyan et al., 2015)
Methanotrophic consortium from landfill cover (96% *Methylomicrobium* sp.)	30% CH_4	25ºC	Continuous	CSTR, Copper and iron 1:1 (5 μM)	4.8% (47.88 mg/g DCW)	(Chidambarampadmavathy et al., 2015a)
Methylocystis parvus OBBP	50% CH_4	30ºC	Batch	Copper (5 μM) and calcium (7.2 μM)	49.4%	(Sundstrom and Criddle, 2015)
Methanotrophic consortium from landfill cover (dominated by *Methylosarcina* sp.)	40% CH_4	25ºC	Batch	-	2.5% (24.6 mg/g DCW)	(Chidambarampadmavathy et al., 2017)
Methylocystis parvus OBBP	CH_4:O_2 1:1.5 molar ratio	30ºC	Batch	3-hydroxybutyrate as co-substrate (1.2 mM)	59%	(Myung et al., 2017a)
Methylosinus trichosporium OB3b	50% CH_4	30ºC	Batch	Copper (5 μM)	51%	(Zhang et al., 2017)
Methanotrophic-heterotrophic cultures (dominated by heterotrophic microorganisms)	50 % CH_4 (0.5 atm)	30ºC	Batch	N_2 as N source for growth (0.3 atm) Copper (5 μM), O_2 (0.2 atm)	48.7%	(Zhang et al., 2018)
Methylosinus trichosporium OB3b	30% CH_4 (of C source)	30ºC	Batch	Methanol – 70% of carbon source	52.5%	(Zaldívar Carrillo et al., 2018)
Methylocystis hirsuta	49.8 g CH_4 /m^3 h	25ºC	Continuous	Internal gas-recycling BCB Nitrogen feast-famine cycles	34.6%	(García-Pérez et al., 2018)
Methanotrophic consortium (dominated by *Methylocystis* sp.)	50 % CH_4	25ºC	Batch	Copper (10 μM)	51%	(Fergala et al., 2018c)
Methylosinus trichosporium OB3b	50% CH_4 (0.5 atm)	30ºC	Batch	N_2 (0.3 atm), O_2 (0.2 atm)	55.5%	(Zhang et al., 2019)
Enriched culture (dominated by *Methylocystis* sp. ~ 30%)	195 ± 7 g CH_4 /m^3	30ºC	Batch	-	35.1%	(Pérez et al., 2019)
Methylocystis hirsuta CSC1	Synthetic biogas, O_2:CH_4 2:1	25ºC	Batch	CH_4:O_2:CO_2 29.2:58.3:12.5%	45.3%	(Rodríguez et al., 2020a)
Methylocystis hirsuta CSC1	Synthetic biogas (86 g CH_4 /m^3 h)	25ºC	Continuous	BCB, Nitrogen feast-famine cycles	14.5%	(Rodríguez et al., 2020b)
Methanotrophic sludge enriched for nitrogen removal	50% CH_4 (CH_4:O_2 1:1)	27ºC 27ºC	Batch Batch	- Phosphorus limitation	21% 11%	(Bishoff et al., 2021)
PHBV						
Methanotrophic consortium (dominated by *Methylocystis* sp.)	CH_4:O_2 molar ratio of 1:1.5	30ºC	Batch	Copper (7 μM) Valeric acid as co-substrate (100 mg/L)	44% 20 mol% of 3HV fraction	(Myung et al., 2015)

Methylocystis sp. WRRC1	50% CH_4	30ºC	Batch	Valeric acid as co-substrate (0.34%)	78% 58 mol% of 3HV fraction	(Cal et al., 2016)
Methylosinus trichosporium OB3b	CH_4:O_2 molar ratio of 1:1.5	30ºC	Batch	Copper (7 μM) Valeric acid as co-substrate (100 mg/L)	50 % 20 mol% of 3HV fraction	(Myung et al., 2016a)
Methylocystis parvus OBBP	CH_4:O_2 molar ratio of 1:1.5	30ºC	Batch	Copper (7 μM) Valeric acid as co-substrate (1.2 mM)	54 % 25.0 mol% of 3HV fraction	(Myung et al., 2017a)
Methanotrophic consortium (dominated by *Methylocystis* sp.)	50 % CH_4	25ºC	Batch	Copper (10 μM) Valeric acid as co-substrate (100 mg/L)	52 % 33 mol% of 3HV fraction	(Fergala et al., 2018c)
Thermophilic enrichment	90% CH_4	55ºC	Batch	Copper (2.5 μM) Valeric acid as co-substrate (100 ppm)	10% 6 mol% of 3HV fraction	(Luangthongkam et al., 2019)
Other PHAs						
Methylocystis parvus OBBP	CH_4:O_2 molar ratio of 1:1.5	30ºC	Batch	Copper (7 μM) Co-substrates: - 4-hydroxybutyrate (1.2 mM) - 5-hydroxyvalerate (1.2 mM) - 6-hydroxyhexanoate (1.2 mM)	- 50% P(3HB-co-4HB) 9.5 mol% of 4HB fraction - 48% P(3HB-co-5HV-co-3HV), 1.4 mol% of 3HV and 3.6% mol% 5HV fraction - 48% P(3HB-co-HHxco-4HB), 1.0 mol% of 4HB and 1.4 mol% of 6HHx fraction	(Myung et al., 2017a)
Methylosinus trichosporium OB3b/SYOI mutant strain	40% CH_4	30ºC	Batch	Copper (5 μM)	7.01% P(3HB-co-4HB) 3.08 mol% of 4HB fraction	(T. T. Nguyen and Lee, 2021)

Table 2.2. Methanol production by methanotrophs since 2015 expressed in titers (mM). Abbreviations: AD – anaerobic digestion, CSTR – continuous stirred-tank reactor.

STRAIN	METHANE	TEMP.	pH	CULTIVATION TYPE	PROCESS DETAILS	TITER	REFERNECE
Methylosinus trichosporium OB3b	30% CH_4	30ºC	6.3	Batch	EDTA (0.5 mM), Na-formate (40 mM) Copper (5 μM)	12.28 mM (0.393 g/L)	(Hwang et al., 2015)
Encapsulated *Methylosinus sporium*	20% CH_4	30ºC	7.0	Batch	Encapsulation in silica gel Na-formate (40 mM), $MgCl_2$ (20 mM) Copper (5 μM), Iron (10 μM)	3.73 mM	(Patel et al., 2016a)
Methylosinus sporium	20% CH_4 (raw biogas)	30ºC	7.0	Batch	$MgCl_2$ (20 mM) Copper (5 μM), Iron (10 μM) 62.3% CH_4, 36.7% CO_2, 0.13% H_2S Supplementation with H_2 (10%)	6.68 mM	(Patel et al., 2016b)
Methylocystis bryophila	50% CH_4	30ºC	6.8	Batch	Na-formate (100 mM), $MgCl_2$ (50 mM)	4.63 mM	(Patel et al., 2016c)

					Copper (5 μM), Iron (10 μM)		
Methylocella tundrae	50% CH_4	30ºC	7.0	Batch	Na-formate (50 mM), $MgCl_2$ (50 mM) Copper (5 μM), Iron (10 μM)	5.18 mM	(Mardina et al., 2016)
Strain SAD2 belonging to *Methylocaldum* sp. isolated from H_2-S-rich AD	Biogas:air 1:2	37ºC	6.7	Batch	Na-formate (100 mM), Copper (5 μM)	10.72 mM (0.343 g/L)	(Zhang et al., 2016)
Strain 14B isolated from SS-AD reactor (similar to *Methylocaldum* sp.)	Biogas:air 1:2.5	37ºC	6.6–6.8	Batch	70.6% CH_4, 28.6% CO_2, 0.5% N_2, 0.3% O_2 Formate (80 mM), Copper (1 μM) 69.8% CH_4, 28.8% CO_2, 1.0% N_2, 0.4% O_2	13.44 mM (0.43 g/L)	(Sheets et al., 2016)
Methylomonas sp. DH-1	40% CH_4	30ºC	7.0	Batch	Na-formate (40 mM), EDTA (0.5 mM)	41.86 mM (1.34 g/L)	(Hur et al., 2017)
Mixed culture dominated by *Methylocaldum* sp. 14B	Biogas:air 1:2.5				Trickle-bed reactor Liquid circulation 50 mL/min Gas circulation 80 mL/min 67.7 ± 2.8% CH_4, 29.9 ± 4.1% CO_2, 3.2 ± 3.0% N_2, 1.2 ± 1.0% O_2	28.13 mM/d (0.9 g/L/d)	(Sheets et al., 2017a)
Immobilized *Methylocystis bryophila*	30% CH_4 (simulated biogas CH_4:CO_2)	30ºC	7.0	Batch	Repeated batches (up to 8) Immobilized with chitosan Formate (100 mM), $MgCl_2$ (50 mM) Copper (5 μM), Iron (10 μM)	25.75 mM	(Patel et al., 2018a)
Co-culture of *Methylocella tundrae* and *Methylomonas methanica*	30% CH_4 (simulated biogas CH_4:CO_2)	30ºC	7.0	Batch	Encapsulated in silica gel Formate (100 mM), $MgCl_2$ (50 mM) Copper (5 μM), Iron (10 μM)	9.65 mM	(Patel et al., 2018b)
Strain AS1 isolated from active anaerobic sludge	50% CH_4	28ºC	n/a	Batch	Sequencing mode in external-loop airlift bioreactor, Recycling flow of 0.1 L min^{-1}	50 mM (1.6 g/L)	(Ghaz-Jahanian et al., 2018)
Mixed culture enriched from activated sludge (dominated by type I methanotrophs)	80% CH_4 (CH_4:O_2 4:1 v/v)	RT	7.0	Batch	Formate (120 mM), $MgCl_2$ (10 mM) Copper (5 μM)	15.16 mM (485.08 mg/L)	(Alsayed et al., 2018)
Encapsulated *Methylosinus trichosporium* OB3b	20% CH_4	30ºC	7.0	Continuous	Column reactor Immobilized in alginate (2%) Cyclopropane 100 μM (pre-treatment of immobilized cells)	3.7 mM	(Taylor et al., 2018)
Methylacidiphilum fumariolicum SolV	70 ml/min CO_2-argon (5%:95% v/v), 10 ml/min CH_4-CO_2 (95%:5% v/v), and 6 ml/min H_2	55ºC	3.0	Continuous	CSTR 1% of air saturation Oxygen-limited and lanthanide-depleted cultivation	4.1 mM	(Hogendoorn et al., 2020)
Encapsulated *Methylomicrobium album*	30% CH_4 (simulated biogas CH_4:CO_2 at 4:1)	30ºC	7.0	Batch	Encapsulated in chemically modified chitosan Formate (100 mM), $MgCl_2$ (20 mM) Copper (5 μM), Iron (10 μM)	4.96 mM	(Patel et al., 2020a)
	30% of raw biogas (65.4% CH_4, 34.6% CO_2)					6.92 mM	

Immobilized co-culture of *Methylocystis bryophila* and *Methyloferula stellata*	30% CH_4	30ºC	7.0	Batch	Immobilized with polyvinyl alcohol (10%) in 3:2 ratio of *M. bryophila* and *M. stellate* Formate (100 mM), $MgCl_2$ (20 mM) Copper (5 µM), Iron (10 µM)	5.37 mM	(Patel et al., 2020b)
Encapsulated *Methylomicrobium album*	30% CH_4 (simulated biogas; CH_4:CO_2 at 2:1)	30ºC	7.0	Batch	Encapsulated in polyvinyl alcohol (10%) Formate (100 mM), $MgCl_2$ (50 mM) Paraffin oil (5% v/v)	7.46 mM	(Patel et al., 2020d)
Encapsulated *Methyloferula stellate*						7.14 mM	
Methylocella tundrae	30% CH_4 (raw biogas)	30ºC	6.8–7.0	Batch	Formate (100 mM), $MgCl_2$ (20 mM) Iron (10 µM), Copper (5 µM)	4.97 mM	(Patel et al., 2021)
Methylocella tundrae immobilized on banana leaves						6.05 mM	
Methyloferula stellata						3.36 mM	
Methylosinus trichosporium OB3b *ΔmxaF* mutant strain	CH_4:air 1:4 (v/v)	30ºC	7.0	Batch	Copper (10 µM)	2.6 µmol/mg DCW x h	(Ito et al., 2021)

Table 2.3. Microbial protein (SCP) production expressed as accumulation of microbial protein in cells (%DCW) or as SCP yield on methane (g DCW/g CH_4). Abbreviations: CSTR – continuous stirred-tank reactor, DCW – dry cell weight.

STRAIN	METHANE	TEMP.	CULTIVATION TYPE	PROCESS DETAILS	ACCUMULATION / YIELD	REFERNECE
Methylococcus capsulatus	60% CH_4	35ºC	Batch	Nitrate as N source	52%	(Steinberg et al., 2017)
Methylococcus capsulatus (Bath)	60% CH_4	37ºC	Batch	Batch, Ammonium as N source	52.52%	(Rasouli et al., 2018)
Mixed-culture enriched in *Methylococcales* and *Methylophilales*	O_2:CH_4 2:1 v/v (biogas)	RT	Batch	Batch, filtrated-digestate as medium Biogas; CH_4:CO_2 60:40%	0.87 g DCW/g CH_4	(Khoshnevisan et al., 2019)
Mixed methanotrophic culture (dominated by *Methylophilus* sp.)	40% CH_4	25ºC	Continuous	CSTR Filtrated-digestate as medium	40.91 % 0.79 g DCW/g CH_4	(Tsapekos et al., 2019)
Methylocapsa acidiphila	60% CH_4	24ºC	Batch	pH 5.7	58.6%	(Xu et al., 2020)
Enriched methanotrophic and hydrogenotrophic culture	26% CH_4 (biogas)	28ºC	Batch	Ammonium as N source Electrochemically upgraded biogas	66.60%	(Acosta et al., 2020)
Enriched methanotroph culture (dominated by *Methylophiluls* sp. >70%)	O_2:CH_4 2:1 v/v	25ºC	Batch	N_2 extracted from anaerobically digested organic wastes as N source	0.88 g DCW/g CH_4	(Khoshnevisan et al., 2020)
Mixed methanotrophs culture (dominated by *Methylomonas* and *Methylophilus* spp.)	Upgraded biogas	25ºC	Semi-continuous	Copper (100 µg/L)	49%	(Tsapekos et al., 2020)
	40% CH_4			N_2 extracted from anaerobically digested biowastes as N source	53%	

Enriched methanotroph culture (dominated by *Methylomonas* sp. or *Methylosinus* sp. depending on culture period)	CH_4 15.78 mL/h I period 31.57 mL/h IV period	RT	Continuous	Bubble-free membrane bioreactor Ammonium as N source	51%	(Valverde-Pérez et al., 2020a)
Mixed methanotrophic culture (dominated by *Methylomonas* and *Methylophilus* sp.)	2:1 O_2: CH_4 v/v (biogas)	25ºC	Batch	Filtrated-digestate as medium	41% 0.66 g DCW/g CH_4	(Zha et al., 2021)

Table 2.4. Ectoine production by methanotrophic cultures since 2015, expressed as accumulation in cells (mg/g DCW) for intracellular ectoine production (IC) and as titer (mg/L) for extracellular ectoine production (EC). Abbreviations: CSTR – continuous stirred-tank reactor, DCW – dry cell weight.

STRAIN	METHANE	TEMP.	CULTIVATION TYPE	PROCESS DETAILS	ACCUMULATION / TITER	REFERNECE
Methylomicrobium alcaliphilum 20Z	4% CH_4	25ºC	Batch	6% NaCl, Copper (0,05 µM)	66.9 mg/g DCW (IC)	(Cantera et al., 2016)
	20% CH_4	30ºC	Batch	6% NaCl, Copper (50 µM)	40.7 mg/g DCW (IC) 4.7 mg/L (EC)	
Methylomicrobium alcaliphilum 20Z	4% CH_4	25ºC	Continuous	CSTR, 6% NaCl, Copper (25 µM)	37.4 mg/g DCW (IC) 8.3 mg/L (EC)	(Cantera et al., 2017a)
Methylomicrobium alcaliphilum 20Z	4% CH_4	25ºC	Continuous	Two sequential CSTR Bio-milking process	70.4 mg/g DCW	(Cantera et al., 2017b)
Methylomicrobium alcaliphilum 20Z	0.060 L/min CH_4-air emission containing 25.6 g CH_4/m^3	25ºC	Continuous	BCB, pH 9.0 Magnesium (0.2 g/L)	94.2 mg/g DCW	(Cantera et al., 2018b)
Enrichment of haloalkaliphilic bacteria					79.7 mg/g DCW	
Halomonas sp. strain PGE1	20% CH_4	30ºC	Batch	pH 9.0, 6% NaCl	91.7 mg/g DCW	(Cantera et al., 2019b)
Methylomicrobium alcaliphilum 20Z	31.5% CH_4 in H_2S-free upgraded biogas For Stage 5 and 6 CO_2 replaced by He	20ºC	Continuous	BCB CH_4:O_2:CO_2:He 31.5:55.0:13.3:0.23%	Stage 1: 2.9 mg/g DCW Stage 2: 4.0 mg/g DCW Stage 6: 108.7 mg/g DCW	(Cantera et al., 2020)
Enriched haloalkaliphilic consortium (dominated by *Methylomicrobium* and *Methylophaga* sp.)						

Table 2.5. Fatty acids and lipids production by methanotrophs since 2015, expressed as accumulation in cells (% DCW). Abbreviations: CSTR – continuous stirred-tank reactor, DCW – dry cell weight.

	STRAIN	METHANE	TEMP.	CULTIVATION TYPE	PROCESS DETAILS	ACCUMULATION	REFERNECE
FAME	*Methylomicrobium buryatense* 5GB1	20% CH_4	30ºC	Continuous	CSTR, pH 8.8 Oxygen limitation	10.7%	(Gilman et al., 2015)

Lipids	*Methylomicrobium buryatense* 5GB1	20% CH_4	30°C	Batch	pH 9.0 Increased nitrate in the medium	9.5%	(Dong et al., 2017)
Fatty acids	*Methylomicrobium buryatense* 5G(B1) mutant strain	50% CH_4	30°C	Batch	-	11% (111 ± 2 mg/g DCW)	(Demidenko et al., 2017)
FAME	*Methylomicrobium buryatense* 5GB1S engineered strain	20% CH_4	30°C	Batch	Increased nitrate, phosphate, and trace elements in the medium	9%	(Henard et al., 2017)
Lipids	*Methylomicrobium buryatense* 5GB1 mutant strain AP18	20% CH_4 (CH_4:O_2 5:4)	30°C	Batch	pH 9.0 Increased nitrate, phosphate, and trace elements in the medium	9.3%	(Fei et al., 2018)

Table 2.6. Organic acids production by methanotrophic bacteria since 2015, expressed in titers (g/L). Abbreviation: BCB – bubble column bioreactor.

	STRAIN	METHANE	TEMP.	CULTIVATION TYPE	PROCESS DETAILS	TITER / PRODUCTIVITY	REFERNECE
Lactic acid	*Methylomicrobium buryatense* 5GB1S *pLhldh* mutant strain	20% CH_4	30°C	Batch	Increased nitrate, phosphate, and trace elements in the medium	0.808 g/L	(Henard et al., 2016)
Lactic acid	*Methylomicrobium alcaliphilum* 20Z mutant strain	20% CH_4 (33% mock biogas in air)	30°C	Continuous	BCB	0.027 g/g DCW/h	(Henard et al., 2018)
Lactic acid	*Methylomicrobium buryatense* 5GB1 mutant strain pAMR4	21% CH_4	30°C	Batch	Ammonium as N source	0.50 g/L	(Garg et al., 2018a)
Crotonic acid	*Methylomicrobium buryatense* 5GB1C mutant strain pCA09	25% CH_4	30°C	Batch	-	0.06 g/L	(Garg et al., 2018b)
Butyric acid						0.08 g/L	
D-Lactic acid	*Methylomonas* sp. DH-1, LA-tolerant strain JHM80	20% CH_4	30°C	Batch	Increased nitrate, Kanamycin (10 μg/mL)	1.19 g/L	(Lee et al., 2019)
Acetic acid	Mixed culture dominated by *Candidatus 'Methanoperedens nitroreducens'*	90% CH_4	RT	Batch	Nitrogen limitation	0.097 g/L (1620 μmol/L)	(Cai et al., 2019)
Muconic acid	*Methylomicrobium buryatense* 5GB1 mutant strain pMUC	20% CH_4	30°C	Semi-continuous	CSTR Continuous gas supply	0.012 g/L	(Henard et al., 2019)
Succinic acid	*Methylomonas* sp. DH-1 mutant DS-GL strain	30% CH_4	30°C	Batch	pH 6.9	0.195 g/L	(Nguyen et al., 2019)
3-HP acid (hydroxypropionic)	*Methylosinus trichosporium* OB3b mutant MCRMP strain	30% CH_4	30°C	Batch	pH 7.0	0.061 g/L	(D. T. N. Nguyen et al., 2020)
4-HB acid (hydroxybutyric)	*Methylosinus trichosporium* OB3b 4HB-SY4 mutant strain	40% CH_4	30°C	Batch	-	0.011 g/L (10.5 mg/L)	(T. T. Nguyen and Lee, 2021)

Table 2.7. Other new products of methanotrophs achieved by engineered strains since 2015, expressed as accumulation in cells (mg/g DCW) for internally produced compounds or as titer (g/L) for externally produced compounds.

	STRAIN	METHANE	TEMP.	CULTIVATION TYPE	PROCESS DETAILS	ACCUMULATION / TITER	REFERNECE
2,3-butanediol	*Methylomicrobium alcaliphilum* 20ZM3/pNBM-Re mutant strain	20% CH_4	30°C	Batch	pH 8.8 Oxygen-limitation	0.086 g/L (86.2 mg/L)	(Nguyen et al., 2018)
Putrescine	*Methylomicrobium alcaliphilum* 20ZE4-pACO mutant strain	30% CH_4	30°C	Batch	Kanamycin (50 μg/mL) Supplementation with ammonium (2 mM)	0.098 g/L (98.08 ± 2.86 mg/L)	(Nguyen and Lee, 2019)
α-humulene (sesquiterpenoid)	*Methylomicrobium alcaliphilum* 20Z SQ08 mutant strain	50% CH_4	30°C	Batch	Two-phase cultivation 20% (v/v) dodecane as an organic phase	0.75 mg/g DCW	(A. D. Nguyen et al., 2020)
Cadavarine	*Methylosinus trichosporium* OB3b/cad4 mutant strain	30% CH_4	30°C	Batch	pH 7.5	0.284 g/L (283.63 mg/L)	(T. T. Nguyen et al., 2020)
α-humulene (sesquiterpenoid)	*Methylotuvimicrobium alcaliphilum* 20Z pDXP-07 mutant strain	50% CH_4	30°C	Batch	Two-phase cultivation 20% (v/v) dodecane as an organic phase	0.56 mg/g DCW	(Nguyen et al., 2021)
α-bisabolene (sesquiterpenoid)	*Methylotuvimicrobium alcaliphilum* 20Z pBs-02 mutant strain					12.24 ± 0.43 mg/g DCW (24.55 ± 0.86 mg/L)	

2.3. Bioproducts

The biotechnological potential of methanotrophic bacteria has been a subject of studies for many years and is already industrially exploited for commercial production of a couple of products: animal feed and PHA pellets. The growing interest in the possible application of methanotrophs is reflected in the increasing, through the years, number of scientific articles on methane conversion to bioproducts. The scale of conducted studies on methanotrophic production for specific products is shown in Table 2.1-2.7. In the second half of the last decade, researchers have focused mostly on achieving and improving the efficient production of biopolymers and methanol as well as single-cell proteins using methane as a carbon source. Additionally, the production of ectoine and the use of genetic engineering of strains for the synthesis of other valuable commodity chemicals and precursors became a significant part of conducted studies, creating a new trend for future research in this field.

2.3.1. Biopolymers

One of the most intensively studied methanotrophic product due to its potential value as a replacement for synthetic plastics are polyhydroxyalkanoates (PHA). PHAs are thermoplastic polyesters produced by various bacteria as a storage compound under nutrient-deficient conditions when a carbon source is present in excess. Microbial production of these biopolymers became the target of many researchers due to their plastic-like properties combined with high biodegradability, biocompatibility, sustainability and eco-friendly production process (Anjum et al., 2016). Recent advances in PHA production by different microorganisms were summarized by Koller (2020, 2017). Possible applications of PHAs are dependent on specific properties of produced polyester and span from industrial purposes as an alternative to petroleum-based plastic for packaging, to biomedicine and pharmacy as well as agriculture (Liu et al., 2020; Wendlandt et al., 2005). However, due to high production cost, low achieved production volume and relatively slow crystallization rates with high brittleness, the popularization of PHAs for commercial use is restricted (Samui and Kanai, 2019). The high cost of commonly used carbon sources (e.g. glucose) along with the possibility to mitigate climate change by using methane as a C_1 source for bacteria metabolism makes methanotrophs' application for biopolymer production very attractive. To date, there exists only one company that commercially produces PHAs from methane, Mango Materials based in the USA.

Polyhydroxybutyrate (PHB) is the first discovered and most studied PHA with mechanical properties similar to polypropylene or polyethylene. Accumulation of PHB, although occurs in all methanotrophs in suitable conditions, is much more efficient in the type II methanotrophs that utilize the serine pathway than in the type I methanotrophs (Rahnama et al., 2012). Pathway for PHB synthesis in methanotrophic bacteria cells (Figure 2.2) begins from acetyl-CoA which after series of enzyme-mediated reactions leads to the formation of PHB. Three essential enzymes catalyze reactions in this metabolic pathway; β-ketothiolase, acetoacetyl-CoA reductase and PHA synthase (Cantera et al., 2019a). These enzymes are encoded by *phaA*, *phaB* and *phaC* genes respectively and are commonly used for screening microorganisms for PHB-accumulators (Strong et al., 2016b). When conditions change and cell switches for restoring biomass growth, depolymerization and catabolism of PHB to acetyl-CoA is activated again through enzyme-mediated reactions. Catabolism of accumulated PHB, which provides intracellular reducing equivalents for cells, is catalysed by PHB depolymerase, β-hydroxybutyrate dehydrogenase, and acetoacetate succinyl-CoA transferase (Karthikeyan et al., 2015a).

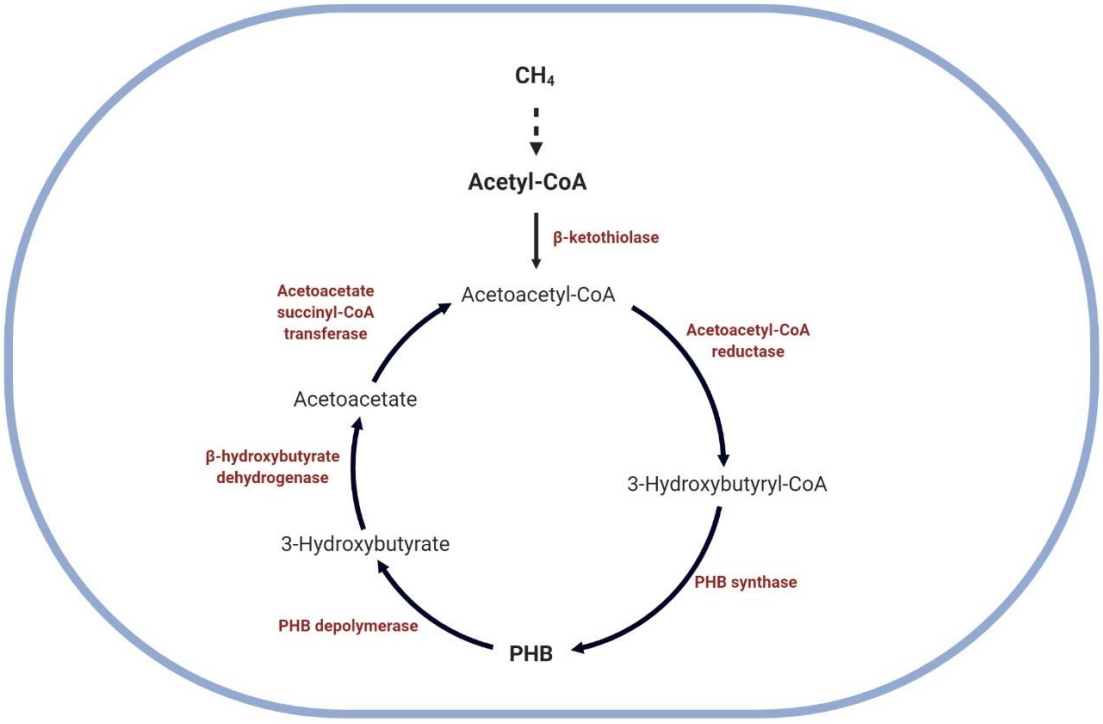


Figure 2.2. PHB synthesis cycle in methanotrophic bacteria

As the technology for methane-based productions advances, the need for improved processes and product properties created new trends in current methanotrophic biopolymer research. Nearly half of the studies reported for PHA production in the last few years (Table 2.1) focused on using mixed methanotrophic consortium rather than pure strain. Application of mixed cultures in microbial processes has a lot of advantages, mainly owning to interactions occurring within the microbial community, in this case, improving methanotrophs growth by removing toxic products and overproduced metabolites from the medium by co-inhibiting bacteria. Additionally to their stability in prolonged cultivations, the use of consortia enables to omit the need for sterile culture conditions and achieving higher accumulations of PHA when dominated by the type II methanotrophs (Chidambarampadmavathy et al., 2015a). While trying to improve the quality of produced biopolymers, the generation of PHAs copolymer (co-PHA) with more advantageous properties over PHB became of interest in many studies. PHB, even though commonly produced, has limited applications due to some disadvantageous properties e.g. narrow melting processing window, low thermal stability or lack of flexibility. The introduction of volatile fatty acids as a co-substrate during cultivation enables the production of co-PHA with decreased melting temperature and crystallinity along with increased flexibility (Myung et al., 2015). Most quantitative results for co-PHA production were achieved with the addition of valeric acid that leads to the accumulation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) a copolymer generated by incorporating 3-hydroxyvalerate (3HV) into PHA polymer (Fergala et al., 2018c). The properties of produced co-PHA differ depending on the monomer fraction incorporated into the copolymer (Myung et al., 2016a). Development in this field shows a promising perspective and is an important aspect to consider for future studies. The growing interest in the valorization of biogas as a feedstock for the production of valuable compounds influences also the field of bioplastic production. Studies conducted by Rodríguez et al. (2020a, 2020b) considered the possibility of growing *Methylocystis hirsuta* for PHB production on synthetic biogas as an alternative for commonly used pure methane. Assessment on the effect of major culture conditions for PHB synthesis under nitrogen limitation in batch processes resulted in PHB content at 45%, which is comparable to the average accumulation of this biopolymer achieved in cultures based on pure methane in air mixtures (Rodríguez et al., 2020a). A constant issue in biopolymer production remains the need to conduct optimization research for every specific inoculum used in the studied process as production is organism-specific (Rostkowski et al., 2013).

2.3.2. Methanol

Another highly popular and often exploited bioproduct of methane conversion is methanol. The production of methanol is widely studied in various processes due to its valuable possible uses as biofuel and as a precursor in the production of other commodity chemicals such as organic acids, alcohols, formaldehyde and olefins (Bjorck et al., 2018). Methanotrophs produce methanol at the first step of methane oxidation and extracellular accumulation of this alcohol can be achieved by inhibiting the MDH enzyme which normally would convert methanol to formaldehyde (Figure 2.3). The activity of MDH is dependent on Ca^{2+} concentration and becomes inhibited by lowering calcium content, most effective inhibitors usually added to the medium are phosphate, $MgCl_2$ or NaCl, cyclopropanol and EDTA (Ge et al., 2014; Hwang et al., 2015). Inhibition of MDH disrupts the following steps of methane metabolism which leads to a decline in bacterial growth and shortage of electrons needed for methane uptake. To prevent the negative effects of enzyme inhibition supplying an external source of reducing power is imperative for maintaining cell activity, this objective is generally achieved by the addition of formate (Alsayed et al., 2018).

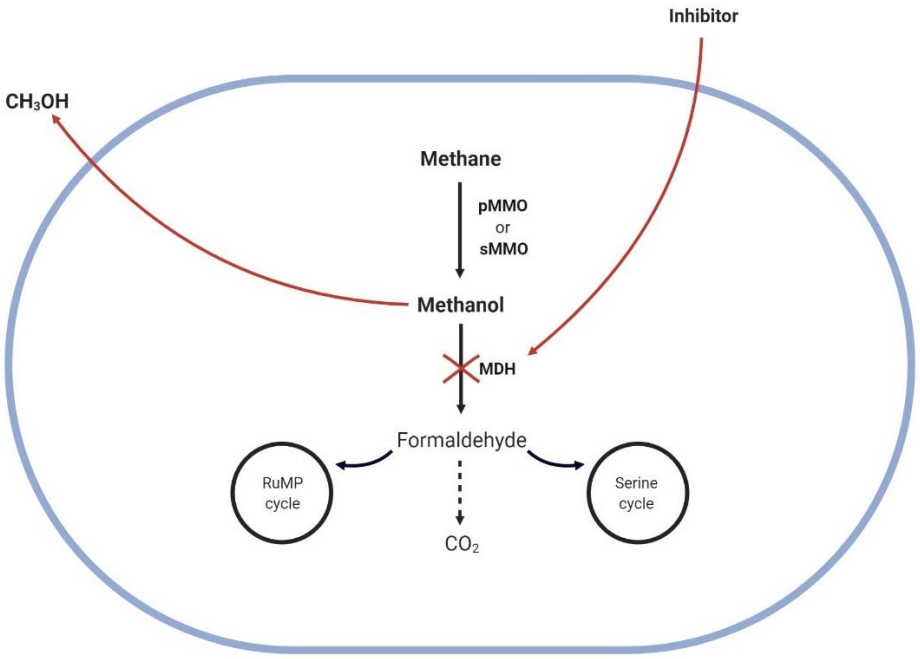


Figure 2.3. Methanol production in methanotrophic bacteria

In recent years two distinctive directions on studies for methanol production could be distinguished i) the use of biogas as a carbon source and ii) immobilization of cells for

process improvement. Comparative investigations on different sources of methane from pure methane to synthetic biogas and raw biogas from different sources (Table 2.2) were conducted mostly by the research team from Konkuk University, Republic of Korea, and showed advantages of using biogas over pure methane (Patel et al., 2020a, 2020d, 2018b, 2016b). The presence of CO_2 in biogas may contribute to higher stability of methanol production, the same effect is observed in immobilized cells cultures (Patel et al., 2018b). As observed by some researchers CO_2 may have an inhibiting effect on MDH activity and obstruct the complete oxidation of methanol to formaldehyde (Patel et al., 2018a; Xin et al., 2004). Noticeably, the main target of the conducted research remains the assessment of how various process conditions and MDH inhibitors addition effect methanol accumulation by different strains (Ghaz-Jahanian et al., 2018; Hur et al., 2017; Hwang et al., 2015; Mardina et al., 2016; Taylor et al., 2018) or mixed culture (Alsayed et al., 2018; Sheets et al., 2017a).

2.3.3. Microbial protein

In the late 1960s, single-cell protein (SCP) achieved from methanotrophic biomass became the first product of methane conversion to be industrially commercialized and used as an animal feed additive. It is currently produced from natural gas by Calysta as FeedKind® protein and by Unibio as Uniprotein®. SCP refers to microbial protein biomass that can be produced by various microorganisms but the application of methanotrophs is more advantageous as they have higher protein content compared to fungi and yeast while also having cell walls more digestible than that of algae (Khoshnevisan et al., 2019). As of now methanotrophic biomass with high protein content and favorable amino acids profile is produced as an ingredient of mostly fish feed, but also for other animals such as pigs, chickens and even cats and dogs. Microbial protein is produced by bacteria in growth-optimal conditions (Figure 2.4) reaching even to 70% of dry weight for *Methylococcus capsulatus* which for years was the most often considered methanotroph for SCP production processes (Pieja et al., 2017). Strategy for SCP production aims to maximize the cell yield on methane while minimizing yield from ammonium as nitrogen source in the medium - when biomass is produced from ammonium, the cells accumulate lower protein content (Khoshnevisan et al., 2019).

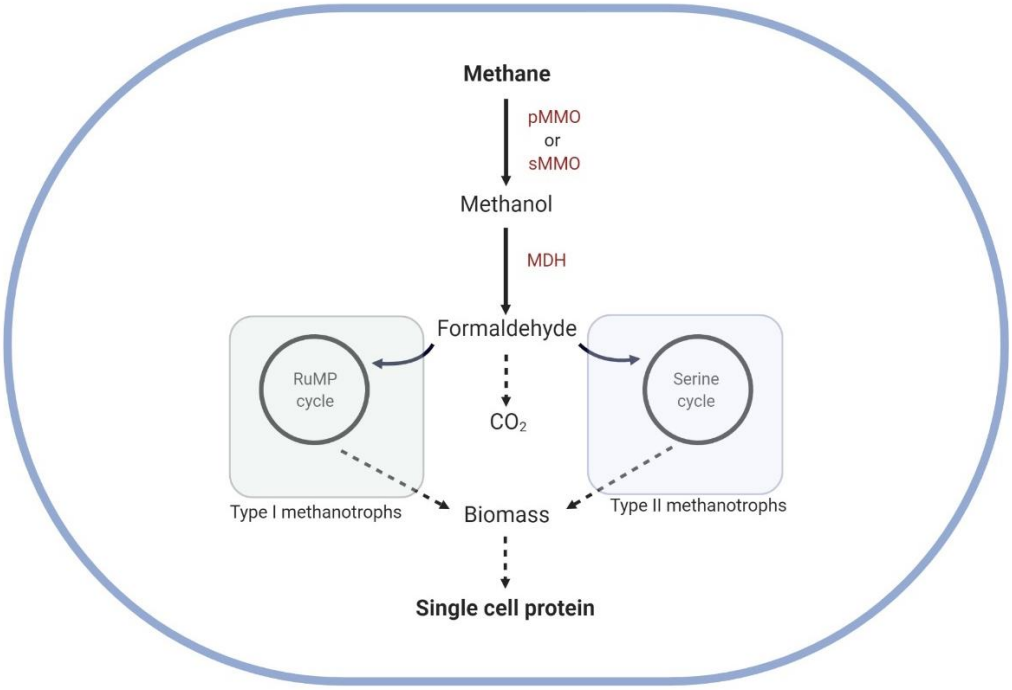


Figure 2.4. Single cell protein production in methanotrophic bacteria

Similar to biopolymers and methanol production, valorization of biogas also became a considered option in studies on SCP production. Research on biogas utilization for microbial protein production by methanotrophs has begun to appear mostly since 2019. Studies conducted on raw biogas containing H_2S reported lower yields compared to pure methane, as the presence of sulfides in the medium reduced amino acids content (Tsapekos et al., 2019; Xu et al., 2020). Better results could be achieved with upgraded biogas as showed by Acosta et al. (2020) who achieved almost 70% protein content in the process conducted on enriched methanotrophic and hydrogenotrophic culture. Operating microbial protein production process on mixed microbial cultures has been the main focus of studies for the past couple of years. Although symbiosis of methanotroph with other microorganism does not always positively affect protein accumulation in the culture as in the case of co-culture of *Methylococcus capsulatus* and *Chlorella sorokiniana* (Rasouli et al., 2018), cultivations of mixed methanotrophic cultures usually give better or comparable results to the production of SCP by pure strains. Produced protein content ranged from 40% up to 70% while for most cases it was higher than 50% (Table 2.3). Except for the differences in protein content also amino acids profile may differ depending on the used microorganism and process conditions but in most cases achieved profiles were suitable for substitution of conventional protein sources.

2.3.4. Ectoine

A relatively new methanotrophic bioproduct of high value is a cyclic imino acid, ectoine, which production has been extensively studied in the last few years primarily by one research team from Valladolid University, Spain (Table 2.4). Ectoine is found in a wide range of halotolerant bacteria as it is a compound naturally produced in high salinity environments for the protection of cells and to maintain cell osmotic integrity. Due to its exceptional properties in stabilizing enzymes, DNA-protein complexes and nucleic acids, ectoine can be applicable in medicine and nutrition but is mostly used in the pharmaceutical and cosmetic industry (Cantera et al., 2018a; Strong et al., 2016a). Pathway for ectoine synthesis (Figure 2.5) begins from acetyl-CoA and aspartate as a side branch of the aspartate family amino acids synthesis pathway. In the series of enzyme-mediated reactions that convert aspartate into L-ectoine, there are three key enzymes encoded by ectABC cluster: diaminobutyric acid aminotransferase (EctB), diaminobutyric acid acetyltransferase (EctA) and ectoine synthase (EctC) (Cantera et al., 2019a; Khmelenina et al., 2015). Although ectoine is produced intracellularly it can be released into the medium when the environment changes for lower salt concentration, due to hypoosmotic shock (Strong et al., 2016a). Cantera et al. focused their research on optimization of major factors affecting ectoine production in batch and continuous processes (Cantera et al., 2017a, 2016) as well as on continuous production by well-established ectoine-accumulating strain *Methylomicrobium alcaliphilum* 20Z (Cantera et al., 2020, 2018b, 2017a, 2017b, 2016). They have observed that copper added to the medium could have mediated excretion of produced ectoine up to 20% (Cantera et al., 2017a) and that apart from major factors such as NaCl concentration, also increased magnesium concentration resulted in higher ectoine yields (Cantera et al., 2018b). Using pure cultures and methane as a carbon source they were able to produce up to 100 mg ectoine/g of biomass (Cantera et al., 2018b). Following the trend of operating microbial methane conversion processes by mixed methanotrophic cultures and on biogas researchers were recently able to obtain slightly higher (up to 110 mg ectoine/g biomass) product accumulation in a continuously run bubble column bioreactor without the supplementation with Mg or Cu ions (Cantera et al., 2020).

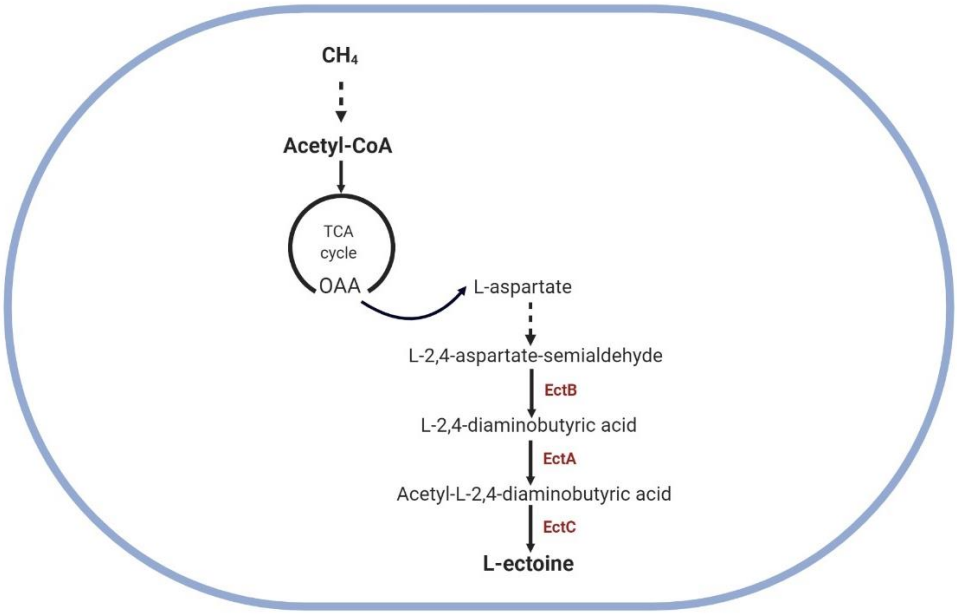


Figure 2.5. Ectoine synthesis pathway in methanotrophic bacteria

2.3.5. Biodiesel precursors

Besides producing biomethanol as a biofuel, methanotrophs could be also indirectly used for biodiesel production by directing methanotrophic metabolism to fatty acids and lipids accumulation. Fatty acids and glycerolipids that are produced from acetyl-CoA (Figure 2.6) during cell growth on CH_4 and are incorporated into membranes can be precursors for biodiesel fuel blendstock production (Fei et al., 2018). *Methylomicrobium buryatense* is the main methanotroph to be considered as a workhorse for this process due to its robust growth and high lipid content (Gilman et al., 2015). Some progress in this field has been made in recent years mainly with the use of genetic tools to improve *M. buryatense* strain for fatty acids and lipids yield. Gilman et al. (2015) showed that maximum accumulation of lipids from wild-type strain was limited to 10.7% even under continuous growth conditions and such level of production has not yet been significantly improved in studies with engineered *M. buryatense* (Table 2.5). Conducted modifications of wild-type strains aimed to overcome bottlenecks for higher fatty acids and lipids production such as fatty acid degradation and acetyl- and malonyl-CoA levels in cells as the precursor for lipids production (Demidenko et al., 2017; Henard et al., 2017). *M. buryatense* strain engineered by Henard et al. (2017) aimed at overexpression of phosphoketolase pathway which resulted in improved lipid yield due to the increase in acetyl-CoA level but total FAME (fatty acid methyl esters) content was similar to that of a wild-type strain. On the other hand, Fei et al. (2018) targeted glycogen synthesis limitation and achieved a 90%

enhancement in lipid content but its overall accumulation in this study was still no more than 10% of dry weight. Only the strain engineered by Demidenko et al. (2017), due to its modified fatty acid biosynthesis regulation, was able to produce slightly over 11% of extractable FAME by dry weight. Production of fatty acids is restricted by metabolic fluxes upstream of fatty acid biosynthesis and low carbon conversion efficiency, these are crucial metabolic issues that need to be addressed in the future prior an industrial application of this process (Demidenko et al., 2017; Fei et al., 2018).

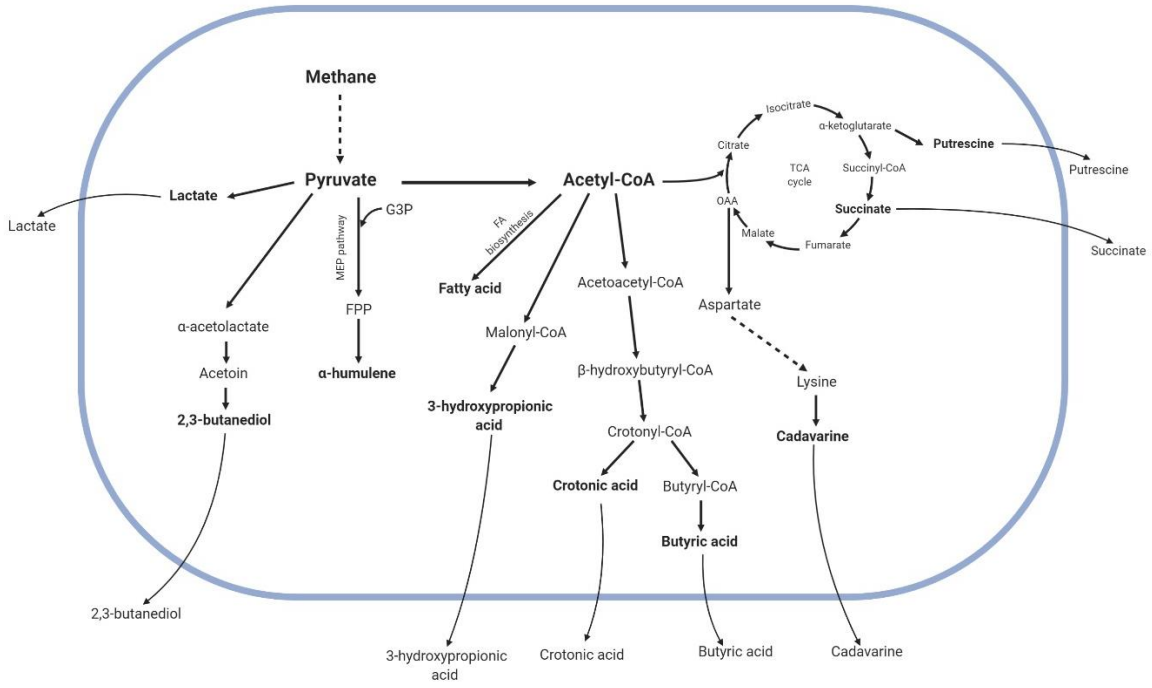


Figure 2.6. Secondary metabolites synthesized through the pyruvate or acetyl-CoA node in methanotrophs

2.3.6. Organic acids

Metabolism of methane is also considered for the production of other commodity chemicals such as various organic acids (Figure 2.6), some produced naturally by the strain and some obtained by using genetic tools (Table 2.6). With advances in discovering detailed metabolic pathways and genome sequencing, genetic modification of wild methanotrophic strain for improvement of yield or synthesis of non-native organic acids becomes popular. Especially of interest in recent years was the production of lactic acid which is natively produced from pyruvate by lactate dehydrogenase (LDH) but at low yields due to the toxic effect of lactate concentration on the cells (Henard et al., 2016)(Henard et al., 2016). Constructing lactate-tolerant strains and improving production yield on methane as well as

biogas was studied and general limitations for industrial applications such as low carbon fluxes to targeted product, low methane uptake due to gas-to-liquid transfer limitations and product toxicity were defined (Garg et al., 2018a; Henard et al., 2018, 2016; Lee et al., 2019). Potential improvement of process efficiency may result from removing pyruvate conversion to acetyl-CoA which could boost carbon flux to lactate and other engineering strategies for increased lactate tolerance of the strain should be targeted in the future (Garg et al., 2018a; Henard et al., 2018). Other possible organic acids achieved through the acetyl-CoA node include acetate, C-4 carboxylic acids such as crotonic and butyric acids, succinate, and 3-hydroxypropionic acids (Cai et al., 2019; Garg et al., 2018b; D. T. N. Nguyen et al., 2020; Nguyen et al., 2019). The most distinctive trend for studies on the synthesis of this type of secondary metabolites is assessing the feasibility of using different genetical tools for green chemicals production from methane feedstock.

2.3.7. Other products

In the last few years, a research team from Kyung Hee University, Republic of Korea, has begun actively working on genetic modification of methanotrophs for heterologous production and improvement of existing metabolic pathways (Figure 2.6) of various chemical compounds from aforementioned organic acids to other new products such as 2,3-butanediol, putrescine, cadaverine or sesquiterpenoids on an example of α-humulene and α-bisabolene (Table 2.7). These compounds could possibly be used in a wide range of applications e.g. as biofuels (2,3-butanediol), monomers for bioplastic (putrescine, cadaverine), agrochemicals (putrescine), in medicine (putrescine, cadaverine, sesquiterpenoids) or industry (cadaverine, sesquiterpenoids) (A. D. Nguyen et al., 2020; Nguyen et al., 2021, 2018; Nguyen and Lee, 2019; T. T. Nguyen et al., 2020). Scientists conducted primary research on exploring the potential of methanotrophic bacteria as a cell factory for the production of novel and valuable bioproducts from methane contained in waste streams. *M. alcaliphilum* 20Z and *M. buryatense* 5GB1, as well as *Methylosinus trichosporium* OB3b, are the most optimal strains for genetic manipulations due to their growth rate, metabolic capacities and existing genetic tools (A. D. Nguyen and Lee, 2021). Comprehensive reviews on genetic engineering of methanotrophs for commodity chemicals production were recently neatly presented by Hwang et al. (2018) and A. D. Nguyen and Lee (2021). It is a relatively new field of studies for MOB and there is still a need for a more in-deep investigation, mostly focusing on resolving limitations such as low

carbon flux toward target products, methane oxidation efficiency and expression levels of key enzymes.

2.4. Challenges and future perspectives

Biological processes are constrained by some metabolic and technological limitations that prevent them from achieving maximum theoretical production yield. Table 2.8 summarizes the main challenges for commercially successful methane to product conversion processes. Efficient production is strictly linked to substrate availability for the biocatalyst, i.e. cells, which in the case of gas fermentation depends highly on the solubility of used gases. For all methanotrophic methane to product conversion processes, there exists one universal problem arising from using sparingly soluble gases. Utilization of methane as gaseous substrate and oxygen needed for oxidation, both of which have poor solubility in the medium, limits mass transfer affecting substrate consumption rate, cell concentration and consequently lowering production yield (Yasin et al., 2015). Primary research studies on methanotroph-based production are usually conducted in bottles or in continuously stirred tank reactors (CSTR) which are not able to support sufficient mass transfer. Although it is possible to enhance the mass transfer of methane to the aqueous phase by increasing the agitation rate, this approach is detrimental to the cells as it causes high mechanical stress and severe damage to the cell (Cantera et al., 2017a). To overcome this limitation, different bioreactor designs for high gas-liquid mass transfer were tested throughout the years, such as bubble column reactors, airlift bioreactors, trickle bed reactors, vertical loop reactors, pressurized bioreactors and fluidized bioreactors (Chidambarampadmavathy et al., 2015a; Ghaz-Jahanian et al., 2018; Rodríguez et al., 2020b; Sheets et al., 2017a). Designing the optimal bioreactor which could ensure higher substrate availability and cell concentrations, and hence high product concentration under no substrate limitation or inhibition is a fundamental technological challenge to tackle for economical production (Yasin et al., 2015). Jawaharraj et al. (2020) in their review on the valorization of discarded methane in wastewater treatment systems, have also shown two other than biotechnological solutions for enhancing CH_4 conversion efficiency; application of plasma technology and surface modification approaches for enhancing biofilm growth, which shows the various ways in which scientists try to improve the process of methane conversion by MOB.

Table 2.8. Summary of technological and biological challenges and limitations of methane bioconversion

	Challenges and limitations
Technological	Low gas-liquid mass transfer Requirement of sterility for monoculture fermentations The flammable or explosive character of certain methane/oxygen ratios
Biological	Low methane uptake The slow growth of methanotrophs Low carbon fluxes to secondary metabolites

The high specificity of metabolic requirements for particular types of processes combined with organism-specificity depending on the characteristic of strain used, its optimal environmental conditions and other key factors make optimization of microbial conversion a complex issue for researchers. As methanotrophs are a very diversified group of microorganisms that can be found in many different and even extreme environments, conducting large-scale isolation and screening for efficient product accumulators is the first and primary step for enhancing bioproduct production from a metabolic aspect. The traditional and commonly used isolation method is based on enriching environmental samples in a liquid medium under CH_4 in the headspace and pure strain is then isolated by repeated streaking and single colonies picking on agar plates. Furthermore, by changing medium composition and incubation conditions it is possible to screen for unconventional bacteria. Unfortunately, this method is laborious and time-consuming and quite limits the amount and diversity of isolated strains (Kim et al., 2018). Modern industrial microbiology develops rapidly and to follow its progress there is a need for novel isolation techniques that would allow acquiring methanotrophs from environmental samples at much shorter times than traditionally. Some improvements in this field were made in recent years and a couple of unconventional methods of methanotrophic bacteria isolation were suggested. These novel techniques were compiled and reviewed by Kwon et al. (2019) and include the high-throughput extinction cultivation, the soil substrate membrane system and the CSTR screening method for isolation of fast-growing methanotrophs. Although these new approaches to strain isolation have shown their potential there is still some work to be done especially as these methods are not yet optimized. As acquiring fast-growing strains with diversified properties is obligatory for the successful implementation of methanotrophs in industrial production processes, developing and improving isolation techniques should be put under scientists' attention (Kwon et al., 2019).

Except for the isolation of new MOB strains, there also exists a constant need to conduct detailed optimization research for every specific inoculum used in the particular process type to provide the best conditions for high yield production. Differences in metabolic pathways essential for the targeted bioproduct coupled with organism-specific culture requirements for every strain are essential factors to be considered in designing biotechnological processes. For all methanotrophic cultures proper aeration and air:methane ratio are one of the most important factors affecting cell growth (Hwang et al., 2015). When applying raw biogas, its content has to be controlled as the presence of H_2S in the mixture may limit methanotrophs growth and hinder process efficiency (Patel et al., 2016b). Besides basic cultivation parameters affecting microbial processes such as temperature and pH, different environmental requirements for methane to bioproduct conversion define some other important factors to be optimized in the process. Biopolymer production yield is dependent on nutrient limitations conditions which induce PHA accumulation, nitrogen and carbon source along with copper, as an essential ion for PHB synthesis, are all important parameters to be optimized (Chidambarampadmavathy et al., 2015b; Karthikeyan et al., 2015a). High methanol production is correlated to the activity of MMO and inhibition of MDH and as such Cu^{2+} ions concentration, affecting MMO, are also often important in this process (Patel et al., 2016a). Selection of appropriate MDH inhibitor and its concentration along with proper substitution of reducing power by formate are common methods for increasing production yield of methanol (Alsayed et al., 2018; Hur et al., 2017). In processes targeted for SCP production, it is essential to adapt conditions optimal for cell growth in short time periods. Substrate and nutrients availability is crucial and although conventional media can be replaced with cheaper sources e.g. filtrated effluent from anaerobic digestate, but studies have to be conducted to ensure a sufficient level of all nutrients required for the cell growth (Zha et al., 2021). On the other hand, the effectiveness of the ectoine biosynthesis pathway is correlated with the salinity to some degree, and providing sufficient NaCl and CH_4 concentration along with the optimum temperature is the most important factor for intracellular ectoine accumulation (Cantera et al., 2016). Improvement of fatty acids and other commodity chemicals production by methanotrophs relies on finding a way for increasing carbon fluxes to their biosynthesis pathways, as upstream metabolic fluxes restrict their production (Demidenko et al., 2017; Garg et al., 2018a; Henard et al., 2019; Nguyen et al., 2018). Synergistic interactions occurring between organisms in the environment provide attractive advantages that can be implemented into bioprocess. Metabolic limitations of one strain can be overcome by co-

culturing it with another one or in more complex consortia, where intricate interactions between microorganisms improve product synthesis and stability of the production process (Patel et al., 2020b; Singh et al., 2019).

2.5. Conclusions

Methanotrophs play a significant role in the global carbon cycle as the only biological methane sink. However, methane-oxidizing bacteria have also a huge industrial potential to transform methane into valuable products. Review of publications since 2015 indicated the largely growing repertoire of products obtained via biological conversion of methane and in the last years new directions of research can be observed for methane-based synthesis.

On one hand, in the second half of the last decade, researchers have focused on improving the efficiency of production of biopolymers and methanol as well as on single-cell proteins utilizing methane-based feedstocks as a carbon source. On the other hand, it's clear that recent advances in genomics, physiology and methodology for genetic engineering of methanotrophs led to an increased interest in the production of secondary and non-native metabolites. Most of these processes are yet at its infancy and serve as a proof-of-concept of the potential methane-based biorefineries.

Despite methane being considered relevant feedstock of the future and despite significantly increasing the number of research studies in the field in the last years there is still much room for improvement of existing technologies. Much is yet to be discovered, especially in terms of mechanisms controlling CH_4 uptake and assimilation. New bioreactor and process designs to overcome mass transfer limitations will be a major step for the commercialization of potential bioproducts derived from methane. In addition, switching from monocultures to mixed consortia and potential application of diverse methane-based feedstocks are most likely to be the main focus of further development in the near future. Moreover, restricted metabolic fluxes upstream of secondary product biosynthesis and low carbon conversion efficiency are crucial metabolic issues that need to be addressed in the near future for industrial application of these processes.

The broad range of products that can possibly be acquired from low-cost and widely available methane feedstock coupled with a positive effect on the environment is sure to

promote methane bioconversion as one of the key green chemicals production technologies in the future.

2.6. Acknowledgment

This work was supported by the National Science Centre (Poland) under the grant agreement no. 2019/35/D/ST8/03530. Figures 2.1-2.6 were created with BioRender.com.

Chapter 3

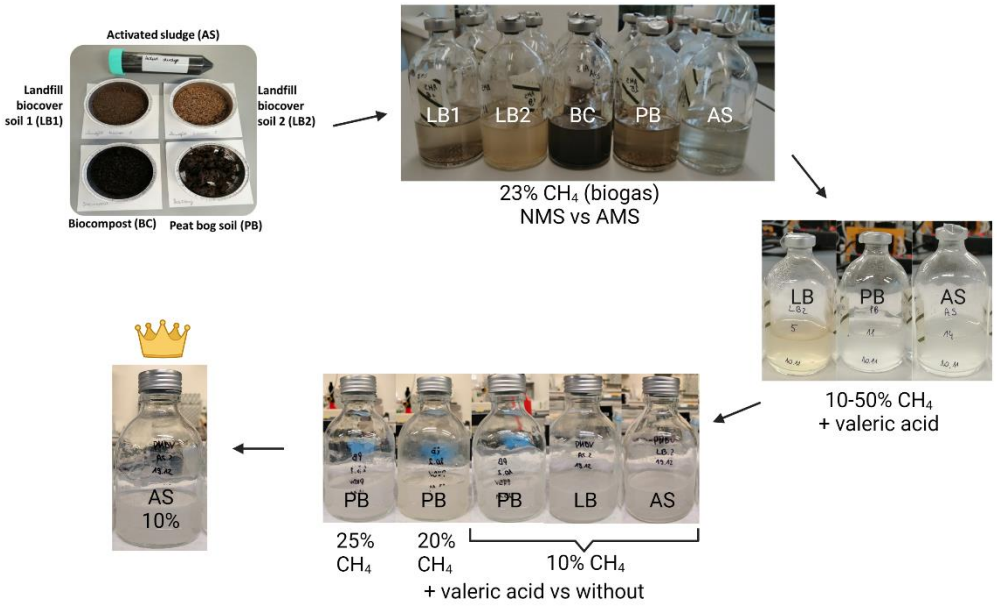
Enrichment of mixed methanotrophic cultures producing polyhydroxyalkanoates (PHAs) from various environmental sources

This chapter is published as:

Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M., 2024. Enrichment of mixed methanotrophic cultures producing polyhydroxyalkanoates (PHAs) from various environmental sources. Science of the Total Environment 912. https://doi.org/10.1016/j.scitotenv.2023.168844

Abstract

Methanotrophic bacteria can use atmospheric methane (CH_4) as a sole carbon source for the growth and production of polyhydroxyalkanoates (PHA). The development of CH4 bioconversion processes relies heavily on the selection of an efficient methanotrophic culture. This research assessed the effect of selected growth conditions, such as nitrogen sources on the enrichment of methanotrophic cultures from various environments for PHA accumulation. Nitrate-based medium favoured the culture growth and selection for PHA-producing methanotrophic cultures with *Methylocystis* sp. as a major genus and accumulation of up to 27% polyhydroxybutyrate (PHB) in the biomass. Three PHB-producing cultures: enriched from waste activated sludge (AS), peat bog soil (PB) and landfill biocover soil (LB) were then tested for their ability to produce PHA copolymer at different CH4:O2 ratios. All enriched cultures were able to utilise valeric acid as a co-substrate for the accumulation of PHA with a 3-hydroxyvaleric (3HV) fraction of 21-41 mol% depending on the inoculum source and CH4 concentration. The process performance of selected cultures was evaluated and compared to the culture of reference strain Methylocystis hirsuta DSM 18500. All mixed cultures irrespective of their inoculum source had similar levels of 3HV fraction in the PHA (38 ± 2 mol%). The highest poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) production was observed for AS culture at 10% CH4 with an accumulation of 27 ± 3% of dry cell weight (DCW), 3HV fraction of 39 ± 2 mol% and yield of 0.42 ± 0.02 g-PHA/g-substrate.



3.1. Introduction

Throughout the 21st century, global greenhouse gas (GHG) emissions have been steadily rising, experiencing a significant 40% increase during the first two decades (ClimateWatch, 2022) and further increasing global warming with the passing years (NOAA, 2022). CH_4, a potent GHG with a higher global warming potential than CO_2, was reported to be responsible for around 30% rise in the global temperature since the industrial revolution (IEA, 2022). Human activities are responsible for 60% of global CH_4 emissions primarily stemming from agriculture, the energy sector, and waste management, while natural sources are mainly associated with CH_4 emissions from wetlands (IEA, 2022). CH_4 released from major anthropogenic sources such as livestock farms, landfills, and wastewater treatment plants can be collected and transferred through pipeline systems and then burned or used for power generation. Such captured gas could potentially also be used as a carbon source in the CH_4-based production processes driven by methanotrophic bacteria in a pure or mixed culture systems (Jawaharraj et al., 2020).

Methanotrophic bacteria are able to directly utilise atmospheric CH_4 as an only carbon source and convert it into various valuable products through different metabolic pathways (Gęsicka et al., 2021). One class of the main products of interest in CH_4 conversion are biopolymers that could replace conventional plastics, polyhydroxyalkanoates (PHA) commonly in the form of polyhydroxybutyrate (PHB) and its co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with enhanced mechanical properties (Strong et al., 2016a). Most aerobic methanotrophs are a part of Proteobacteria phyla and can be classified as Alphaproteobacteria (type II methanotrophs) or Gammaproteobacteria (type I methanotrophs) mainly based on their carbon fixation metabolism (Hanson and Hanson, 1996). Type I methanotrophs assimilate CH_4-derived carbon through a RuMP pathway and are known for their higher growth rates as compared to type II methanotrophs, which fixate carbon through the serine cycle and are known for PHA synthesis (Karthikeyan et al., 2015a). Methanotrophic bacteria are ubiquitous and can be sourced from a wide range of habitats, while methanotrophic communities for PHB production are usually sourced from activated sludge (Fergala et al., 2018a; Myung et al., 2015; Pérez et al., 2019b; Salem et al., 2021; Zhang et al., 2018) but also from other reservoirs such as landfills, marine sediments, rice fields, and other environments associated with CH_4 emission (Chidambarampadmavathy et al., 2015a; Karthikeyan et al., 2015b; Kulkarni et

al., 2022; Luangthongkam et al., 2019a, 2019b). Screening and enrichment of environmental samples in the search for methanotrophic microorganisms with high PHA-producing potential is crucial for the development of an efficient CH_4-to-biopolymer process. Most research is focused on the enrichment of PHA-accumulating methanotrophic cultures from one or two sources. In the present study, the tested inoculum range was widened to five niches to see how microbial communities from various environments develop when grown on CH_4 alone and to compare their potential for polymer accumulation.

Furthermore, previous studies have indicated that PHB accumulation is primarily observed in type II methanotrophs (Pieja et al., 2011). Therefore, the enrichment of mixed methanotrophic cultures capable of producing PHA requires the application of additional selective pressures that promote the growth of type II over type I methanotrophs. These selective pressures can include various factors such as different nitrogen sources, the CH_4:O_2 ratio, or copper concentration (Fergala et al., 2018a). In addition, methanotrophic cultures can be utilised for copolymer production when supplied with fatty acids as co-substrates. However, there have been only a limited number of studies investigating PHA copolymer production by mixed methanotrophic cultures to date (Fergala et al., 2018c; Luangthongkam et al., 2019a, 2019b; Myung et al., 2015). To achieve optimal conditions for PHA accumulation with a high fraction of incorporated 3-hydroxyvaleric (3HV) monomers in the mixed culture process, it is crucial to evaluate several key parameters related to CH_4 utilization. This evaluation should include an assessment of the impact of cosubstrate addition on the microbiome structure, substrate utilisation and PHA yield. Such a comprehensive understanding of these factors will contribute to the development of efficient CH_4-to-PHA conversion processes by mixed methanotrophic cultures.

This study aimed to enrich PHB-producing mixed methanotrophic cultures from various environmental origins using diluted biogas as a carbon source and to evaluate the effect of the type of nitrogen source on the culture development and the biopolymer accumulation potential. Furthermore, it aimed to assess the effect of CH_4:O_2 ratios on microbial culture structure and PHBV accumulation with added co-substrate and to determine the most optimal conditions for PHA production by diverse mixed methanotrophic cultures. Finally, this work also compared the efficiency of PHA production solely from CH_4 and with a co-substrate to select the most promising microbial culture for further applications in biological PHA copolymer production.

3.2. Methods

3.2.1. Inoculum source, medium, and cultivation conditions

Five environmental samples were sourced for methanotrophic bacteria enrichment. Landfill biocover soil from a freshly formed (LB1) and aged (LB2) landfill from a landfill facility at Poznań, Poland, as well as four-week-old biocompost (BC) (sampled from a compost pile at the same landfill facility), were collected in an air-tight bags. Soil from a peat bog (PB) located north of Poznań, Poland, and waste activated sludge (AS) from the Central Municipal Wastewater Treatment Plant (WWTP) (Poznań area, Poland) were all similarly collected on the same day and used immediately for starting the enrichment cultures. The reference culture of *Methylocystis hirsut*a DSM 18500 was purchased from DSMZ (Braunschweig, Germany).

Minimal salt media for the growth of methanotrophic mixed cultures were prepared based on Whittenbury et al. (1970) with slight modifications. If not otherwise specified, all cultures were grown in nitrate mineral salt (NMS) medium containing (per litre): KNO_3, 1 g; $MgSO_4 \cdot 7H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg; chelated Fe solution, 2 mL, molybdate solution, 0.1 mL and trace elements solution, 1 mL. Chelated Fe solution contained (per litre) $FeCl_3 \cdot 6H_2O$, 0.8 g; EDTA sodium salt, 2 g and concentrated HCl, 3 mL. Trace elements solution contained (per litre): $FeSO_4 \cdot 7H_2O$, 500 mg; 400 $ZnSO_4 \cdot 7H_2O$, 400 mg; $MnCl_2 \cdot 4H_2O$, 20 mg; $CoCl_2 \cdot 6H_2O$, 50 mg; $NiCl_2 \cdot 6H_2O$, 10 mg; H_3BO_3, 15 mg and EDTA sodium salt, 250 mg. The medium was sterilized by autoclaving at 121^{o}C for 20 min. Phosphate stock solution (pH 6.8) containing (per litre): KH_2PO_4, 26 g and $Na_2HPO_4 \cdot 12H_2O$, 71.6 g; was prepared and autoclaved separately. 10mM $CuSO_4 \cdot 5H_2O$ solution and 10x concentrated vitamin stock solution containing (in per litre): biotin, 20 mg; folic acid, 20 mg; thiamine HCl, 50 mg; calcium pantothenate, 50 mg; vitamin B_{12}, 1 mg; riboflavin, 50 mg, and nicotinamide, 50 mg; were prepared separately by cold sterilization. Sterile NMS medium was supplemented (per litre) with 2 mL of phosphate stock solution, 0.2 mL vitamin stock solution, and 0.5 mL copper solution. For the biopolymer accumulation test, an NMS medium without a nitrogen source (N-free NMS) was used to induce cells in nitrogen depletion conditions.

If not otherwise specified, all cultures were grown in batch modes in 120 mL serum bottles in 40 mL cultures sealed with butyl septa and aluminium caps. Headspace gas composition

was renewed every 2-3 days with defined gas composition by flushing the bottles for 2 min. Cultures were incubated in an orbital shaker at 30°C and 130 rpm. All cultures were done in triplicates.

3.2.2. Culture enrichment on biogas

Enrichment cultivations were done under biogas with CH_4 content of 56% mixed with air as a sole carbon source and by using two media differentiated by the nitrogen source to assess the impact of nitrate and ammonium on culture development and polymer production. Biogas was acquired from the municipal WWTP (Poznan area, Poland) installation, after desulfurization. Ammonium medium (AMS) was prepared the same as NMS medium with one modification where 1 g/L of KNO_3 was changed for 0.5 g/L of NH_4Cl. Collected environmental samples were used as initial inoculum as follows; 5 g of each sample were suspended in 25 ml (20% w/v) of NMS or AMS medium and mixed, 3 mL of the suspension were then diluted 5x, shaken for 10 min and used as an initial inoculum (4% w/v). Serum bottles with 36 mL NMS or AMS medium were inoculated with 4 mL of prepared inoculum (10% v/v), sealed, and then headspace composition was defined by flushing the bottles with synthetic air, then taking out 32 mL of air with an air-tight syringe and exchanging it with 32 mL of biogas, giving final CH_4 concentration of 23% (1.8 CH_4:O_2). Enrichment was conducted as a series of six one-week culture transfers when at the end of every week 4 mL of culture was used to inoculate fresh 36 mL of medium. After seven weeks enriched cultures biomass were collected by centrifuging at 6000 rpm for 8 min (MPW-352R, MPW, Poland), biomass pellets were then resuspended in 40 mL N-free NMS to induce PHB accumulation. Gas headspace was defined as previously. After two days of accumulation, biomass was again collected by centrifugation, then washed twice with sterile distilled water and stored at -20°C until lyophilization. Figure S3.1 shows a simplified scheme of conducted enrichment. Headspace gas composition was analysed in a representative bottle of every culture variant before every gas renewal. Samples for microbial composition analysis were collected from the initial inoculum and before every transfer. To compare the PHB-producing potential of enriched cultures with a pure culture of methanotrophic bacteria *M. hirsuta* DSM 18500 was cultured for a week in both media in the same conditions and then induced for PHB accumulation.

3.2.2. PHAs production assays at different CH_4 concentrations

To test the effect of the CH_4:O_2 ratio on the culture structure and ability to accumulate PHAs with added co-substrate, three chosen enriched cultures were cultivated for two weeks in six different CH_4 concentrations (Fig. S3.2). Serum bottles with 36 mL NMS medium were inoculated with 4 mL of LB2 (from here on named as LB), PB, or AS culture (10% v/v) and were cultured under six CH_4 concentrations: 10, 20, 25, 30, 50 and 90% in the air, equivalent to CH_4:O_2 ratio of 1:2, 4:3, 5:3, 2:1, 5:1 and 45:1. After a week, 4 ml of culture were transferred to a fresh medium and culture for another week, after which biomass was collected and resuspended in an N-free NMS with 100 mg/L of valeric acid (≥99% Sigma-Aldrich) to induce accumulation of PHBV. After two days biomass with accumulated polymer was collected washed twice with distilled water and stored at -20^{o}C for further processing. Samples for microbial analysis were collected at the end of the growth phase. Gas mixtures used for flushing were fixed by using a mass flow controller (DPC, Aalborg, USA). CH_4 (≥99.995) was purchased from Air Liquide S.A. (Paris, France) and synthetic air (≥99.999) was purchased from Air Products and Chemicals, Inc. (Allentown, USA).

3.2.4. Copolymer production studies

To further study the copolymer production in the most promising cultures from CH_4 concentration trials, five cultures were grown in larger volumes (200 mL): PB at 10, 20 and 25%, AS at 10% and LB at 10% of CH_4. 4 mL of frozen culture from the previous stage were used to inoculate 36 mL of NMS media and grown for a week in respective CH_4 concentrations. Additionally, a pure strain of *M. hirsuta* DSM 18500 was cultured as a reference at 10% of CH_4. Biomass was then collected and used to inoculate 580 mL serum bottles filled with 200 mL of NMS (5% v/v) so that the initial OD_{600} of cultures was 0.02-0.03, except for the PB 10% and PB 25% cultures which due to their floccular nature could not be adjusted to the same OD_{600} level. Cultures were grown in a batch mode for 2 weeks after which 40 mL of grown culture was centrifuged and resuspended in 40 mL of N-free NMS to assess PHB production without added co-substrate and the biomass from 120 mL was resuspended in 160 mL N-free NMS with 100 mg/L of valeric acid to study PHA copolymer production. Samples for OD_{600}, biomass growth (dry cell weight (DCW)), metabolites, and gas composition were collected regularly with every gas renewal. The

biomass with accumulated polymer was collected by centrifugation, washed twice with distilled water, and stored at -20^{o}C for PHA analysis. Samples for microbial analysis were collected at the end of the growth phase. Process parameters such as: productivity (mg/L·d), PHA accumulation (% DCW), 3HV fraction (mol%), PHA yield (g-PHA/g-substrate), P(HB) yield (g-P(HB)/g-CH_4), P(HV) yield (g-P(HV)/g-VA), biomass growth (g/L), and CH_4 utilisation rate (mmol CH_4/g-DCW·d), and valeric acid utilisation rate (mmol VA/g-DCW·d) as well as CO_2 production rate (mmol CO_2/g-DCW·d) were determined.

3.2.5. Analytical methods

The concentration of CH_4 and CO_2 in the headspace was analysed by injecting 0.2 mL of the gas phase from batch cultures into a Shimadzu GC-2014 gas chromatograph equipped with the Porapak N packed column and the TCD. Nitrogen at a flow rate of 15 mL/min was used as a carrier gas. The injector, column, and detector temperatures were 110, 50, and 80^{o}C, respectively. All gas volumes were reported at 1 atm and 273.15 K. Valeric acid and other organic acids concentrations in this study were measured using a Shimadzu GC-2014 gas chromatography equipped with Zebron ZB-FFAP column and the FID. Helium was used as a carrier gas and a ramp temperature program was applied; the initial oven temperature was 70^{o}C for 3 min, then raised to 185^{o}C at 10^{o}C per min, and held at the 185^{o}C for 8 min. The injector port and detector temperature were both set to 250^{o}C. Before analysis, samples were acidified using H_3PO_4 and isopropanol was added as an internal standard, then samples were filtered through 0.45 μm pore size syringe filters.

Bacterial growth was monitored by observing the optical density at 600 nm (OD_{600}) using Spectroquant® Prove 100 from Merck (Darmstadt, Germany) and measuring the dry cell weight (DCW). DCW was measured gravimetrically by weighing the freeze-dried biomass. Before freeze-drying biomass was stored at -80^{o}C and then dried at -85^{o}C for 24 h using LyoQuest -85 from Azbil Telstar Technologies S.L.U. (Barcelona, Spain).

For the PHA to be analysed, first esterification step was needed using the hydrochloric acid propanolysis method described by (Wendlandt et al., 2001) with modification i.e., 1-6 mg of freeze-dried biomass was suspended in 2 mL of chloroform in a 16x100 mm screw cap glass tube, 1 mL of propanol containing 20% (v/v) concentrated hydrochloric acid, and 10 μL of internal standard (25 g/L benzoic acid in propanol) were added. The tubes were sealed, mixed, and heated in a thermoreactor (TR320, Merck, Darmstadt, Germany) at

100°C for 3 h. After cooling down overnight 1 mL of distilled H_2O was added and after mixing the tubes were left for a couple of hours to allow phase separation. 1.5 mL of the bottom organic phase was sampled with a syringe and filtered through 0.45 μm pore size syringe filters to a vial for GC analysis. The quantification of PHA concentration was conducted on the Shimadzu GC-2014 gas chromatograph equipped with a capillary column Zebron ZB-FFAP (30 m × 0.53 mm × 1 μm) (Phenomenex) and coupled with the FID. The temperature of the injector and the detector was set to 250°C while the oven temperature was initially held at 70°C for 2.5 min, then programmed to rise to 185°C at 11°C min -1 and maintained at 185°C for 10 min. The injection volume was 1 μL. The calibration was based on reference standards of biologically sourced PHBV copolymer with 8 mol% of PHV content (Sigma-Aldrich) and 3-hydroxybutyric acid (95%, Sigma-Aldrich). The calibration standards were prepared according to the same procedure. PHA accumulation was reported as a percentage of PHA in the DCW (%), and the content of 3HV monomer in PHAs copolymer was reported as a mol% fraction of the polymer.

All results, except for the microbial analysis from biogas enrichment tests, are presented as the arithmetic mean values of triplicates with standard deviation. Statistical analysis was performed using one-way ANOVA with a significance $p \leq 0,05$.

3.2.6. Microbial composition analysis

In order to analyse the composition of microbial communities, biomass samples from the last day before induction of PHA accumulation were collected by centrifugation of 1mL of culture broth and stored frozen at -20°C until processing.

Total metagenomic DNA was isolated using GeneMATRIX Soil DNA Purification Kit (Eurx, Poland) according to the manufacturer's recommendations. ZymoBIOMICS Microbial Community Standard D6300 (Zymoresearch, USA) was used as a mock microbial community to evaluate the analysis pipeline. The preparation of the 16S rRNA gene amplicon library was carried out with 16S Barcoding Kit 1-24 (SQK-16S024), utilizing primers sequences targeting the full-length 16S rRNA genes (5′-AGAGTTTGATCMTGGCTCAGATCGCCTACCGTGAC - barcode - AGAGTTTGATCMTGGCTCAG-3’) and (5′-ATCGCCTACCGTGAC - barcode - CGGTTACCTTGTTACGACTT-3’).

Resulting libraries were sequenced on FLO-MIN106D (R9.4.1) flow cell utilizing MinION Mk1C sequencer (Nanopore Technologies), to the depth of ca. 40-60k full-length amplicons per sample. Acquired raw data was subsequently basecalled using super-accurate dna_r9.4.1_450bps_sup model with GPU version 6.4.6 of guppy basecaller. For each sample, the resulting read sequences after barcoding were processed and clustered individually utilizing the Nanoclust pipeline. Qiime2 (Bolyen et al., 2019) package was used for the taxonomy assignment of representative sequences using Qiime's hybrid vsearch-sklearn classifier and Silva v138 database (Quast et al., 2013). Downstream analyses and visualisations were carried out using the Phyloseq package (Mcmurdie and Holmes, 2013).

Raw sequences obtained in this study were submitted to NCBI Sequence Read Archive (SRA) database and are available under BioProject ID PRJNA994503 (BioSample SAMN36430227-SAMN36430157).

3.3. Results and discussion

3.3.1. Enrichment of environmental samples on biogas

Cultures enriched for methanotrophic bacteria using a biogas-air mixture as a carbon source after seven weeks of cultivation showed different microbial compositions (Fig. 3.1) and potential for PHB production (Table 3.1). The impact of the nitrogen source on culture development and biopolymer accumulation was observed, as there were significant differences in PHB production between cultures enriched in NMS and AMS media. Growth in AMS media seemed to favour biopolymer accumulation in nitrogen-limited conditions, as almost all enrichments had higher values of PHB accumulation than their counterparts in NMS media (Table 3.1). AMS cultures were able to accumulate from 20% up to nearly 50% PHB (LB2, BC, and PB), whereas the highest PHB accumulation observed in NMS cultures ranged between 19% and 27% PHB (LB2, PB, AS, and reference strain) (Table 1). However, cultures grown on AMS had more restricted growth as compared to those grown on NMS, and due to their lower biomass densities (Fig. S3) AMS cultures had a much lower potential for PHB production as compared to NMS cultures (Table 3.1). Apart from BC culture, growth on NMS supported higher production of PHB, reaching the highest PHB productivity in given conditions of 16 ± 1 mg/L·d and 12 ± 1 mg/L·d PHB for AS NMS and LB2 NMS, respectively (Table 3.1).

Table 3.1. Comparison of PHB production potential (PHB productivity and accumulation) of enriched cultures depending on the nitrogen source.

Inoculum source	Abbr.	PHB productivity (mg/L·d)		PHB accumulation (% DCW)	
		NMS	AMS	NMS	AMS
Freshly formed landfill biocover	LB1	2 ± 1	1 ± 1	9 ± 6	26 ± 5
Aged landfill biocover	LB2	12 ± 1	5 ± 1	19 ± 2	20 ± 4
Biocompost	BC	4 ± 3	7 ± 2	12 ± 8	35 ± 1
Peatbog soil	PB	9 ± 3	8 ± 1	19 ± 6	49 ± 7
Waste activated sludge	AS	16 ± 1	1 ± 1	27 ± 2	5 ± 4
Methylocystis hirsuta DSM 18500	Mh	20 ± 1	-	26 ± 2	-

The negative effect of AMS medium in given conditions was especially noticeable for *M. hirsuta* culture, which at the end of the growth phase did not have a sufficient amount of biomass to transfer for accumulation tests, which was a surprising finding as *Methylocystis* sp. are generally known to grow well on ammonium (Rodríguez et al., 2020a; Tays et al., 2018). A similar inhibitory effect of ammonium and the favourable effect of nitrate as a nitrogen source for biomass growth and polymer synthesis were observed by Kulkarni et al. (2022). In their work, nitrate was found to be essential for the growth of *Methylocystis* dominant mixed culture and PHB production, reaching its maximum at a content of 0.5 g/L KNO_3. On the other hand, Salem et al. (2021), in similar studies, have found AMS medium to be favourable for PHB synthesis, where enriched waste activated sludge cultures had similar biomass yields irrespective of the nitrogen source while the AMS cultures accumulated almost four times higher amounts of PHB (38 ± 4% and 9 ± 2% of DCW in the AMS and NMS respectively). As ammonium has a structure similar to that of CH_4 its presence in the medium creates a competitive inhibition of an enzyme essential in CH_4 oxidation, methane monooxygenase (Stein and Klotz, 2011). Additionally, the toxicity of the end-products of ammonium oxidation, i.e., hydroxylamine or nitrite, adds to the bacterial growth inhibition (He et al., 2017). Even though ammonium was reported to select for type II methanotrophs due to their better adaptability to the inhibitory effects (Fergala et al., 2018b), results of comparative studies between different representatives of type I and

type II ammonia-oxidising ability and sensitivity to the inhibition of methane-oxidising ability have suggested that physiological responses and environmental adaptations of methanotrophs to the nitrogen source are organism-specific and not type-specific (Nyerges et al., 2010). Also, other research has shown that different strains of the same type II *Methylocystis* genus have different nitrogen source preferences (Tays et al., 2018).

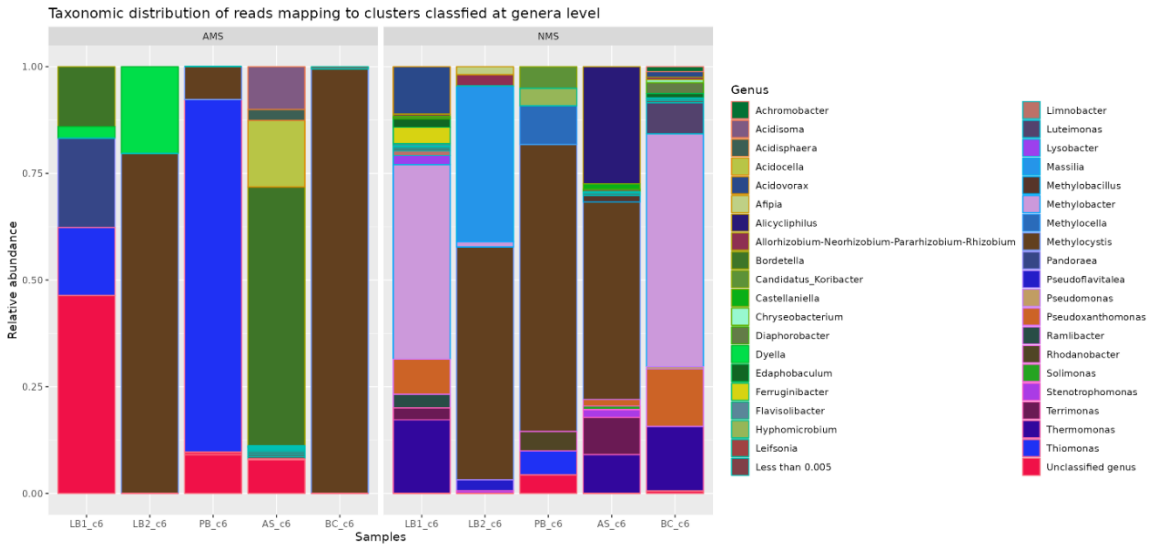


Figure 3.1. Microbial analysis at the genus level of samples enriched from five different environmental sources with AMS and NMS as nitrogen source.

This strain-specific preferences for nitrogen source could explain why, in the conducted enrichment, there was no common way in which cultures differentiated in AMS and NMS media during the enrichment (Fig. S3.4). Microbial community analysis of the samples at the final stage of enrichment (Fig. 3.1) showed that all NMS cultures were enriched in various methanotrophic bacteria, mainly *Methylocystis* and *Methylobacter* sp., while BC AMS and LB2 AMS had the highest relative abundance of reads mapping to *Methylocystis* sp. in all enriched cultures. Some other major genera of AMS-enriched cultures were *Thiomonas* sp. (PB AMS), *Bordetella* sp. (AS AMS), *Dyella* sp. (LB2 AMS), and *Pandoraea* sp. (LB1 AMS). NMS cultures were more diverse in their microbial community, and apart from methanotrophic bacteria that were dominant species in all cultures, some other major genera, including *Alicycliphilus* sp. (AS NMS), *Massilia* sp. (LB2 NMS) and *Thermomonas* sp., were present in three enriched cultures (Fig. 3.1). Cultures with substantial PHB-producing potential had a high presence of *Methylocystis* sp., a known PHB-producing methanotroph genus (Rodríguez et al., 2020a; Sundstrom and Criddle, 2015). While in the case of the best PHB-producing culture, AS NMS, the relative abundance of reads mapping to methanotrophic bacteria was slightly lower than in LB2

and PB NMS cultures, it was also enriched in *Alicycliphilus* sp., a genus from which some strains are known to also accumulate PHB (Cheema et al., 2012; Oosterkamp et al., 2015). To the authors' best knowledge, it was the first time that the presence of *Alicycliphilus* sp. was reported for mixed cultures producing PHA from CH_4. The observed accumulation of PHB in cultures with low-to-no presence of type II methanotrophs may have been due to the presence of other bacterial genera from which some strains have been reported to accumulate PHB, such as *Thiomonas* sp. (Arsène-Ploetze et al., 2010), *Bordetella* sp. (Izac et al., 2015), or *Acidisoma* sp. (Belova et al., 2009).

Although none of the enriched cultures reached the same PHB productivity as *M. hirsuta* DSM 18500 (20 ± 1 mg/L·d), for AS NMS the PHB accumulation was slightly higher than for pure strain culture, i.e., 27 ± 2% and 25 ± 2% of DCW, respectively. Two other inocula that showed high potential for biopolymer production were an aged landfill biocover soil and soil from a peat bog, which differed in PHB productivity but had similar accumulation levels of 19 ± 6% and 19 ± 2% of DCW PHB, respectively. The NMS-grown AS, LB2, and PB mixed cultures were able to accumulate similar or higher PHB levels when compared to other similar studies: 8.5% (Karthikeyan et al., 2015b), 15.93 ± 1.9 % (Cardoso et al., 2022), and 22.20% (Kulkarni et al., 2022), but the achieved accumulation was still two times lower than the highest 54.3 ± 3% PHB reported in the literature (Salem et al., 2021), though in a different experimental design approach. A screening of environmental samples for methanotrophic culture enrichment showed the highest potential for the use of waste activated sludge grown on nitrate-based media. It is in agreement with the previous studies that reported the highest PHB accumulation in cultures enriched from activated sludge (Fergala et al., 2018a; Salem et al., 2021; Zhang et al., 2018). The three most promising cultures for PHB production were chosen for the next stage of screening to investigate the effect of different CH_4:O_2 ratios on PHA production with additional carbon sources.

3.3.2. PHBV production at different CH_4 concentrations

A valeric acid concentration of 100 mg/L was chosen to investigate and assess the co-polymer production by mixed culture as it was previously reported to support higher PHBV accumulation (Fergala et al., 2018c; Myung et al., 2015). Results of the PHA production potential of three cultures at CH_4 concentrations ranging from 10% to 50% are shown in Fig. 3.2. A high CH_4 concentration of 90% in the air inhibited culture growth (data not

shown), and cultures at this CH_4:O_2 ratio were not subjected to PHA accumulation tests. Each mixed culture developed differently in response to the initial CH_4 concentrations in the headspace.

Increasing CH_4 concentration from 10% to 25% had a detrimental effect on the PHA production by LB cultures, decreasing maximum PHA productivity and accumulation from 17 ± 2 mg/L·d and 33 ± 3% of DCW PHA at 10% CH_4 to 6 ± 1 mg/L·d and 11 ± 2% of DCW PHA at 25% CH_4. CH_4 concentrations higher than 25% did not seem to have any further effect on the PHAs production, as the achieved PHA productivities were similar and the degree of PHBV accumulation in the cells, with the exception for LB25% culture, was comparable to that at 20% (Fig. 3.2). Copolymer composition was not affected by the CH_4:O_2 ratios, as the 3HV fraction of accumulated PHBV did not differ significantly ($p>0.05$) with the levels from 21 to 25 mol%. In contrast to the LB cultures, CH_4 concentration did not significantly impact the PHA accumulation by PB-enriched cultures ($p>0.05$), but seemed to affect the 3HV fraction of the produced polymer ($p\leq0.05$) (Fig. 3.2). Increasing CH_4 concentration from 10% to 25% did not have a definite effect on PHA accumulation as it ranged from 21% to 26% PHA; however, it seemed to have an impact on the 3HV fraction as it decreased from 41 ± 1 mol% to 24 ± 3 mol% between 10% and 25% CH_4. A slight dependence of the ability to incorporate valeric acid into the PHBV copolymer on the CH_4 concentration in the range of 10-25% was observed. At higher CH_4 levels (30% and 50%), 3HV fractions in the polymer were similar to this at 20%. PHA productivity did not show any significant differences ($p>0.05$) and was stable (from 12 to 15 mg/L·d) in the cultures with initial CH_4 concentrations up to 30% and decreased at 50% CH_4 (Fig. 3.2). Cultures enriched from AS showed a low response to the varied CH_4 concentrations in terms of PHA accumulation and 3HV fraction, while increasing the initial CH_4 concentrations in the headspace had an adverse effect on the PHA production, decreasing PHA productivity from 19 mg/L·d at 10% to 8 mg/L·d at 30% CH_4. AS cultures showed the highest PHA productivity (19 ± 2 mg/L·d at 10% CH_4) and accumulation (37 ± 2 % of DCW PHA at 30% CH_4) from all tested cultures, while the 3HV fraction was also generally higher than for LB and PB cultures (Fig. 3.2).

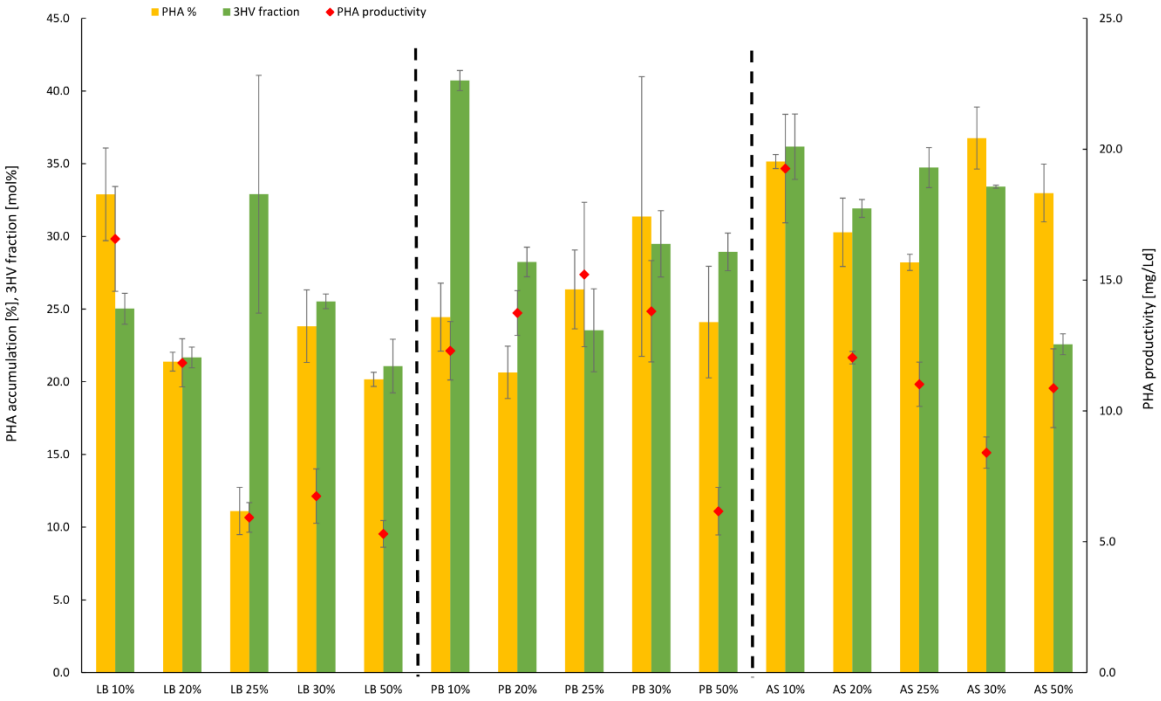


Figure 3.2. Effect of CH_4 concentration [%] in the headspace on the PHA productivity, accumulation and 3HV fraction of PHBV of three different mixed cultures; LB (previously LB2), PB and AS. Error bars show standard deviation (n=3).

For two out of three tested cultures (LB and AS), the highest PHA accumulation and productivity occurred for cultures grown at 10% initial CH_4 concentrations, which corresponded to a CH_4:O_2 ratio of approximately 1:2. Such conditions favour CH_4 metabolism, which requires two molecules of O_2 to oxidise one molecule of CH_4 (Karthikeyan et al., 2015b). Wei et al. (2016) noticed that better C_1 oxidation at O_2-sufficient conditions in methanotrophic mixed cultures supported a higher conversion rate of CH_4 to CO_2 and biomass In the present work this effect was observed to be culture-dependent and was true for AS culture that reached the highest biomass density (Fig. S3.5) and PHA productivity (Fig. 3.2) at 10% CH_4. While for LB cultures 10% CH_4 in the headspace also supported the highest PHA productivities, the biomass density was comparable in the range of 10-25% of CH_4 (Fig. S3.5). The difference in the effect of CH_4 concentration on the PHA productivity for PB cultures as compared to the LB and AS cultures may have stemmed from strain-specific requirements of bacteria in the microbial community that may have led to the preference of 25% CH_4 concentration in the case of PB cultures. Previous reports (Karthikeyan et al., 2015b; López et al., 2014) showed that

PHA accumulation generally did not appear to be directly correlated to the CH_4:O_2 ratios. However, as PHA productivity in the culture is greatly dependent on biomass growth, the applied CH_4 and O_2 availability in the headspace were crucial for evaluating the potential use of those cultures for efficient copolymer production. The fraction composition of PHBV was not correlated to the applied gas conditions but was rather culture-dependent, with AS cultures being able to incorporate the 3HV fraction at a level of around 35 mol%, PB cultures close to 30 mol%, and LB cultures up to 25 mol%. The highest results achieved for the 3HV fraction of PHA copolymer in this experiment (41 ± 1 mol% for PB 10%) were one of the highest previously reported for *Methylocystis*-dominated cultures with similar cosubstrate additions: 21 ± 3 mol% (Myung et al., 2015) and 35 ± 3 mol% (Fergala et al., 2018c). On the other hand, PHBV accumulation levels achieved here were generally lower than those previously reported; PHA of 45 ± 2% DCW (Myung et al., 2015) and PHBV of 47 ± 4% DCW (Fergala et al., 2018c), which may have been due to the difference in the nitrogen source, copper availability, or microbiome structure.

All cultures showed the presence of type II methanotroph *Methylocystis* sp., although the relative abundance of reads mapping to this genus in the culture differed between inocula and CH_4 concentrations (Fig. 3.3). The taxonomic distribution of AS cultures was generally similar and differed only in the ratios of specific genera. The major genera included *Methylocystis* sp., *Alicycliphilus* sp., *Methylobacillus* sp., and *Terrimonas* sp. At higher CH_4 concentrations, cultures were enriched in *Alicycliphilus* sp., while the abundance of *Methylocystis* sp. reads decreased, and the relative abundance of methylotrophic bacteria from the *Methylobacillus* genus was not affected by the difference in CH_4:O_2 ratios. *Methylocystis* sp. was a dominant genus in most of the LB and PB cultures, while for LB cultures, its abundance of reads decreased at a CH_4 concentration higher than 10%. All LB and PB cultures also showed a relative abundance of reads mapping to *Pseudoflavitalea* sp. Additionally, for LB cultures at 20-30% of the initial CH_4 concentration in the headspace, *Massilia* sp. emerged as a major genus. At 50% CH_4, all cultures, irrespective of the inoculum, were enriched in *Alicycliphilus* sp.

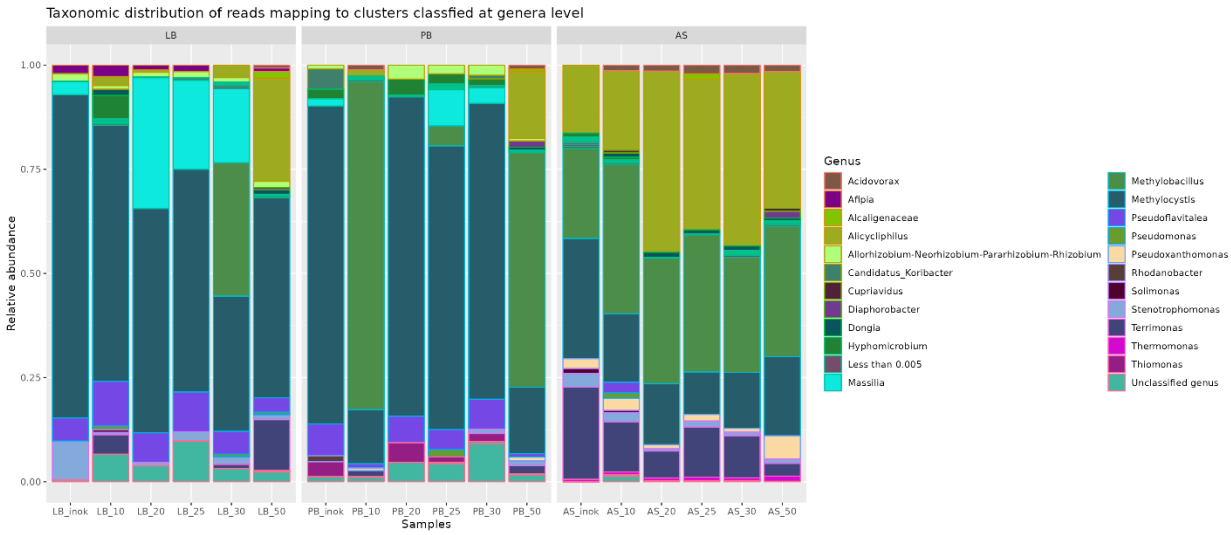


Figure 3.3. Influence of initial CH_4 concentration (10-50%) in the headspace on the community composition at the genus level of the cultures enriched from activated sludge (AS), aged landfill biocover soil (LB) and peat bog (PB). Shown as the average from the triplicate.

The use of a higher CH_4:O_2 ratio creates an O_2 tension that may affect the structure of the microbial community and the distribution of CH_4-derived carbon (Wei et al., 2016). AS culture seemed to adapt well to the different CH_4:O_2 ratios (including O_2 limitations), as it did not have much of an impact on their microbial composition or ability to produce PHAs. The observed high PHA accumulation observed in all AS cultures, despite their relatively low abundance of *Methylocystis* sp. reads compared to other cultures, could be attributed to several factors. One possibility is that there might be differences on the bacterial strain level of *Methylocystis* sp. that were not determined in the study. Another possibility is that additional polymer accumulation could be occurring by other non-methanotrophic bacteria that are utilising CH_4-derived carbon, such as *Alicycliphilus* sp. (Oosterkamp et al., 2015). The microbial composition of PB cultures remained stable for CH_4 concentrations ranging from 20% to 30% in O_2-sufficient conditions and shifted for methylotrophic bacteria dominance at 50% CH_4, indicating higher CH_4-derived carbon flux into the environment, allowing for the growth of the non-methanotrophic bacteria. For LB cultures, a decrease in the relative abundance of reads mapping to *Methylocystis* sp. at higher CH_4:O_2 in favour of bacteria not capable of PHA synthesis correlated to lower PHA production (Fig. 3.2 and 3.3). Wei et al. (2016) observed an increased microbial community richness at higher CH_4:O_2 ratios. However, taking into account calculated diversity measures (Table S3.1) and variability within sample replicates we cannot support such conclusion.

The conducted study showed AS and LB cultures grown at 10% CH_4 to be the most promising for high PHA production. Furthermore, the observed susceptibility of 3HV incorporation into the PHBV to the CH_4:O_2 ratio for PB cultures may be used as a factor for modelling copolymer composition. To further study the potential of enriched cultures for the production of PHA copolymer, AS and LB cultures were grown at 10%, and PB cultures in the range of 10-25% CH_4 were compared to the culture of *M. hirsuta* DSM 18500 at 10% CH_4.

3.3.3. Selection of PHAs producing mixed culture

Two weeks-long cultivation of promising PHA-producing mixed methanotrophic cultures was carried out to assess their ability to produce co-polymers from CH_4 and valeric acid as a co-substrate. Contrary to expectations and the results from the previous stage, the developed cultures showed no significant difference in the fraction of 3HV incorporated into the PHAs between the tested cultures ($p>0.05$). All mixed methanotrophic cultures with added cosubstrate, irrespective of the microbiome composition and applied conditions, had similar fractions of 3HV in the synthesised biopolymer, with an average of 38 ± 2 mol% (35-41 mol%) (Table 3.2). However, there was a significant difference in the PHA productivity and polymer accumulation in the cells ($p\leq0.05$) as they ranged from 12 to 39 mg/L·d and from 9% to 27% of DCW, respectively (Table 3.2). As previously shown, 10% CH_4 concentration favoured higher biopolymer production, and all mixed cultures grown in these conditions accumulated similar levels of PHAs of around 25-27% and a fraction of 3HV of 35-39 mol% (Table 3.2). The highest co-polymer synthesis from CH_4 and valeric acid, as a co-substrate, was observed for AS culture at 10% CH_4, with productivity of 39 ± 2 mg/L·d, PHA accumulation of 27 ± 3% of DCW, and PHA yield of 0.42 ± 0.02 g-PHA/g-substrate. PHA yield on substrate for AS culture was also the closest to the yield of reference strain *M. hirsuta* (0.45 ± 0.01 g-PHA/g-substrate) (Table 3.2). The obtained yields were lower than the maximum of 0.63-0.67 g-PHA/g-substrate reported in the literature for PHA production from CH_4 and valeric acid by *Methylocystis* sp. (Cal et al., 2016; López et al., 2018). Low PHA productivity and yields may be improved by changing the feeding strategy to increase CH_4 availability during cultivation and optimising the feast-famine regime for PHA accumulation (Rodríguez et al., 2022). No correlation between PHA production and biomass growth during an accumulation phase was observed, as cell growth was primarily dependent on the CH_4 concentrations (Table 3.2). To improve

biomass accumulation and subsequently, PHA production, preventing CH_4-limited conditions through more frequent headspace renewal or continuous gas flow may prove to be beneficial for the process efficiency. An important limiting factor for CH_4-based PHA accumulation process is the low solubility of CH_4 in the water (0.0023 g CH_4/100 g of water at 293K and 1 atm, compared to 0.169 g CO_2/100 g of water (Kaye and Laby, 1986)) which may results in constrained biomass growth and PHA production when CH_4 is supplied as the only carbon source and substrate utilization is limited due to its lower accessibility (Yasin et al., 2015). Changing the culture system from shaken bottles to bioreactors designed for better gas-liquid mass transfer could potentially lead to increased substrate availability and boost the process efficiency (Gęsicka et al., 2021).

It was previously observed that CH_4 oxidation is directed towards 3HV monomer synthesis until reaching its maximum at the expanse of the overall PHA accumulation when conditions favour valeric acid uptake (Fergala et al., 2018c). A similar effect was observed in the present study for PB cultures, which, despite the difference in PHA accumulation, did not show a significant difference in the 3HV fraction or yields for PHV synthesis ($p>0.05$) (Table 3.2). Although in the work by Fergala et al. (2018c), this occurrence was stated to be dependent on the O_2 availability, it was not evident in this study. The CH_4:O_2 ratio did, however, have an impact on the valeric acid utilisation by mixed cultures, as PHBV synthesis is an energy-intensive process that increases oxygen demands to generate additional energy from CH_4 oxidation pathways (Myung et al., 2016a). Higher O_2 availability at 10% CH_4 concentrations supported valeric acid uptake and incorporation into the 3HV monomer synthesis pathway, yet CH_4 consumption decreased in cultures supplied with cosubstrate as compared to the control on CH_4 alone (Fig. 3.4A). Although some studies have shown the ability to synthesise PHBV solely on valeric acid without the need for CH_4 oxidation for energy generation (López et al., 2018; Luangthongkam et al., 2019b), this work could not assess this hypothesis as a control on only valeric acid was not conducted. Cosubstrate addition significantly increased PHA accumulation in the cultures at 10% CH_4 while only slightly increasing it at higher concentrations of CH_4, with almost no impact on the PHA yields for these cultures (Table 3.2). The utilisation of CH_4 and valeric acid differed between tested cultures (Fig. 3.4) and cosubstrate addition shifted the metabolism towards higher carbon flux for PHA accumulation at the expense of biomass and other metabolites (Fig. S3.6). At 10% CH_4, all cultures grown without a cosubstrate utilised all of the provided CH_4, while PB at 20% CH_4 used more than 60% of the available

CH_4 (Fig. 3.4A). This level of utilisation at double the CH_4 dose could imply that at 10% CH_4, the carbon source was utilised completely before the end of the accumulation phase, and limited availability of CH_4 could lower the PHB yield. During the accumulation phase with added cosubstrate, PB cultures generally had higher CH_4 utilisation rates (16-18 mmol CH_4/g-DCWd) compared to other mixed cultures (12 mmol CH_4/g-DCWd for AS and LB), while valeric acid utilisation rates were the highest at 10% CH_4 in LB and PB cultures (Fig. 3.4B). Despite these differences, CO_2 production rates were generally similar, and the carbon distributed for CO_2 generation was around 50% of the total carbon used (Fig. S3.6). Interestingly, in the case of *M. hirsuta* culture, there was no CO_2 generated while a very small part of the provided CH_4 was consumed when valeric acid was added, yet the PHA accumulation was still a little higher than in the culture on CH_4 only where the substrate was completely consumed (Fig. 3.4). Very low CH_4 utilisation by *M. hirsuta* (2 ± 1 mmol CH_4/g-DCW·d) during the accumulation phase, along with high PHA and poly(hydroxyvalerate) (P(HV)) yields (Table 3.2), might indicate that for this strain, valeric acid uptake and P(HV) synthesis were not strictly linked to the CH_4 assimilation, which is in line with the findings of López et al. (2018). Additionally, the PHA accumulation observed here for *M. hirsuta* was the lowest of the reported PHBV accumulations in pure cultures, although the 3HV fraction was the highest (Cal et al., 2016; López et al., 2018; Myung et al., 2017, 2016a) which would also suggest that the applied conditions did not support PHA accumulation by methanotrophic bacteria. On the other hand, this might have been a strain-specific response and not a general trend for all *Methylocystis* sp. bacteria.

Table 3.2. Comparison of PHA accumulation, 3HV fraction, PHA productivity, PHA, P(HB), and P(HV) yield on substrate, and biomass growth during growth and accumulation phase of six cultures fed with only CH_4 and with valeric acid as a cosubstrate during the accumulation phase.

Culture		PHA (% DCW)	HV (mol%)	PHA productivity (mg/L·d)	PHA yield (g-PHA/g-substrate)	P(HB) yield (g-P(HB)/g-CH_4)	P(HV) yield (g-P(HV)/g-VA)	Biomass growth (g/L)	
								Growth phase	Accumulation phase
LB 10%	+ VA	26 ± 2	35 ± 1	26 ± 1	0.33 ± 0.01	0.46 ± 0.08	0.28 ± 0.01	0.33 ± 0.00	0.12 ± 0.02
	-VA	12 ± 1	0	54 ± 6	0.23 ± 0.03	0.22 ± 0.02	ND		ND
PB 10%	+ VA	25 ± 1	36 ± 1	32 ± 1	0.27 ± 0.01	0.25 ± 0.01	0.36 ± 0.09	0.27 ± 0.04	0.13 ± 0.01
	-VA	1 ± 2	0	5 ± 1	0.02 ± 0.01	0.02 ± 0.01	ND		ND
PB 20%	+ VA	9 ± 1	41 ± 3	12 ± 1	0.14 ± 0.02	0.10 ± 0.03	0.40*	0.42 ± 0.03	0.14 ± 0.01
	-VA	6 ± 3	0	32 ± 3	0.14 ± 0.02	0.12 ± 0.01	ND		ND
PB 25%	+ VA	19 ± 2	37 ± 1	21 ± 2	0.21 ± 0.01	0.22 ± 0.01	0.34 ± 0.02	0.19 ± 0.01	0.05 ± 0.01
	-VA	13 ± 2	0	48 ± 1	0.26 ± 0.05	0.20 ± 0.01	ND		ND
AS 10%	+ VA	27 ± 3	39 ± 2	39 ± 2	0.42 ± 0.02	0.38 ± 0.02	0.52 ± 0.01	0.33 ± 0.01	0.12 ± 0.01
	-VA	11 ± 2	0	61 ± 9	0.25 ± 0.05	0.19 ± 0.04	ND		ND
Mh 10%	+ VA	9 ± 1	88 ± 1	12 ± 1	0.45 ± 0.01	0.45 ± 0.01	0.47 ± 0.01	0.37 ± 0.02	0.12 ± 0.01
	-VA	6 ± 1	0	43 ± 2	0.21 ± 0.03	0.19 ± 0.01	ND		ND

ND – not determined
* data from only one sample

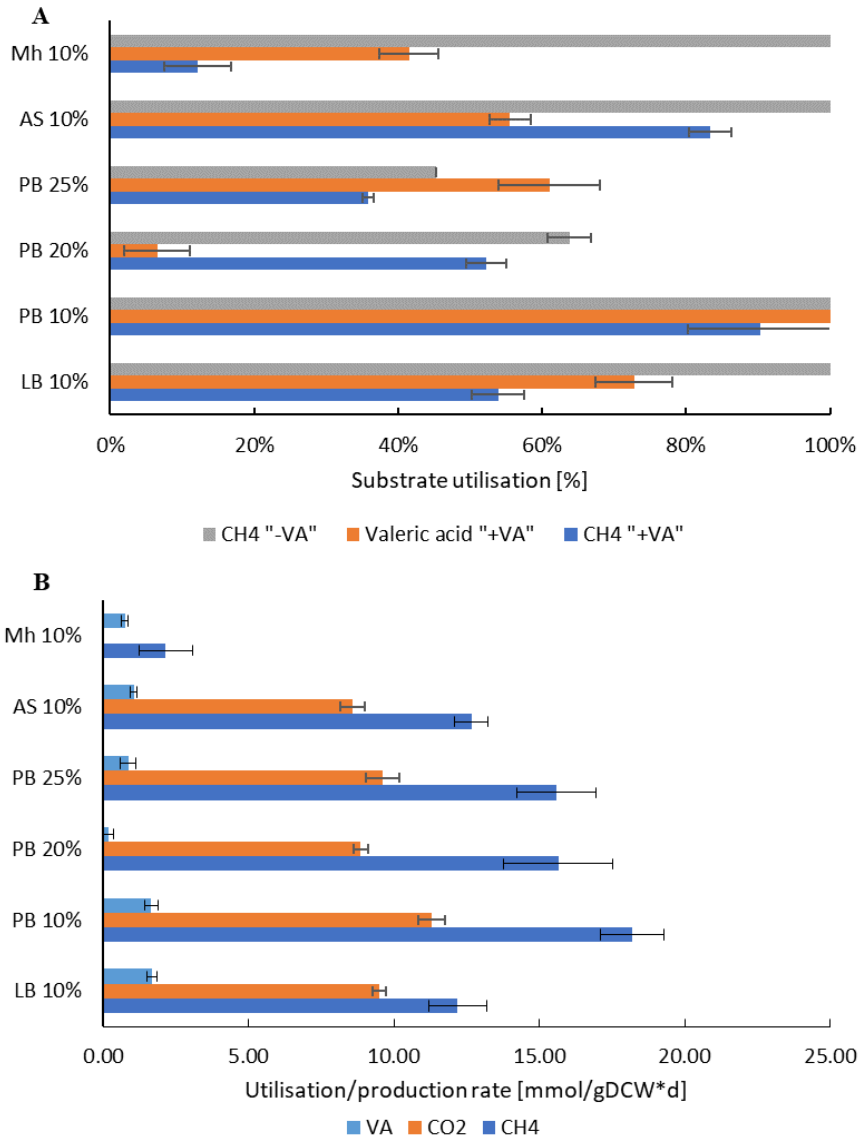


Figure 3.4. Comparison of percentage of substrate utilisation of six tested cultures with (+VA) and without (-VA) valeric acid addition (A). CH_4 and valeric acid (VA) utilisation and CO_2 production rate (B) during the accumulation phase of valeric acid-fed cultures (B). Error bars show standard deviation (n=3).

The microbial composition of AS culture at 10% remained relatively stable during the two-week cultivation, as relative abundances of reads of major genera were similar to those from inoculum culture (Fig. 3.5), with a slight shift towards methanotrophs, showing that the culture, in the given conditions, has achieved a stable state. Other cultures were still susceptible to the microbial composition changes and were shifting from the high abundance of *Methylocystis* sp. reads in the favour of other non-methanotrophic bacteria, such as *Methylobacillus* sp., *Terrimonas* sp., *Pseudoflavitalea* sp., *Hyphomicrobium* sp., and *Dongia* sp. (Fig. 3.5), that may grow on CH_4-derived carbon, metabolites of other

bacteria, or cell debris (Baldani et al., 2014; Chistoserdova et al., 2007; Lawson et al., 2020; Zhao et al., 1993). Despite differences in the relative abundance of methanotrophs in the different cultures at 10% CH_4 in the headspace, the PHA accumulation was similar, suggesting that microorganisms other than *Methylocystis* sp. were involved in the biopolymer accumulation. This hypothesis was further supported by the fact that the PB 20% culture, which showed the highest relative abundance of reads mapping to *Methylocystis* sp., and the reference culture of *M. hirsuta* DSM 18500 had the lowest PHBV accumulation in the cells. The presence of other heterotrophic bacteria capable of accumulating PHA, such as *Alicycliphilus* sp., *Hyphomicrobium* sp., and *Pseudoxanthomonas* sp., had presumably influenced the PHA-producing potential of those cultures (based on a positive match for PHA synthase in the NCBI Protein database).

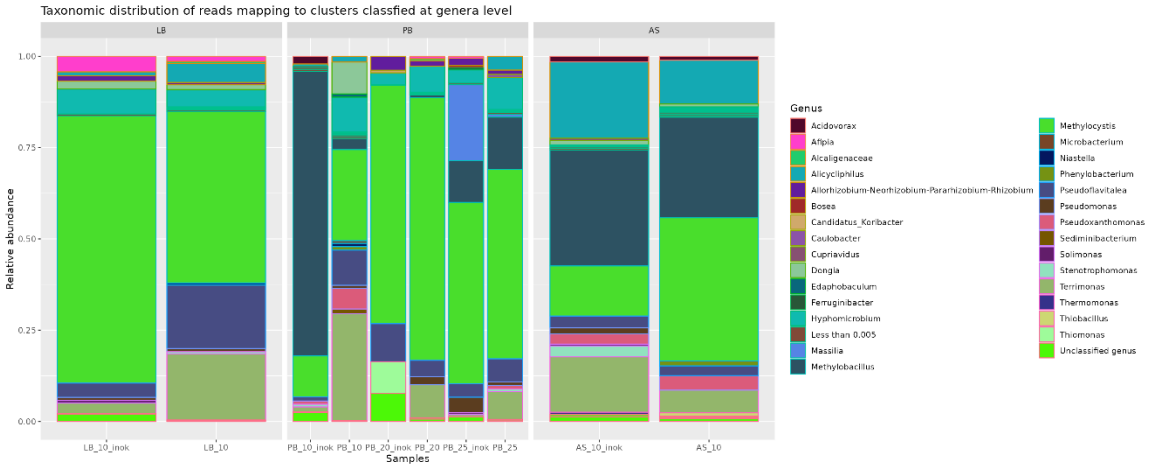


Figure 3.5. Average (n=3) microbial community composition at the genus level of the selected mixed methanotrophic cultures.

From all tested cultures, AS at 10% CH_4 showed to be the most promising for an application for biopolymer production due to the highest PHA accumulation and yield as well as the high P(HV) yield on the cosubstrate and 3HV fraction of the synthesised polymer. Additionally, AS cultures were shown to be the most promising for further optimisation based on the possibility of reaching a cut-off value for PHA extraction and purification for industrial applications. Although there is not much data on the cut-off values for PHA production from methanotrophic cultures, available data from a different PHA production process suggested that PHA content higher than 32% w/w of lyophilised samples was good enough for further downstream processing and PHA extraction (Mineo et al., 2023). AS cultures were the only ones from the tested cultures that reached the proposed cut-off value (AS10%, AS30%, and AS50% in the study at different CH_4

concentrations) which further confirms the industrial potential for PHA production by AS mixed methanotrophic culture.

3.4. Conclusions

Enrichment of environmental samples for PHA production from CH_4 was studied, and the effect of different nitrogen sources and CH_4:O_2 ratios on biopolymer accumulation and microbial structure was investigated. Mixed methanotrophic cultures enriched in *Methylocystis* sp. had the highest biomass growth in the NMS media and were capable of accumulating PHAs from a wide range of CH_4 concentrations in the air, with 10% CH_4 supporting the highest biomass and PHA production. Co-substrate addition during the accumulation phase resulted in an approximately two-fold increase in the PHA accumulation by AS and LB mixed cultures. The environmental source of the inoculum influenced the efficiency of PHA accumulation in enriched cultures due to the differences in microbial composition. PHA-producing cultures were not only enriched in *Methylocystis* sp. but also in other bacterial species that could take part in the PHA accumulation, mainly *Alicycliphilus* sp., *Hyphomicrobium* sp., and *Pseudoxanthomonas* sp. that were able to grow on CH_4-derived carbon. The culture enriched from waste activated sludge demonstrated the highest potential for biopolymer accumulation, demonstrating excellent PHA productivity, yields, and stability across various CH_4 concentrations. Even though more research is needed in order to better understand the particular mechanisms and improve the overall yields, the presented results showcase another novel approach for methane utilisation and conversion.

3.5. Acknowledgements

This work was financed by the National Science Centre, Poland under the grant agreement no. 2019/35/D/ST8/03530. Figures S3.1 and S3.2 were created with BioRender.com.

3.6. Supplementary material

Table S3.1. Three alpha-diversity metrics (Observed features, Simpson, Shannon), summarizing richness and evenness of microbial communities, calculated for selected samples in this study.

Sample/diversity metric	Observed features	Simpson	Shannon	Observed features	Simpson	Shannon
	Average			Std deviation		
2_LB_inok	7	0,384	1,251	-	-	-
LB_10	15,667	0,564	2,023	3,512	0,101	0,322
LB_20	7,667	0,562	1,613	1,155	0,072	0,174
LB_25	8	0,64	1,926	0	0,028	0,095
LB_30	9,667	0,579	1,801	2,887	0,081	0,178
LB_50	12	0,672	2,175	1,732	0,084	0,253
2_PB_inok	9	0,406	1,417	-	-	-
PB_10	10,667	0,376	1,219	3,215	0,037	0,114
PB_20	6,5	0,387	1,266	0,707	0,224	0,648
PB_25	9,333	0,45	1,508	4,163	0,21	0,697
PB_30	7	0,434	1,39	1	0,124	0,365
PB_50	13	0,641	2,017	4	0,063	0,162
2_AS_inok	17	0,793	2,599	-	-	-
AS_10	21,333	0,789	2,79	2,517	0,023	0,096
AS_20	10	0,688	2,04	1,414	0,03	0,107
AS_25	10,667	0,732	2,278	0,577	0,011	0,056
AS_30	12,333	0,707	2,186	1,528	0,043	0,109
AS_50	14,667	0,743	2,411	0,577	0,014	0,038
3_LB_10_inok	12	0,454	1,662	-	-	-
3_LB_10	14,333	0,707	2,325	3,512	0,002	0,106
3_PB_10_inok	12	0,379	1,314	-	-	-
3_PB_10	18	0,814	2,923	3,606	0,029	0,248
3_PB_20_inok	7	0,550	1,503	-	-	-
3_PB_20	9,333	0,454	1,528	2,082	0,048	0,148
3_PB_25_inok	14	0,692	2,313	-	-	-
3_PB_25	15,333	0,666	2,341	1,528	0,098	0,278
3_AS_10_inok	21	0,810	2,901	-	-	-
3_AS_10	18,333	0,71	2,449	0,577	0,095	0,267

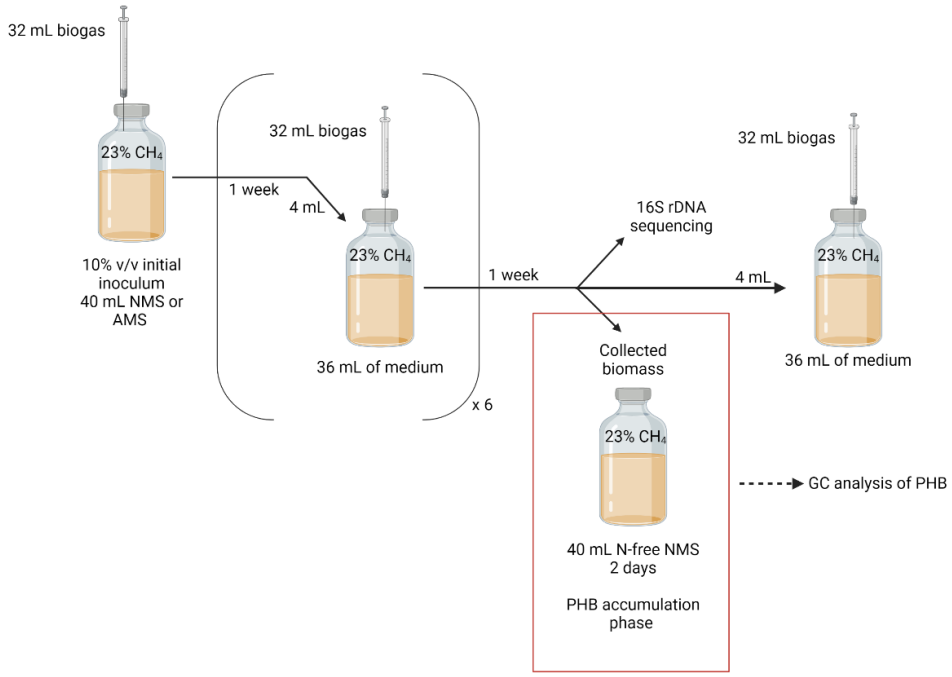


Figure S3.1. Scheme of the conducted enrichment of environmental samples and PHB accumulation assessment tests.

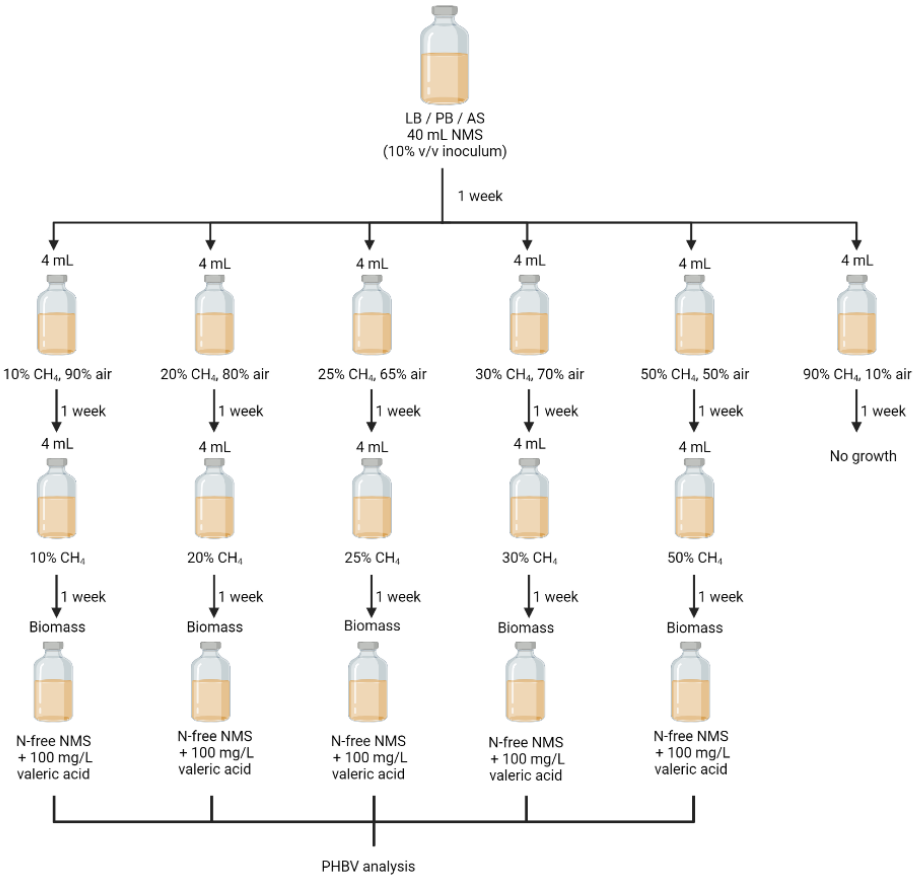


Figure S3.2. Scheme of the PHAs production assays at different methane concentrations.

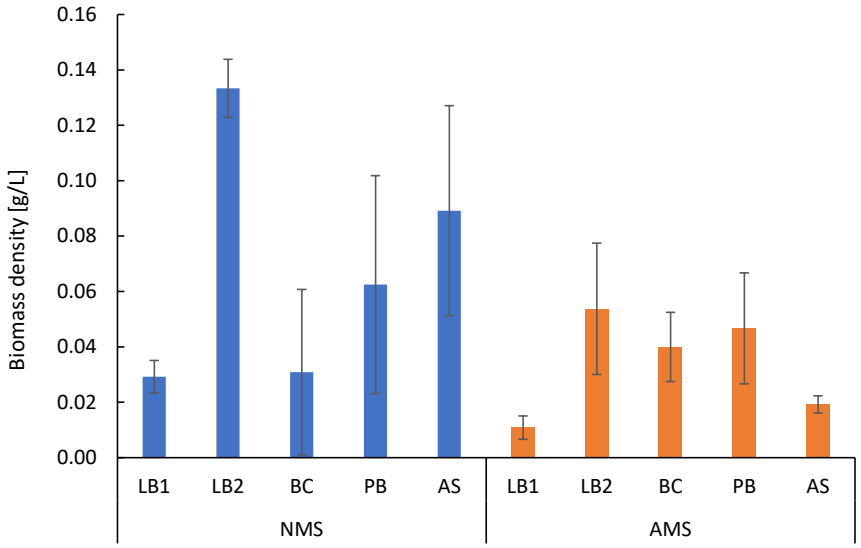


Figure S3.3. Comparison of biomass density (g/L) of enriched cultures at the end of the accumulation phase grown on different nitrogen sources.

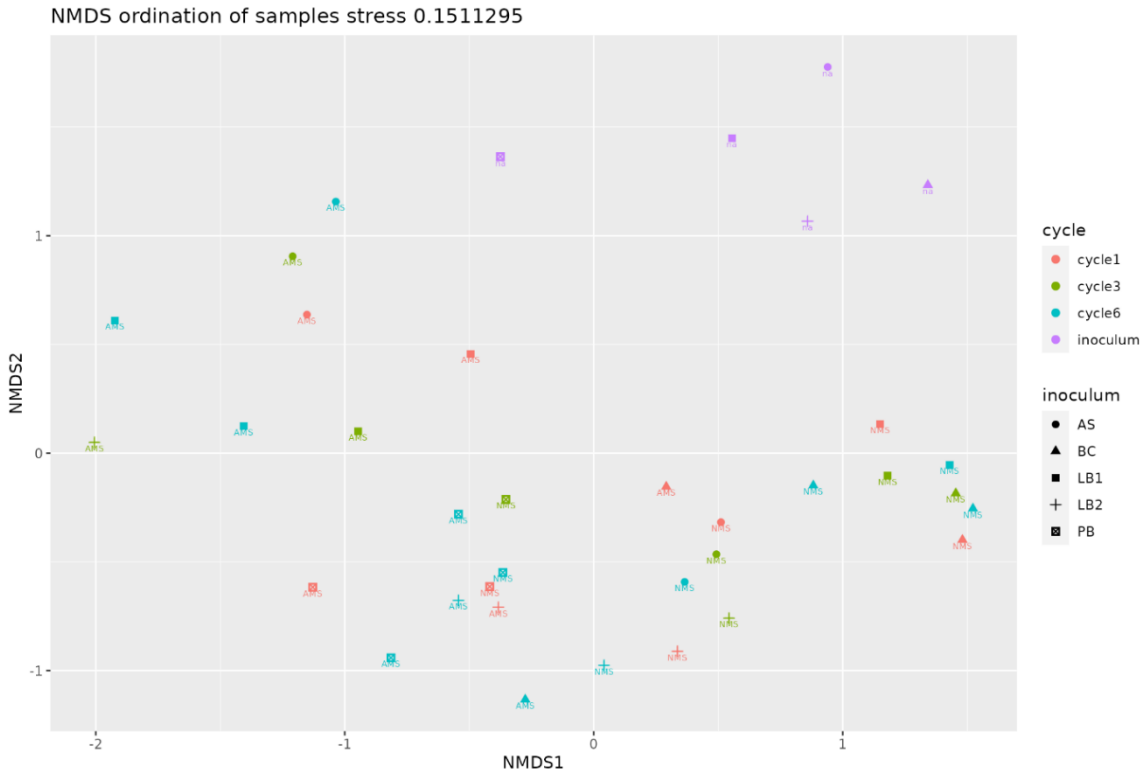


Figure S3.4. NMDS ordination of microbial community composition development during the enrichment of five environmental samples grown on AMS and NMS under methane as the only carbon source.

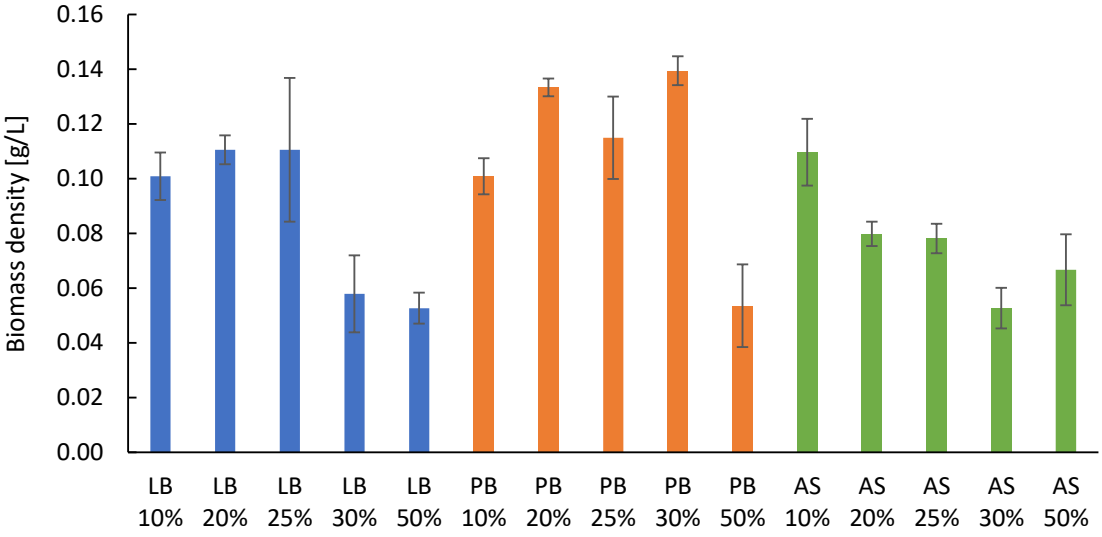


Figure S3.5. Comparison of biomass density (g/L) at the end of the accumulation phase of three methanotrophic cultures grown at different initial CH_4 concentrations (10-50%).

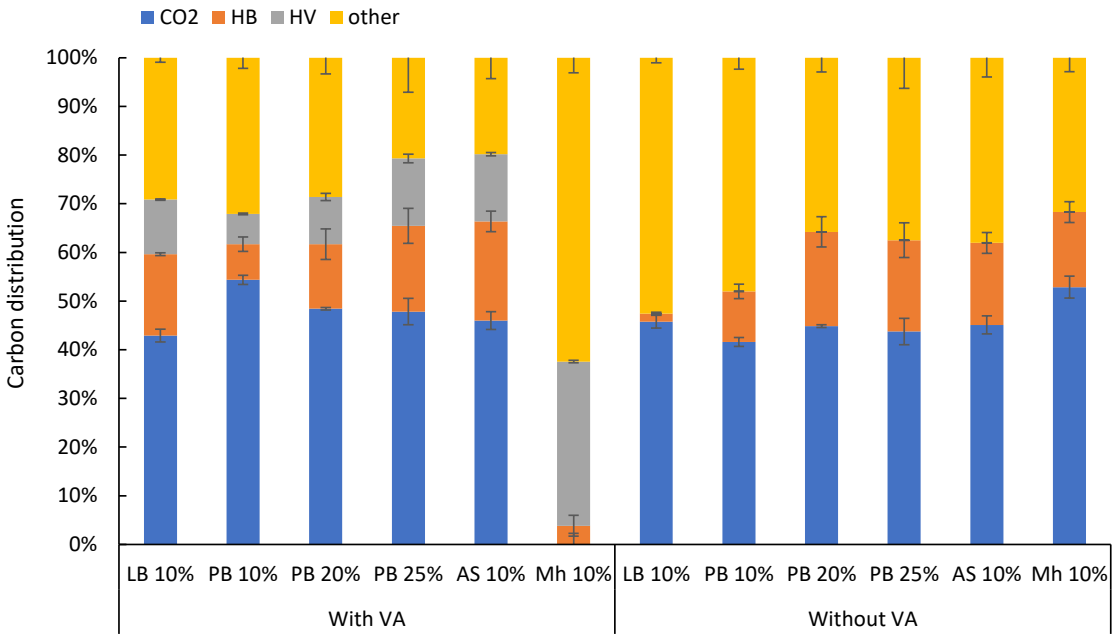


Figure S3.6. Comparison of carbon distribution to major products during the accumulation phase in cultures with and without valeric acid (VA) addition. Error bars show standard deviations (n=3).

Chapter 4

Sequential feast-famine process for polyhydroxyalkanoates production by mixed methanotrophic culture under different carbon supply strategies

This chapter is under preparation for publication as:

Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M., 2024. Sequential feast-famine process for polyhydroxyalkanoates production by mixed methanotrophic culture under different carbon supply and pH control strategies.

Abstract

Microbial synthesis of biopolymers from methane presents an environmentally friendly alternative to traditional plastics production. The production of polyhydroxyalkanoates (PHAs) from methane was assessed, along with the impact of various cosubstrates on PHA copolymer composition. Valeric acid, as the most effective cosubstrate for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) accumulation, was used for the first time to study PHAs production from methane by mixed methanotrophic culture in a sequential feast-famine process. The influence of various gas flow rates, cosubstrate feeding strategies, and pH on culture growth and PHA accumulation was investigated. A high 3-hydroxyvalerate fraction of approximately 60 mol% at 0.1 standard litre per minute (slpm) and 40 mol% at 0.2 slpm was observed. The maximum biomass concentration of 2.25 g/L and a PHA concentration of about 0.5 g/L was achieved. A pH change led to a microbial shift from *Methylocystis hirsuta* to *Cupriavidus metallidurans*, without impacting the PHA accumulation capacity.

4.1. Introduction

The biological production of polymers for industrial applications is an intensely studied topic, largely because it presents a promising alternative to the traditional, environmentally harmful fossil fuel-based plastic production. The research on microbial synthesis of biopolymers is particularly focused on polyhydroxyalkanoates (PHAs), a group of thermoplastic polyesters that are biocompatible, biodegradable and sustainable. PHAs are naturally stored as carbon and energy source by various bacterial groups when there is an excess of carbon sources in nutrient-limited conditions (Kumar et al., 2020). The commercialization of this biopolymer production is still hindered by high production costs, which could be partially mediated by the use of waste streams as carbon source. In this context, the use of waste streams, including C1 gas emissions for PHAs production, could contribute to the global move towards a zero-waste bioeconomy supporting sustainable development (Vlaeminck et al., 2022). Notably, the use of methane (CH_4), a potent greenhouse gas, as the sole carbon source for bacterial PHA production presents an environmentally friendly and promising method to reduce CH_4 emissions. This process is facilitated by methanotrophic bacteria, which can directly utilise emitted CH_4 and convert it into various compounds, including PHAs, in pure culture cultivation as well as in microbial consortia processes (Gęsicka et al., 2021).

Various studies have shown the ability of the methanotrophic cultures to produce poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with varied 3-hydroxyvalerate fraction (3HV) as well as more complex PHA copolymers depending on the secondary source of carbon used and the cultivation conditions (López et al., 2018; Myung et al., 2017b, 2016; Zuñiga et al., 2013). The production of tailor-made PHAs copolymers could expand the range of possible industrial applications of the biopolymers as their properties change depending on the polymer composition. The most commonly studied PHA copolymer, PHBV, is produced when odd carbon number compounds such as propionic and valeric acid are introduced to the culture during the accumulation stage (Myung et al., 2016). When CH_4 is used as a sole carbon source under nitrogen limitation, PHB is the primarily accumulated type of PHA and different types of cosubstrates can become precursors for 3-hydroxybutyrate (3HB) or 3HV monomers and concomitantly affect the polymer composition and properties. Understanding the general relationship between the cosubstrate used and the final product structure creates an

opportunity to produce a customized biopolymer with the desired properties. Most studies for PHBV accumulation in methanotrophic cultures were done batchwise on a small scale (Amabile et al., 2024a) with only a few in a bioreactor system (Lee et al., 2023; Zuñiga et al., 2013). Traditionally, PHA production is a two-stage process. During the first phase, the culture is allowed to grow in nutrient-rich conditions. This culture is then collected and biomass is transferred to nitrogen-deficient conditions to induce PHA accumulation. However, this two-stage system has limitations for industrial production due to its complexity and equipment requirements. Therefore, the development of a single-vessel continuous process for PHBV production could enhance its potential for industrialization. Previous studies focused mostly on the continuous PHB production in continuously stirred tank reactors (CSTR) (Rodríguez et al., 2023a, 2022; Sabale et al., 2023) or in a bubble column bioreactors (BCB) (García-Pérez et al., 2018; Rodríguez et al., 2020c) using specific methanotrophic strains. To the authors' best knowledge, only one study has been conducted on the use of methanotrophic enrichment for PHB production in a CSTR. However, this yielded relatively low accumulations (up to 5% of dry cell weight (DCW)) (Chidambarampadmavathy et al., 2015a). This suggests that the potential of mixed cultures in gas fermentation for PHA production in bioreactors is yet to be fully explored. The potential benefits, such as no problem with contamination and the possibility of synergistic interactions among consortium members, offer compelling reasons to pursue this line of research. Despite considerable interest in studying PHAs accumulation by mixed cultures (Fergala et al., 2018c; Gęsicka et al., 2024; Luangthongkam et al., 2019a, 2019b; Myung et al., 2015), these studies have been limited to batch processes. Currently, there is a lack of data on the behaviour of methanotrophic enrichments during the PHA copolymer production in reactors fed with CH_4 and cosubstrate.

This study first aimed to evaluate how different cosubstrates affect PHAs accumulation and polymer composition using mixed culture enriched in a *Methylocystis* sp. and then to examine PHAs production in a CSTR under the feast-famine regime. Various alcohols and carboxylic acid additions were tested in batch experiments, and the cosubstrate resulting in the highest 3HV fraction was applied in a bioreactor for PHA accumulation under different carbon supply strategies. This approach was designed to test whether the timing of cosubstrate addition during the accumulation affects the 3HV fraction and could be used to adjust polymer composition. The additional effect of pH control on the process stability

was assessed during the second half of the process. Finally, the molecular weight of the extracted polymer was compared between reactors with different pH conditions.

4.2. Materials and methods

4.2.1. Mixed culture and experimental design

Methylocystis sp. dominant (40%) mixed culture enriched from waste activated sludge (AS10 culture) obtained in a previous study (Gęsicka et al., 2024) was used as an inoculum. As this culture was shown to grow better on nitrate than ammonium all experimental trials were done using a nitrate mineral salt (NMS) medium prepared according to Gęsicka et al. (2024). All cultures were cultivated under 10% CH_4 in the air.

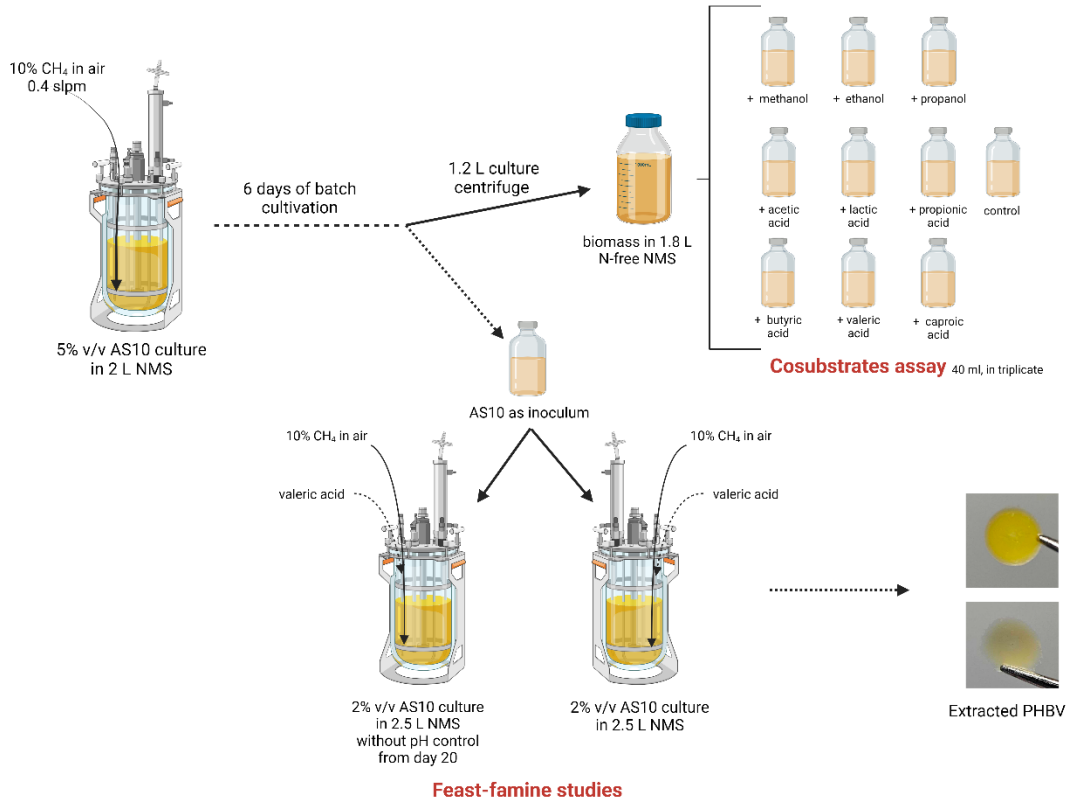


Figure 4.1. Scheme of the conducted studies.

The conducted study included two main experiments: cosubstrate evaluation and PHA production under a feast-famine regime. The scheme of the designed processes is presented in Figure 4.1. Initially, the AS10 mixed culture was grown in a bioreactor under batch cultivation to produce biomass for the cosubstrate assay and inoculum for subsequent bioreactor processes. The cosubstrate evaluation was carried out in small volume batches in triplicates. The most effective cosubstrate for PHBV synthesis was then applied in

sequential feast-famine studies on PHA production by the mixed methanotrophic culture. Finally, accumulated PHA at the end of the feast-famine process was extracted and characterised.

4.2.2. Batch cultivation of mixed culture

The frozen culture of AS10 was first thawed and grown in 20 mL, then in 200 mL of NMS medium under 10% CH_4 in air. It was incubated in an orbital shaker at 30^oC and 130 rpm. The gas in the headspace was renewed every 2-3 days until the culture reached the optical density (OD_{600}) of 0.8. The culture was then used to inoculate 2 L of NMS medium (at 5 % v/v) in a 3 L F0-Baby bioreactor (Bionet, Spain). The bioreactor was equipped with a pH and dissolved oxygen (DO) probe and a gas analyser (DP-28 BIO, Nanosens, Poland) on the gas outlet to periodically measure gas composition. The gas mixture of 10% CH_4 in the air was continuously supplied into the culture through a ring sparger at the bottom of the vessel. This was done at a rate of 0.4 standard litre per minute (slpm) using external gas mass flow controllers (MFC) (DPC, Aalborg, USA). The temperature was set at 30^oC, stirring at 400-450 rpm and pH was maintained at 6.8 with the use of 1 M HCl. The scheme of the bioreactor set-up is shown in Figure S4.1. The culture was grown in a batch mode for 6 days at the end of which 1.2 L of culture was centrifuged to obtain biomass for cosubstrate assays. Part of the culture was frozen in 10% dimethyl sulphide (DMSO) and later used as an inoculum in subsequent bioreactor trials.

4.2.3. Cosubstrate assay

Different alcohols and carboxylic acids were used as potential cosubstrates to assess their effect on PHA synthesis in a small scale batch system. The biomass recovered from 1.2 L of 6-day AS10 culture (section 4.2.2) was resuspended in 1.8 L of N-free NMS medium (NMS medium without the nitrate) to induce PHA accumulation in nitrogen-limited conditions. The culture was divided into 40 mL culture in 120 ml serum bottles, with the initial biomass concentration of 1.12 ± 0.05 g/L, and supplemented with cosubstrate. The concentrations of tested cosubstrates were calculated so that all used chemicals would have a similar carbon loading of 5.3 ± 0.3 mmol C/L. The addition of cosubstrates was as follows [in g/L]; methanol 0.17, ethanol 0.12, propanol 0.12, acetic acid 0.16, lactic acid 0.17, propionic acid 0.13, butyric acid 0.11, valeric acid 0.1 and caproic acid 0.1. In addition, control without any cosubstrate added was also tested. All cultures were run in triplicates,

flushed with 10% CH_4 in air and incubated at 30°C and 130 rpm for 2 days, the headspace gas was renewed after 24 h. After the accumulation the biomass was recovered by centrifugation at 8000 rpm for 10 min (MPW-352R, MPW, Poland), supernatant was used for metabolites analysis, while biomass was washed twice with distilled H_2O and stored at -20°C for DCW and PHA analysis. It is important to note, that the biomass used for the cosubstrates assay came from a nitrogen-depleted culture and already had PHB accumulated at the level of 57% in DCW. The difference in the biomass and PHA concentration at the beginning and end of the process was measured and presented as a biomass and PHA increase (%) during the 2-day accumulation test. Results from triplicates were presented as a mean value with standard deviations and one-way ANOVA with significance $p \leq 0.05$ was performed.

4.2.4. PHA production under a feast-famine regime

Two bioreactor systems operated in sequential feast-famine regime of 48 h:48 h cycles were run under continuous CH_4 supply to evaluate the effect of different carbon supply and pH control strategies on PHA accumulation. The operation of the sequential feast-famine regime herein applied based on the work of Rodríguez et al. (2022), where the process operated under 24 h:24 h feast-famine cycles supported effective PHB production. The 24-hour cycles for each stage were prolonged to 48 hours to allow a longer time for PHA accumulation. The AS10 culture from a 6-day bioreactor batch cultivation (section 4.2.2) was grown to an OD_{600}=0.76 and used to inoculate two reactors of 2.5 L NMS medium (at 2 % v/v). The bioreactor set-up described in section 4.2.2 was upgraded to allow continuous operation conditions by supplying culture with fresh NMS medium and pumping out the effluent adopting a liquid height-based culture volume control with the use of a level sensor. Temperature of 30°C, stirring at 400-450 rpm and pH control with 1 M NaOH and 1 M HCl were applied. 10% CH_4 in air gas mixture flow to the reactor was operated as described in section 4.2.2. The scheme of the applied system is shown in Figure 4.2. During the feast phase, the culture was operated under continuous mode with NMS feeding at 0.5 L/d for 48 h after which it was switched to the famine phase under batch operation to induce nitrogen depletion and PHA accumulation. Valeric acid at the 0.5 g/L concentration was added to the culture during the famine stage as a cosubstrate for PHBV synthesis, and different supply strategies were studied to evaluate if the time point of the cosubstrate feeding had an impact on the PHA composition. During the process run, the gas flow (CH_4

supply) and valeric acid addition were subjected to changes as detailed in Table 4.1. At the beginning of the feast stage on days 13th, 17th, 21st, and 29th, 25 mL of 100 mg/L KNO_3 solution was added to the reactor to instantly supplement the culture with nitrogen at the concentration of 1 g/L after its total depletion in the famine stage. The effect of the pH control strategy on the PHA production was assessed in reactor F1 in which pH control was switched off since day 20. The mixed culture of AS10 was cultivated for 8 sequential feast-famine cycles, while the first feast stage lasted 3 days to allow for culture growth before starting the nitrogen-limited famine stage. Every 24 h samples for OD_{600}, DCW, PHA, nitrate, nitrite, valeric acid, and biomass for microbial analysis were collected. Additionally, gas composition at the outflow was monitored once a day using a gas analyser (DP-28 BIO, Nanosens, Poland). The pH and dissolved oxygen were monitored throughout the process duration (see Supplementary materials). The content of reactor F1 was diluted between days 16th and 20th due to the overdosing of acid and base used by the pH control system.

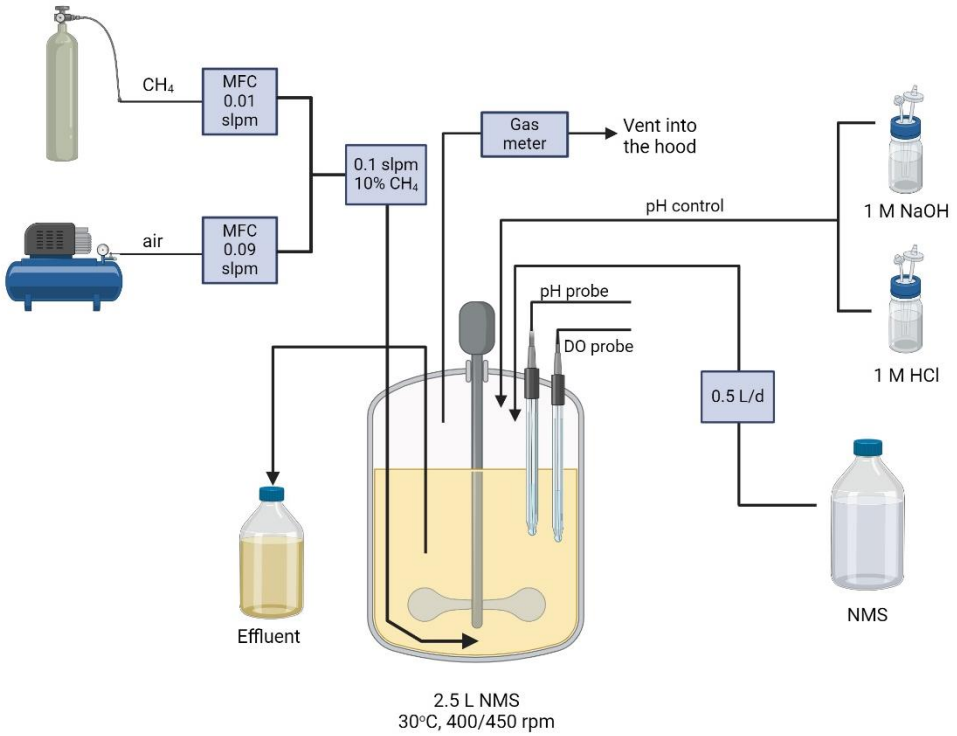


Figure 4.2. Scheme of the bioreactor set-up for feast-famine cycles.

Table 4.1. Gas flow and cosubstrate supply during different stages of the feast-famine process.

Stage	Days	Gas flow [slpm]
Feast	0-2	0.4
	2-3	0.1
Famine	3-5	
Feast	3-7	
Famine + 0.5 g/L VA at 0 h	7-9	
Feast	9-11	
Famine + 0.5 g/L VA at 0 h and 24 h	11-13	
Feast	13-15	
Famine + 0.5 g/L VA at 24 h	15-17	
Feast	17-19	
Famine	19-21	
Feast	21-23	0.2
Famine + 0.5 g/L VA at 0 h and 24 h	23-25	
Feast	25-27	
Famine + 0.125 g/L VA every 6 h	27-29	
Feast	29-31	
Famine + 0.25 g/L VA every 6 h	31-33	

4.2.5. Analytical methods

The concentrations of organic acids and alcohols; especially ethanol, propanol, acetic, propionic, butyric, valeric, and caproic acid, were measured using Shimadzu GC-2014 gas chromatography equipped with Zebron ZB-FFAP column and FID as described previously (Gęsicka et al., 2024). Methanol and lactic acid concentrations were measured with high-performance liquid chromatography (HPLC) (Shimadzu 20AT) equipped with Rezex ROA-Organic Acid column and RID. As an eluent, a 5 mM sulfuric acid was used for elution at the flow rate of 0.6 mL/min at 63^{o}C. Culture growth was monitored by observing the OD_{600} and DCW as described in Gęsicka et al. (2024). Nitrate concentration in the culture was measured photometrically using Spectroquant® Nitrate Tests (1.09713 Supelco, Merck, Germany) according to the provided protocol and analysed on Spectroquant® Prove 300 (Merck, Germany). Similarly, nitrite concentration was monitored using the Spectroquant® Nitrite Test (1.14776 Supelco, Merck, Germany).

PHA composition and quantification were determined on the GC-2014 Shimadzu gas chromatography equipped with a capillary column Zebron ZB-FFAP and coupled with FID, after hydrochloric acid propanolysis of samples as described in detail in Gęsicka et al. (2024). In short 4-12 mg of freeze-dried biomass was suspended in 2 mL of chloroform, 1 mL of propanol with concentrated hydrochloric acid (80:20% v/v), and 10 µL of internal standard (25 g/L benzoic acid in propanol). After heating at 100^{o}C for 3 h and then cooling down, 1 mL of distilled H_2O was added, mixed, and left for phases to separate. The bottom organic phase was used for analysis on the gas chromatography. Biologically sourced PHBV copolymer with 8 mol% of PHV content (Sigma-Aldrich) was used for calibration. PHA accumulation was reported as a PHA content in the DCW (% w/w), and PHA concentration in culture (g/L).

4.2.6. Microbial composition analysis

Microbiome composition analysis was done by Nanopore sequencing of full rRNA operon amplicons. Biomass samples were collected by centrifugation and stored frozen at −20°C until processing. Total metagenomic DNA was isolated using ZymoBIOMICS DNA Microprep Kit (Zymoresearch, Poland) according to the manufacturer's recommendations. ZymoBIOMICS Microbial Community Standard D6300 (Zymoresearch, USA) was used as a mock microbial community to evaluate the analysis pipeline. Amplification of V3 and V4 region of 16S rRNA gene was carried out with Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/µL) and primers 16S-27F (5′- TTTCTGTTGGTGCTGATATTGC AGrGTTTGATyhTGGCTCAG) and 23S-2241R (5′-ACTTGCCTGTCGCTCTATCTTC ACCrCCCCAGThAAACT-3’). The resulting amplicons were indexed during 8 cycles of the second PCR reaction, carried out with LongAmp® Hot Start Taq DNA Polymerase (NEB, M0533S), 50 ng of amplicon and individual forward-reverse barcode primer pair for each sample. After pooling barcoded amplicons in equimolar concentration further library preparation was carried out using Oxford Nanopore Sqk-lsk114 Ligation Kit. Sequencing was done on FLO-MIN114 (R10.4.1) flow cell utilizing MinION Mk1C sequencer (Nanopore Technologies), to the depth of ca. 10k full-length amplicons per sample.

Acquired raw data was subsequently basecalled using super-accurate dna_r10.4.1_e8.2_400bps_sup@v4.3.0 model with dorado v0.5.3 basecaller. The reads were run through Duplex Tools v0.2.9 (Oxford Nanopore Technologies, 2022) to split concatenated reads by Nanopore adapter. Reads were then filtered by average Phred ≥ 10

using NanoFilt v2.8.0 (De Coster et al., 2018) and those in size between 3000bp and 7000bp retained for further analysis. Demultiplexing and removal of synthetic sequences was done with minibar (Krehenwinkel et al., 2019). The reads were then classified using the EMU v3.4.1 (Curry et al., 2022) program by mapping to rrn operon database, ncbi_202006 "NCBI RRN" (Kinoshita et al., 2021). Pseudo tax-table containing all samples and phyloseq object were created as described by (Petrone et al., 2023). Downstream analyses and visualisations were carried out using the Phyloseq package (Mcmurdie and Holmes, 2013). Raw sequences obtained in this study were submitted to NCBI Sequence Read Archive (SRA) database and are available under BioProject ID PRJNA1111824.

4.2.7. Polymer extraction and molecular weight characterisation

The molecular weight of PHA accumulated in reactors F1 and F2 at the end of the process was characterised by gel permeation chromatography (GPC). The polymer was extracted from the biomass collected from 40 mL of culture by applying a modified chloroform extraction method (Vermeer et al., 2022). In short 15-30 mg of freeze-dried biomass was suspended in 5 mL of chloroform and heated at 60^{o}C for 3 h with shaking every 30 min. The reaction mixture was centrifuged at 10000 rpm for 10 min at 20^{o}C and the liquid phase was filtered through 0.45 µm filters to glass test tubes to separate the extracted polymer from the cell debris. The extracted polymer was then left to dry at 60^{o}C for 2 days under the fume hood. The PHBV film was used to determine the molecular weight of the polymer by GPC. To determine the number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI=M_w/M_n), polymer samples were first dissolved in chloroform for 18 h at 35^{o}C, at the concentration of 2.5 mg/mL, filtered through a 0.22 µm PTFE membrane, and then analysed using Agilent 1200 HPLC series with refraction index detector (RID) (Agilent Technologies, Santa Clara, CA, USA). The instrument was equipped with two PLgel 5µm MIXED-C (300x7.5mm) columns (Agilent Technologies). The temperature of analysis was maintained at 35^{o}C and chloroform was used as a mobile phase at the flow rate of 0.7 mL/min. For the calibration curves polystyrene standards with weight in the range of 474 g/mol-1800000 g/mol were used.

4.3. Results and discussion

4.3.1. Effect of different cosubstrate addition on PHA accumulation

The effect of C1-C3 alcohols and C2-C6 carboxylic acids as cosubstrates for CH_4-based PHA accumulation by a mixed methanotrophic culture was evaluated based on the increase in PHA accumulation (%) and the 3HV molar fraction (Fig. 4.3). It is important to note that the culture was transferred from a nitrogen-deficient reactor and the biomass already had PHB accumulated at the level of 57% PHB in DCW. Except for acetic acid, all other cosubstrates were fully utilised by AS10 culture after 48 h of the accumulation phase. The PHA accumulation was distinctively affected by different chemical compounds used as cosubstrates. Of the tested alcohols, the addition of ethanol led to a significant increase in biomass without affecting the PHA accumulation, compared to the control culture ($p \leq 0.05$) (Fig. 4.3). On the other hand, even though methanol can be used by methylotrophic bacteria as a carbon source (Sarwar and Lee, 2023), its addition to the mixed culture did not have a positive impact on biomass increase, however, it significantly reduced the PHA accumulation potential of the culture ($p \leq 0.05$). The use of acetic and lactic acid as cosubstrates proved unfeasible as they resulted in the lowest increase in PHA accumulation (Fig. 4.3). Furthermore, the addition of acetic acid restricted bacterial growth, as indicated by the lowest measured biomass among all tested cultures (Table S4.1). As the trials were conducted without pH adjustment to observe the effect of cosubstrates addition on the mixed culture, the pH changes were analysed showing a similar final pH of 6.8 in all cultures except for the acetic acid (Fig. S4.2). Although the addition of lactic, propionic and butyric acid resulted in a pH decrease to 5.4-6.0 it did not hinder the AS10 culture growth which was similar to or higher than in the control culture (Fig. 4.3). Only the acetic acid supplementation caused a pH drop below 5 creating limiting conditions for mixed culture activity and growth. Contrastingly, López et al. (2018) reported increased PHA accumulation in the *Methylocystis hirsuta* culture supplemented with acetic acid during biogas biodegradation, in culture without pH adjustment. While *M. hirsuta* tolerated low pH (4.2) and was able to grow below 200 mg/L of acetic acid (López et al., 2018), the AS10 culture enriched in *Methylocystis* sp. used in the present study did not exhibit the same tolerance under similar conditions. This discrepancy may be due to the strain-specific differences in response to the pH conditions. When C3-C6 fatty acids were added as cosubstrates, PHA accumulation increased with the carbon chain length of the cosubstrate,

reaching the highest PHA accumulation and 30 ± 2 % of PHA increase when caproic acid was supplied (Fig. 4.3 and S4.3). Conversely, no variations in PHA accumulation were observed when C4, C6, and C8 fatty acids were used as cosubstrates in the *Methylocystis parvus* culture (54-56% PHA in DCW) (Myung et al., 2017b). The greater abundance of possible metabolic pathways in mixed culture, compared to single strain cultures, could account for the observed differences in the effect of various cosubstrate additions on PHA accumulation.

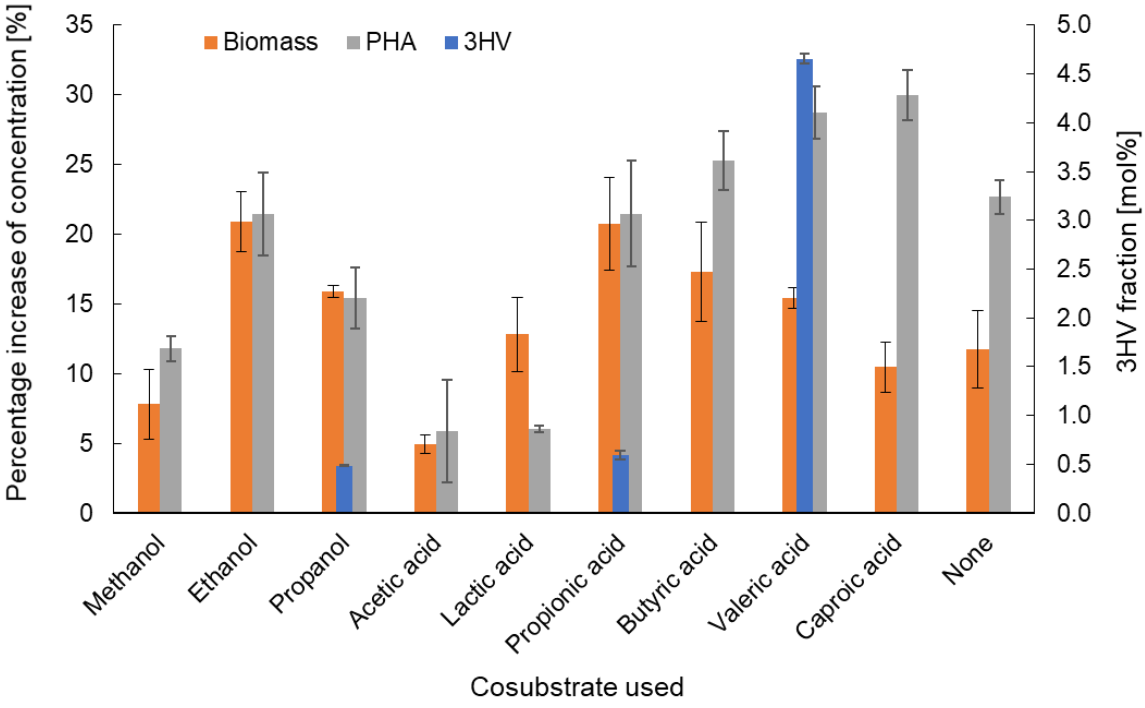


Figure 4.3. Comparison of biomass and PHA increase [%], and 3HV fraction of accumulated PHA after 2 days of accumulation by AS10 culture using different alcohols and organic acids as cosubstrates. The percentage of biomass and PHA increase was calculated based on the changes in their concentrations [g/L] during the accumulation.

The addition of odd-carbon compounds such as propanol, propionic, and valeric acid, resulted in the synthesis of PHBV copolymer with differed 3HV fractions (Fig. 4.3), which is in agreement with the available literature (López et al., 2018; Luangthongkam et al., 2019; Myung et al., 2016). As observed in prior studies, valeric acid addition resulted in the highest 3HV fraction incorporation into PHBV copolymer, though lower than other CH_4-based PHA accumulation studies, which ranged between 14 to 25 mol% (López et al., 2018; Luangthongkam et al., 2019; Myung et al., 2016, 2017). Aside from the culture-specific ability to use valeric acid for PHBV accumulation, the lower 3HV fraction herein achieved might be due to a low cosubstrate to biomass ratio and high PHB content in the

cells at the start of the accumulation assay. In our previous research, the AS10 culture, when supplied with the same concentration of valeric acid, had a 3HV fraction of 39 ± 2 mol% and 3HV yield of 0.52 ± 0.01 g-3HV/g-valeric acid (Gęsicka et al., 2024). In the present study supplied valeric acid was entirely utilised and resulted in a 3HV yield of 0.38 ± 0.03 g-3HV/g-valeric acid. It appears that for a mixed culture with a biomass concentration higher than 1 g/L, and with already accumulated PHB the cosubstrate addition should be adjusted by increasing its concentration or supply frequency to avoid cosubstrate-limited conditions.

4.3.2. Effect of carbon supply strategy on PHA production in a sequential feast-famine process

For the first time, a PHA-producing mixed methanotrophic culture was used to study the PHBV accumulation in a CSTR under a sequential feast-famine regime. As valeric acid has been shown to be the best precursor for 3HV synthesis it was added to the culture during the famine stage to induce PHBV accumulation. To accommodate the increased carbon utilisation by the culture at higher biomass concentration, 0.5 g/L of valeric acid was added to the reactor, instead of 0.1 g/L which was an optimum concentration in batches (Fergala et al., 2018c). Different feeding time-based cosubstrate supply strategies were applied to evaluate their effect on the PHA accumulation. Table 4.1 presents the carbon supply strategy (gas flow and cosubstrate addition) applied at each stage. Figure 4.4 shows the time course of PHA accumulation, the 3HV molar fraction of produced polymer as well as the amount of biomass and PHA concentration therein.

Both reactors had identical starting conditions and the supplied nitrate was similarly depleted after day 7, when the PHA accumulation was first initiated. After supplementing the cultures with nitrate at the beginning of the feast phase, mixed cultures rapidly used the provided nitrogen source, partially converting it to nitrite, which was observed at small concentrations (<70 mg NO_2/L) a few times in the middle of the process (data not shown). During the first famine stage (days 3-5), PHB was not accumulated because nitrate was still readily available above 300 mg NO_3/L (Figure S4.4). After establishing conditions that induce PHA accumulation on day 7, the cultures could produce and maintain PHA for most of the process duration. However, PHA accumulation and composition varied based on the culture conditions at a given time (Table 4.1 and Figure 4.4). After reaching up to 81 mol% of 3HV fraction in accumulated PHA at the end of the second feast-famine cycle, a

consistent 3HV fraction of around 60 mol% was achieved in the next two accumulation stages. This pattern was similar in both reactors. The culture capability to accumulate PHA during the famine stage decreased with each cycle, from a maximum of 29 and 44 % PHA in DCW to 9 and 13 % for F1 and F2, respectively (Figure 4.4A and 4.4C). This diminished PHA accumulation over time by the mixed culture might have resulted from decreased methane availability at 0.1 slpm (0.01 slpm CH_4), when biomass concentration increased in up to 0.81 and 1.07 g/L at day 13, in F1 and F2 respectively. When cultures were not provided with valeric acid in the 5th cycle (19-21 d) the F2 culture metabolized all of its previously accumulated 3HV fraction and were able to only synthesise 3HB in this stage. However, the culture in F1 retained a small concentration of 3HV in the biomass (less than 10 mg 3HV/L, Figure S4.4). To overcome the possible existing limitations for the efficient PHA accumulation the carbon supply to the reactor was doubled by increasing the gas supply from 0.1 to 0.2 slpm from the 21st day. After adjusting the gas flow, the PHA accumulation returned to 20-30% PHA in DCW (Figure 4.4A and 4.4C), and the PHA production reached 281 and 465 mg PHA/L for F1 and F2, respectively. In both cases, the 3HV fraction accounted for nearly half of the produced PHA (Figure 4.4 and S4.4). When CH_4 supply was increased, cosubstrate addition was also kept at higher doses, amounting to overall 1 and 2 g/L during the 2-day famine stage, to enhance the availability of the carbon source for PHA accumulation. This carbon supply strategy resulted in a stable 3HV fraction of around 40 mol% from day 25th to the end of the process for the pH-controlled culture. This stability coincided with the decrease in the biomass concentration from 2.25 to 0.95 g/L (Figure 4.4C and 4.4D). A similar decrease in biomass concentration was observed in a continuous *M. hirsuta* culture in a CSTR, which decreased after reaching a maximum of 1.69 g/L due to a deterioration in CH_4 biodegradation performance (Rodríguez et al., 2023b). On the other hand, in reactor F1 without the pH control, where the initial biomass was low due to culture dilution, the increased carbon supply and cosubstrate feeding strategy led to 3HV fraction fluctuations between 20-50 mol% during the famine stages (Figure 4.4A). Under low CH_4 supply, regardless of the feeding strategy used, the 3HV fraction reached approximately 60 mol%. Meanwhile, when the pH remained unchanged and the gas supply was increased, the 3HV fraction stabilised at around 40 mol%. This is consistent with the results from batch studies with higher valerate concentrations (≥0.4 g/L) (Fergala et al., 2018c; Myung et al., 2016, 2015) and when the AS10 culture was studied with 0.1 g/L of valeric acid (Gęsicka et al., 2024).

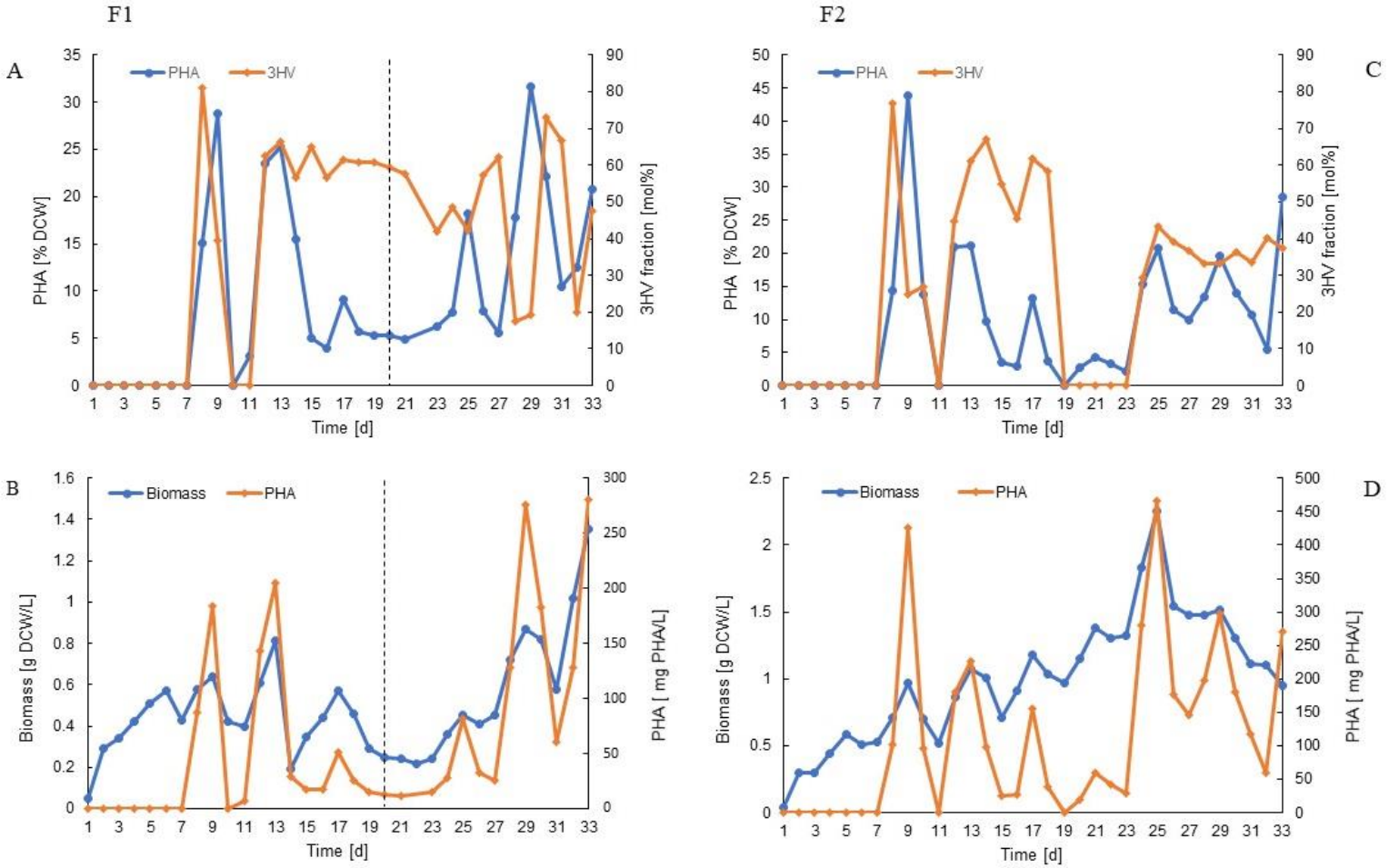


Figure 4.4. Time course of PHA accumulation [% DCW] and 3HV molar fraction [mol%] in reactor F1 (A) and F2 (C), biomass concentration [g DCW/L] and PHA concentration in culture [mg PHA/L] in reactor F1 (B) and F2 (D). Dash line on graphs for F1 reactor marks the day when pH control was switched off.

When valeric acid was added twice during the famine stage (at 0 and 24 h), the cosubstrate was not utilised by the end of the accumulation stage. However, when the same cosubstrate addition strategy was applied at a higher gas supply, valeric acid was completely utilised in both reactors, regardless of the culture density (0.24 vs 1.32 g DCW/L for F1 and F2 respectively) (Figure S4.3). Higher CH_4:air supply allowed for more efficient fatty acid oxidation which is an energy-demanding process (Myung et al., 2016) and higher oxygen supply was beneficial for C1 oxidation required for valeric acid utilisation. Similarly, when valeric acid was only added on the second day of accumulation (day 16), there was leftover cosubstrate in the culture at the end of the famine stage, indicating that these conditions limit efficient cosubstrate utilisation for PHA accumulation (Figure S4.5). These results show that supplying 10% CH_4 in the air at 0.1 slpm did not provide enough energy for the metabolic pathways to utilise 0.5 g/L of provided valeric acid during a 24 h famine stage. This highlights the importance of an optimal CH_4, O_2, and cosubstrate ratio for efficient PHBV accumulation.

The increase in the gas flow to the reactor creates a higher mass transfer of CH_4 and O_2 to the liquid medium, which in turn leads to an increase in both biomass production and the overall PHA concentration in the culture (Sabale et al., 2023). The positive effect of this increased gas supply on culture growth was particularly noticeable in the F1 reactor. Here, biomass quickly rose from 0.24 to 1.35 g/L (Figure 4.4B), and for a few days in F2, where biomass reached a maximum of 2.25 g/L (Figure 4.4D). Although the final biomass concentrations were lower than that achieved in a similar study by (Rodríguez et al., 2022), in which authors used 24 h:24 h feast-famine cycles for PHB production with the *Methylocystis parvus* strain, the PHA content was within a comparable range throughout the one-month process.

Under stable pH conditions (reactor F2), the applied carbon supply strategies had a minimal impact on the mixed culture structure. Throughout the process, the dominant species was *Methylocystis hirsuta* with a relative abundance of 50-60%. The other major species varied over time (Figure 4.5). Initially, the other abundant species in the culture was *Acidovorax delafieldii* (up to day 11), then *Caulobacter segnis* and *Novosphingobium subterraneum* took over (days 15-27). Finally, there was a temporary increase in *Stenotrophomonas acidaminiphila* at the end of the process possibly due to short periods of pH disruption. Most species in the culture can potentially express polyhydroxyalkanoates synthase (PhaC) (based on the positive match for PhaC in the NCBI Protein database) a key enzyme in the

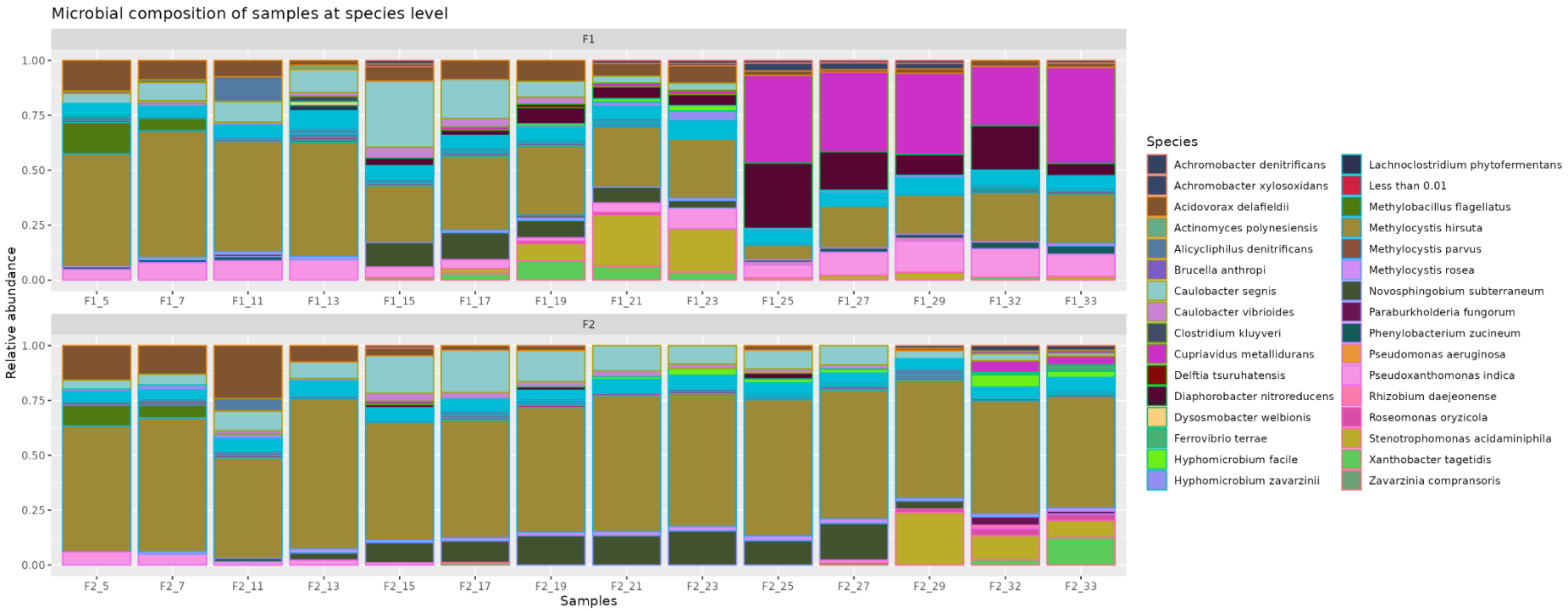


Figure 4.5. The dynamics of microbial community structure changes at the species level in the culture during the course of the process.

PHA synthesis pathway (Zher Neoh et al., 2022). Although many species could theoretically accumulate PHA, it is unclear if they were actively participating in PHA synthesis. The involvement of major bacterial species other than *M. hirsuta* in PHA accumulation cannot be ruled out. This is especially relevant for *C. segnis*, which was present in the culture for most of the process and has proven to be a good PHB producer (Bustamante et al., 2019).

4.3.3. Effect of pH control on PHA production in a sequential feast-famine process

The PHA-producing mixed culture was able to adapt to the changes in pH, from the set neutral conditions, without compromising its ability to grow on CH_4 and to accumulate PHBV (Figure 4.4A and 4.4B). After deactivating the pH control in reactor F1 (day 20), the pH rose to 8.2 within 5 days and stabilised at around 8.0 for the rest of the process (Fig. S4.6). The pH increase did not affect the PHA accumulation capacity, with both reactors reaching the maximum PHA of approximately 18-32 % and 21-29 % PHA in DCW for F1 and F2, respectively. However, while the 3HV fraction in the F2 reactor remained relatively stable, the F1 culture displayed high 3HV fraction fluctuations from 20-50 mol% during the famine stage and up to 73 mol% during the feast stage. Here, the 3HV was not utilised as quickly as 3HB resulting in a higher 3HV molar fraction (Figure 4.4A and S4.4). In the work of Pérez et al. (2024), the mixed methanotrophic culture had comparable biomass productivities at pH 7 and 8.5. Similarly, in this study, the mixed culture growth was not hindered at higher pH levels, and at 0.2 slpm CH_4:air supply the biomass concentration quickly increased (Figure 4.4B). The alternation in pH operating conditions resulted in significant changes in the microbial composition. *M. hirsuta* decreased to approximately 20%, while *Cupriavidus metallidurans* became a major species at 40-50% relative abundance, and *Diaphorobacter nitroreducens* and *Pseudoxanthomonas indica* ranged between 10-30%. Despite the reduced abundance of the primary PHA-producing methanotroph, PHA accumulation remained similar to the culture where *M. hirsuta* was the dominant species. This suggests that other active PHA-producing bacteria were involved in the PHBV synthesis, most likely the dominant *C. metallidurans* which has been previously studied for PHB accumulation (Rogiers et al., 2022), in addition, other species of *Cupriavidus* genus have been shown to grow and accumulate PHBV on valerate (Cai et al., 2023). A similar collaboration among microbial consortia members for PHA accumulation was observed at pH 8.5 when *Methylocystis* sp. was less abundant, and other PHB-producing genera were abundant in the culture (Pérez et al., 2024). Both studies

indicate that operating the CH_4 to biopolymer conversion with a mixed methanotrophic culture at pH 8-8.5 does not significantly impact the PHA accumulation capacity under nitrogen-limited conditions, as the microbial community shifts towards other potential PHB-producers that thrive on CH_4-derived carbon.

The shift in the microbial community due to the pH changes could impact how valeric acid is used for 3HV inclusion in the polymer. This might explain why the distribution of valeric acid into 3HV was 4 times higher in the *M. hirsuta*-dominant culture than in the *C. metallidurans*-rich one (Figure S4.7). For comparison, the incorporation of valeric acid into the PHBV copolymer during the first half of the process was relatively similar in both reactors, at 20-30% (Figure S4.5), but these results were notably lower than the 92-99% reported for pure culture (Lee et al., 2023; Myung et al., 2016). These variations in valeric acid metabolism highlight the complexity of the microbial consortia and the shared carbon utilization pathways. Additionally, the different capacities for PHBV accumulation among specific bacteria in the culture could stem from the type of PHA synthase expressed, as they may differ in substrate specificity (Rehm, 2003). More research is required to understand the roles each microorganism plays in this ecosystem and how their interactions influence the conversion of valeric acid into the PHBV copolymer. This could provide further insight into how to optimise the use of valeric acid for 3HV inclusion in the polymer by mixed methanotrophic cultures.

4.3.4. Molecular weight characterisation of the produced PHBV

The final PHBV accumulated in the culture was extracted, and its molecular weight was determined. The number-average molecular weight of extracted polymers varied slightly between the reactors and were generally lower than PHA produced from CH_4 in previous studies but comparable to the commercial PHBV (Table 4.2). Herein obtained polymers were also close in size to the PHBV with 25 mol% 3HV fraction synthesised by *Methylocystis* sp. MJC1 that was also cultured in a CSTR with higher valerate doses (Lee et al., 2023). The higher molecular weights were generally observed for PHBV accumulated in batch tests on a small scale, while the type of cosubstrate used and its concentration did not have a substantial effect on the PHA copolymers' molecular weight (Myung et al., 2017b, 2016). The slightly higher molecular weight of PHBV with 47 mol% 3HV fraction herein obtained could be the result of variation in the microbial community. Specifically, the prevalence of *C. metallidurans* over *M. hirsuta* may lead to

physicochemical differences in the synthesized polymer. This could result in a polymer with longer molecular chains, and therefore a higher overall molecular weight. Further research into the specific metabolic processes of these microorganisms is needed to confirm this hypothesis.

Table 4.2. Molecular weight of PHB and PHBV produced by methanotrophic cultures

PHA	Strain	Molecular weight		Reference
		M_n [Da]	PDI	
PHBV with 47 mol% 3HV	Methanotrophic enrichment (23 % *Methylocystis hirsuta*)	5.03 x 10^5	2.90	This study
PHBV with 37 mol% 3HV	Methanotrophic enrichment (51 % *Methylocystis hirsuta*)	4.70 x 10^5	3.08	This study
PHB	*Methylocystis* sp. GB 25	2.5 x 10^6	na	(Wendlandt et al., 2001)
PHB	*Methylocystis*-dominated methanotrophic enrichment	1.20 ± 0.20 x 10^6	1.76 ± 0.22	(Myung et al., 2015)
PHBV with 20 mol% 3HV		1.15 ± 0.11 x 10^6	1.88 ± 0.18	
PHBV with 40 mol% 3HV		9.34 ± 0.78 x 10^5	2.14 ± 0.27	
PHB	*Methylosinus trichosporium* OB3b	3.24 ± 0.21 x 10^6	1.67 ± 0.22	(Myung et al., 2016)
PHBV with 22 mol% 3HV		1.83 ± 0.17 x 10^6	1.88 ± 0.19	
PHBV with 37% mol 3HV		1.70 ± 0.19 x 10^6	2.23 ± 0.45	
PHB	*Methylocystis parvus* OBBP	1.48 x 10^6	1.82	(Myung et al., 2017b)
PHBV with 24 mol% 3HV		1.32 x 10^6	2.24	
Commercial PHB	-	7.38 x 10^5	2.02	
Commercial PHBV	-	4.48 x 10^5	2.18	
PHBV with 10 mol% 3HV	*Methylocystis* sp. MJC1	7.78 x 10^5	1.3	(Lee et al., 2023)
PHBV with 25 mol% 3HV		5.75 x 10^5	1.4	

na – not available

4.4. Conclusion

The study evaluated cosubstrates for PHA accumulation by mixed methanotrophic culture, identifying valeric acid as the most effective for 3HV inclusions. It pioneered PHBV synthesis from CH_4 and valeric acid in a long-term CSTR process. Adapting sequential feast-famine cycles facilitated 3HV-rich PHBV production, achieving a 3HV fraction of 60 mol% at 0.1 slpm and 40 mol% at 0.2 slpm. However, adjusting polymer composition was unachievable via carbon supply strategies. pH changes shifted the microbial community from *M. hirsuta* to *C. metallidurans*, without affecting PHA accumulation capacity. It did however affect 3HV molar fraction showcasing a possible way of adjusting polymer composition by manipulating culture composition through changes in culture pH. Produced polymers showed similar properties to commercial PHBV, demonstrating the process potential for CH_4-based PHBV production.

4.5. Acknowledgments

This work was financed by the National Science Centre, Poland under grant agreement no. 2019/35/D/ST8/03530. Figures 1, 2 and S1 were created with BioRender.com.

4.6. Supplementary material

Table S4.1. Comparison of final biomass, PHA and 3HV concentration after 2 days of accumulation using different alcohols and organic acids as cosubstrates. The initial concentrations were at 1.12 ± 0.05 g/L for biomass and 0.64 ± 0.03 g/L for PHA.

Cosubstrate	Biomass [g/L]	PHA [g/L]	3HV [mg/L]
Methanol	1.24 ± 0.03	0.62 ± 0.03	0
Ethanol	1.37 ± 0.02	0.69 ± 0.02	0
Propanol	1.35 ± 0.01	0.67 ± 0.01	4 ± 0.1
Acetic acid	1.17 ± 0.01	0.59 ± 0.02	0
Lactic acid	1.28 ± 0.03	0.60 ± 0.001	0
Propionic acid	1.20 ± 0.03	0.60 ± 0.02	4 ± 0.3
Butyric acid	1.34 ± 0.04	0.71 ± 0.01	0
Valeric acid	1.27 ± 0.07	0.70 ± 0.05	40 ± 2.3
Caproic acid	1.24 ± 0.02	0.72 ± 0.01	0
None	1.21 ± 0.03	0.66 ± 0.01	

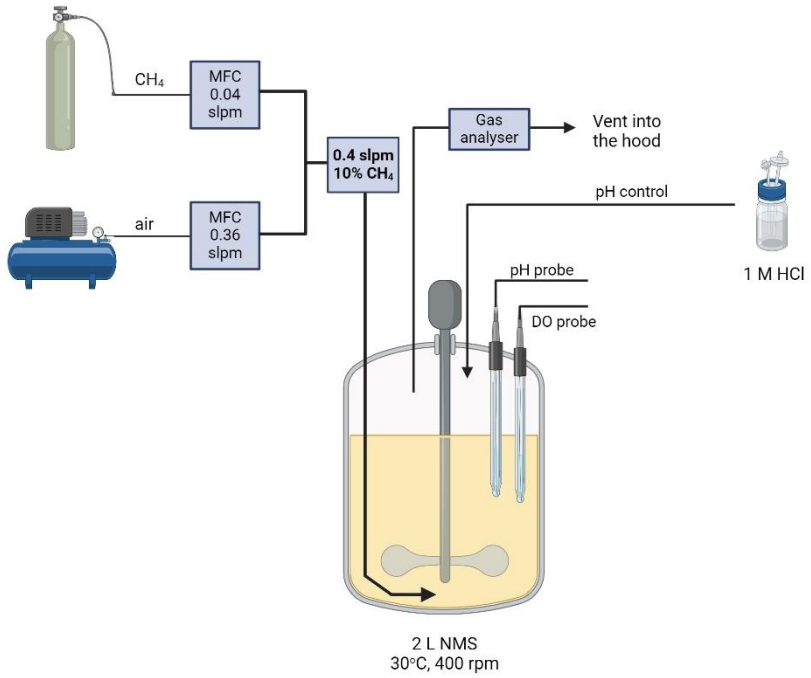


Figure S4.1. Scheme of bioreactor set-up for a batch continuously fed with CH_4.

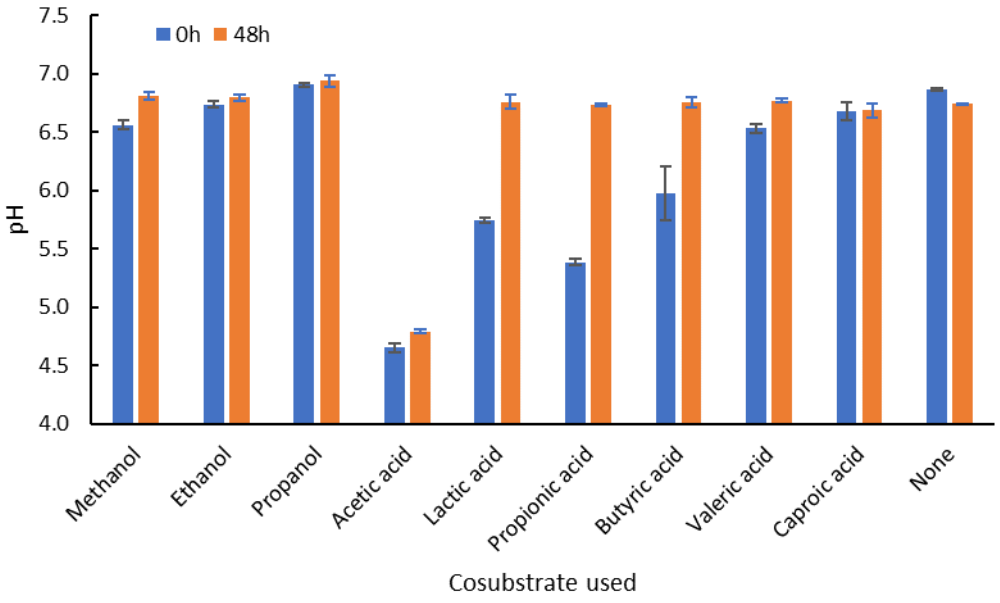


Figure S4.2. pH changes during the 2 days of accumulation in cultures supplied with different cosubstrates.

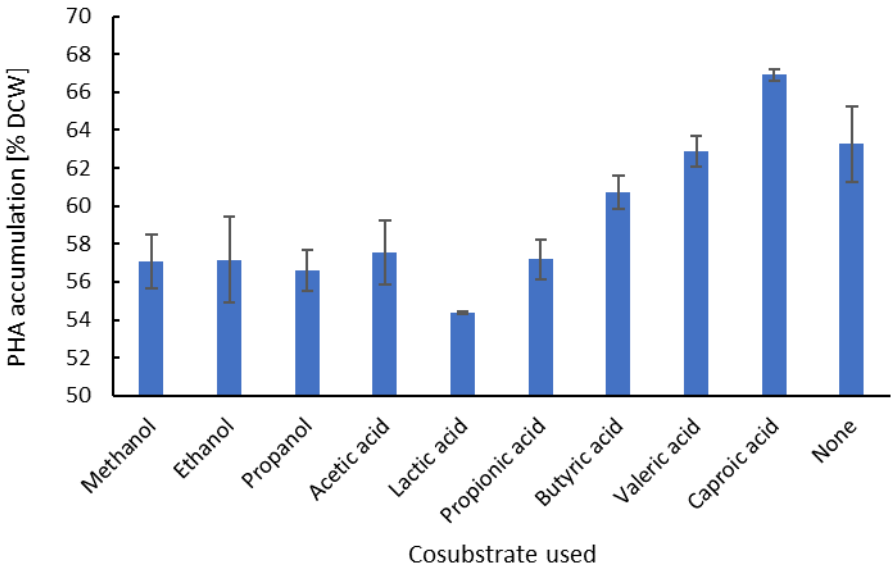


Figure S4.3. Final PHA accumulated [% DCW] by mixed culture supplied with different cosubstrates. Of note, at the start of the accumulation phase, biomass had a PHA content of 57% in DCW.

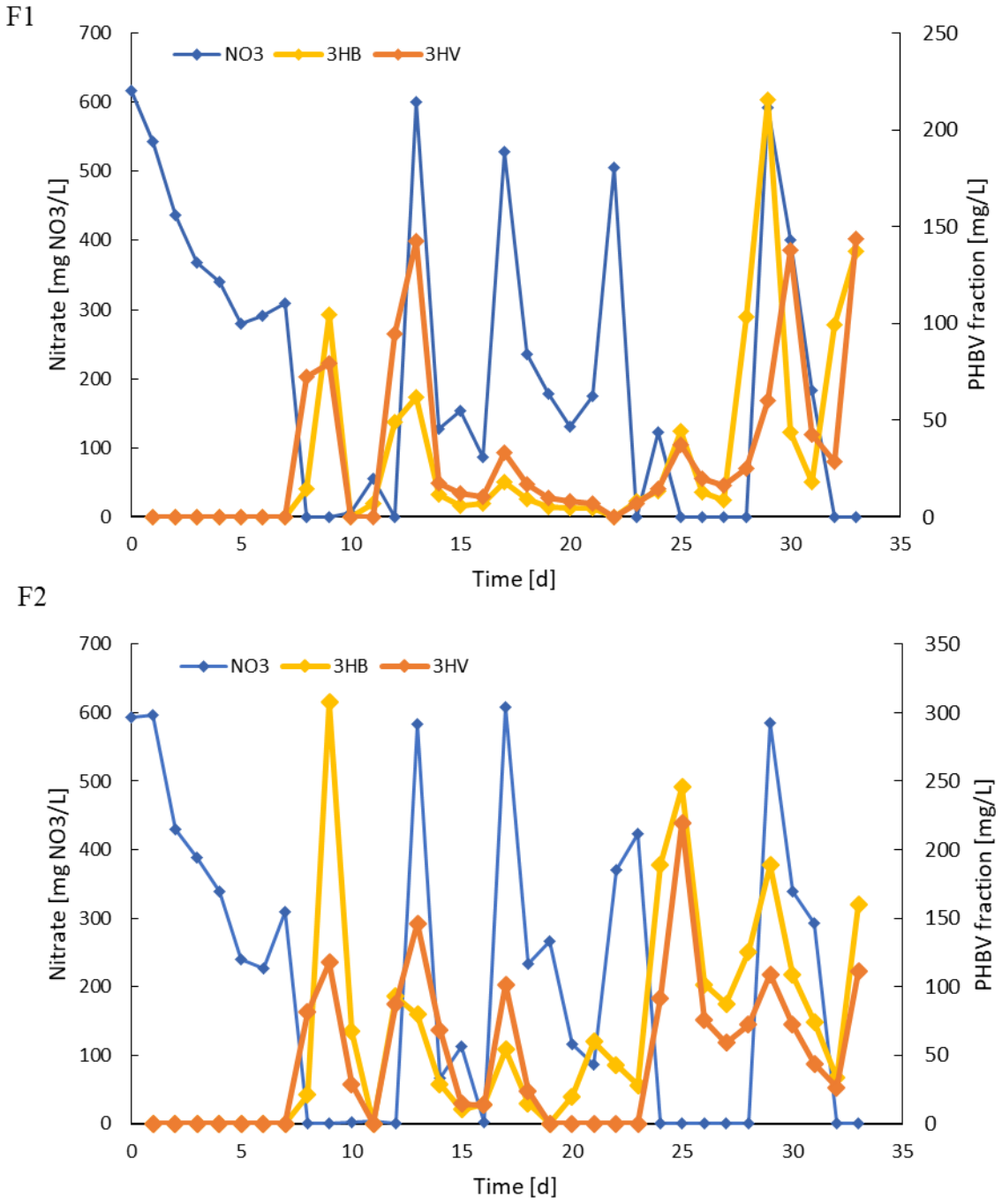


Figure S4.4. The time course of nitrate concentration [mg NO_3/L] and 3HB and 3HV monomer concentration in the culture [mg/L].

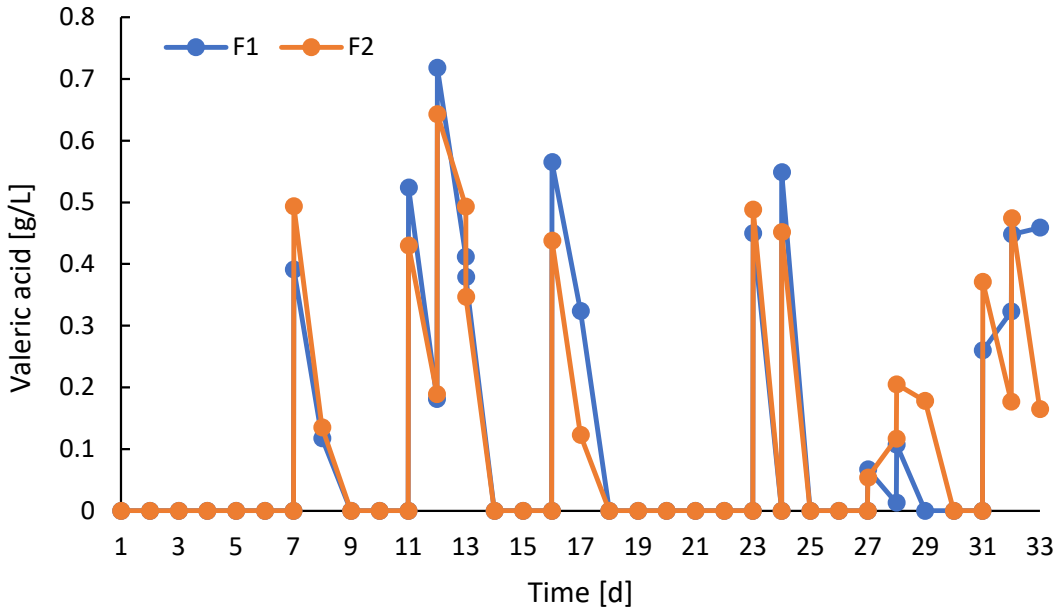


Figure S4.5. The changes in valeric acid concentration [g/L] in the culture during the process.

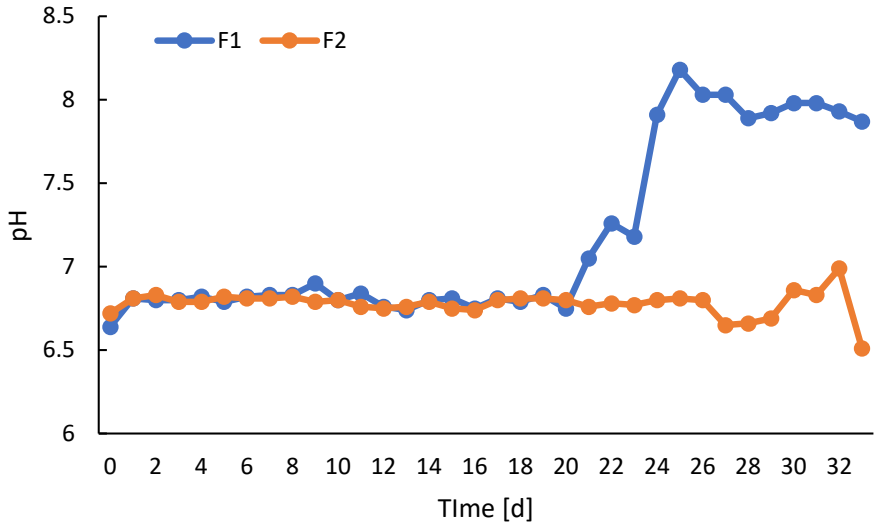


Figure S4.6. The time course of pH in bioreactors F1 and F2.

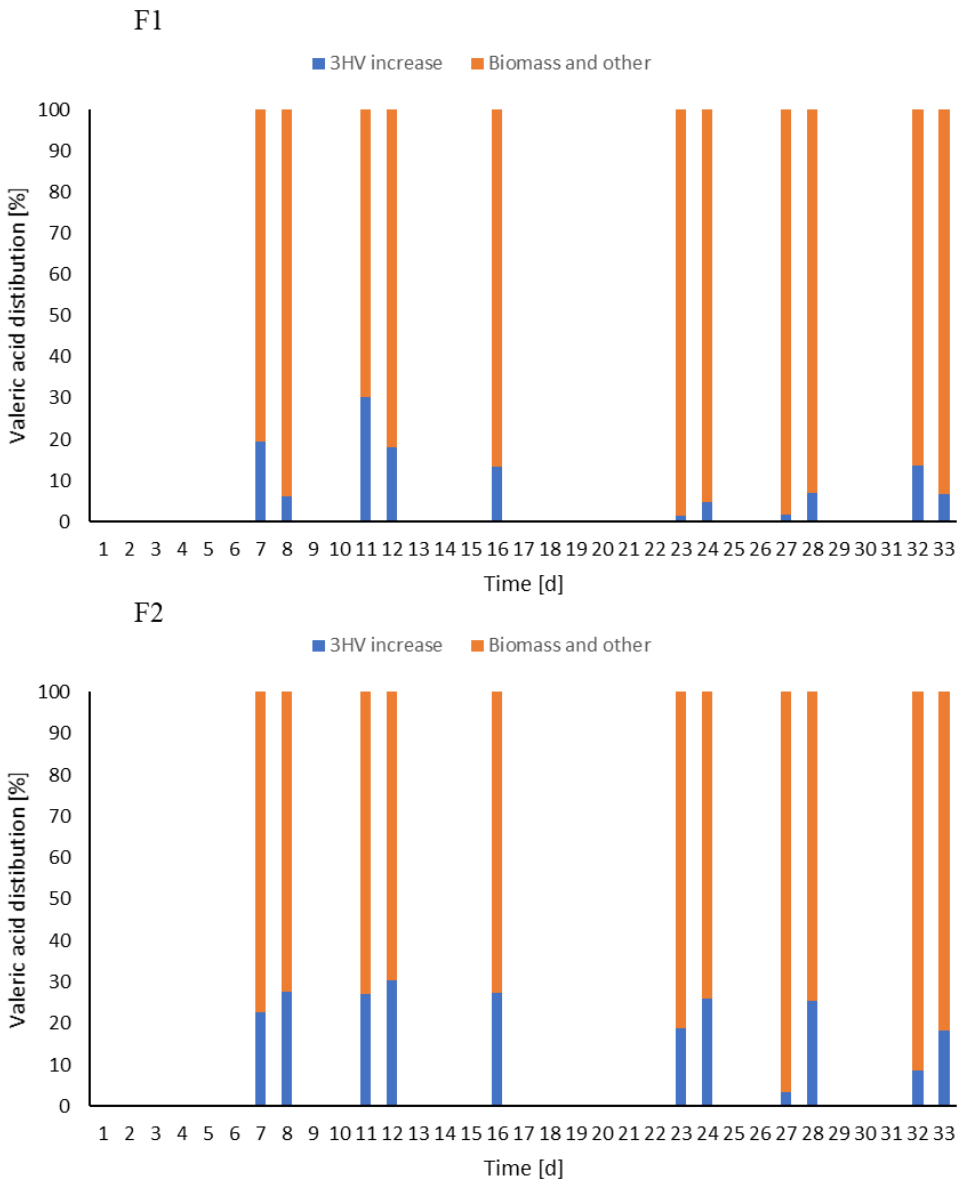


Figure S4.7. Distribution of utilised valeric acid for 3HV increase, biomass and other metabolites.

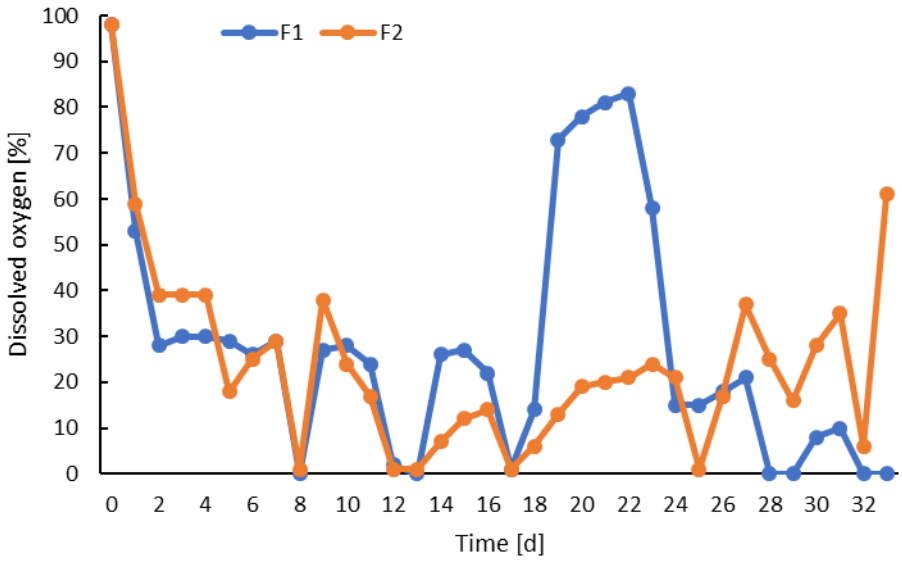


Figure S4.8 – The time course of dissolved oxygen (DO) [%] in the culture during the process operation in bioreactors F1 and F2.

Chapter 5

Influence of valerate addition during fed-batch process for PHBV synthesis by pure and mixed methanotrophic cultures

This chapter is under preparation for publication as:

Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M., 2024. Influence of valerate addition during fed-batch process for PHBV synthesis by pure and mixed methanotrophic cultures

Abstract

The use of methane (CH_4) as a carbon source for biopolymer production in microbial systems offers the opportunity to mitigate CH_4 emissions while simultaneously producing a biodegradable alternative to fossil-based plastics. The synthesis of polyhydroxyalkanoates (PHA) copolymer from CH_4 and valerate as cosubstrate was studied in both pure and mixed methanotrophic cultures. Fed-batch cultures under two valerate feeding rates were compared to observe the effect of low and high cosubstrate concentration on culture dynamics, growth, PHA productivity and monomer composition. The *Methylocystis hirsuta* culture achieved similar biomass concentration regardless of valerate feeding rate, while the AS10 mixed culture had higher density at higher valerate concentration. At low valerate feeding, both cultures produced a maximum of 0.67-0.71 g PHA/L with the AS10 culture accumulating 63% of PHA in dry cell weight (DCW) and a 20 mol% fraction of 3-hydroxyvalerate (3HV), while *M. hirsuta* accumulated 43% PHA in DCW and had a 27 mol% fraction of 3HV. A higher valerate feeding led to decreased PHA production but resulted in a higher 3HV fraction of around 40 mol% for both cultures. The addition of cosubstrate led to a more diverse bacterial growth in the mixed culture with *M. hirsuta*, *Acidovorax delafieldii* and *Novosphingobium subterraneum* becoming the major species. The mixed culture enriched in *M. hirsuta* was equally effective for PHA copolymer production as a pure *M. hirsuta* culture.

5.1. Introduction

Methane (CH_4) is a potent greenhouse gas with a high global warming potential, but it can also be a low-cost and abundant carbon source for bacterial-based production of valuable compounds. The use of methanotrophic bacteria that can directly metabolise CH_4 for biomass growth and production of biopolymers, single-cell proteins, biodiesel and biofuel precursors among others, have garnered significant interest as a means to mitigate CH_4 emission and the development of new sustainable bioprocesses (Cantera et al., 2019a; Gęsicka et al., 2021). Especially of interest is the production of polyhydroxyalkanoates (PHAs) a group of biodegradable polymers with potential applications in various industries, including packaging, biomedical and cosmetic applications, and agriculture (Amabile et al., 2024b).

PHAs are naturally produced by various microorganisms under nutrient-deficient conditions when carbon source is widely available, as a reserve material for carbon and energy. However, the commercial application of PHAs bioproduction is limited due to the production cost, especially the cost of carbon sources. The use of alternative processing with renewable carbon sources such as the use of CH_4 by methanotrophic bacteria could lower the final product cost and increase the biopolymer affordability (Behera et al., 2022). Recent studies have shown that the addition of organic carbon sources, such as valerate, can further enhance PHA production in methanotrophic cultures (López et al., 2018; Myung et al., 2017, 2016a). The volatile fatty acids used as cosubstrates can potentially be derived from the anaerobic digestion of food wastes allowing for further PHA production improvement and cost reduction (Amabile et al., 2023a). When CH_4 is provided as an only carbon source, 3-hydroxybutyrate (3HB) is synthesised and homopolymer poly(3-hydroxybutyrate) (PHB) is accumulated. The odd-chain fatty acid valerate can be incorporated into the PHA polymer, leading to the formation of 3-hydroxyvalerate (3HV) units and the synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (Myung et al., 2016a). The incorporation of 3-hydroxyvalerate units into the PHA chain can enhance the material's properties, such as improved toughness and elasticity, compared to the PHB (Strong et al., 2016b). Valeric acid is the most often used cosubstrate as it supports the highest 3HV inclusions in the copolymer (López et al., 2018; Luangthongkam, et al., 2019; Myung et al., 2016). To maximize PHBV accumulation, the valerate concentration must be optimized. According to Fergala et al. (2018) and Myung et al. (2015), low cosubstrate

concentrations of around 100 mg/L were optimal for high PHA accumulation with a 20-35 mol% 3HV fraction in batch studies. The higher concentrations, while increasing 3HV content, may have a detrimental effect on cell growth and overall polymer yield (Cal et al., 2016; Fergala et al., 2018c; Lee et al., 2023). The degree of 3HV incorporation into polymer greatly affects its properties, with better thermal stability and improved thermomechanical properties as the 3HV fraction increases (Urtuvia et al., 2023). By adjusting the operating conditions, the copolymer composition can be controlled, allowing for the tuning of polymer properties for specific applications. As PHAs are an intracellular product, their efficient production is also greatly dependent on biomass concentration and achieving a high cell-density culture is crucial for the process development on an industrial scale (Bedade et al., 2021). Culture cultivation in a continuous or fed-batch mode and application of cell recycling using membrane systems are some of the strategies applied for high cell-density cultivation (Hong et al., 2024; Lee et al., 2023; Sabale et al., 2023).

Although most of the research on methane-based PHBV accumulation was focused on studying the pure culture systems, the use of mixed cultures enriched in methanotrophic bacteria has also gained considerable attention in recent years as reviewed by Gęsicka et al. (2021). Pure cultures offer specificity, control, and reproducibility but come with challenges related to the risk of contamination and scale-up. On the other hand, mixed cultures demonstrate resilience and cost-effectiveness as they do not require sterile operating conditions, yet they are more complex and prone to variable yield and present analytical challenges. Understanding both systems can help improve the economics and efficiency of PHA production. Up to date, there have been only a couple of studies conducted on PHBV production in a bioreactor system with continuous CH_4 supply and valerate as a cosubstrate (Lee et al., 2023 and Chapter 4). In both cases, the studies focused either on pure or mixed culture use for PHBV accumulation under specific operating conditions. To the authors' best knowledge, there has been no study directly comparing PHBV production by pure and mixed culture under the same conditions in any bioreactor systems.

This study for the first time aimed to evaluate if the use of mixed cultures could match or outperform the monoculture PHA copolymer production. The process was conducted under continuous CH_4 feeding in a fed-batch bioreactor system, and the PHA accumulation was studied under low and high valerate feeding rates. In addition, the effect of CH_4 starvation on PHA production was also considered through a one-day operation with valerate as the

sole carbon source. The physical properties of the extracted polymer were studied based on the molecular weight distribution.

5.2. Materials and methods

5.2.1. Culture

Methylocystis hirsuta DSM 18500 was purchased from DSMZ (Braunschweig, Germany) and mixed methanotrophic culture enriched from waste activated sludge (AS10) cultivated in a bioreactor in a batch mode (Section 4.2.1) was used as an inoculum. All cultures were maintained in a nitrate mineral salt (NMS) medium prepared according to Gęsicka et al. (2024). The cultures were first grown in 40 mL NMS culture and then used to inoculate 200 mL of NMS medium (1% v/v inoculum), maintained under 10% CH_4 in air, renewed every 2-3 days, and incubated at 30^{o}C and 130 rpm until reaching the OD_{600} of 0.5-0.7.

5.2.2. One-step PHBV production

The PHBV accumulation by pure and mixed methanotrophic was studied in a one-step process, where culture growth and PHA production were operated simultaneously in one vessel, and PHA accumulation was initiated by the depletion of initially provided nutrients. The culture was operated in a fed-batch mode where sodium valerate was introduced as a cosubstrate for PHBV accumulation. The trials were conducted in a 3 L F0-Baby bioreactor (Bionet, Spain) equipped with a DO and pH probe. The pH was controlled at 6.8 by the addition of 1 M NaOH and 1 M HCl. The gas flow to the reactor was controlled by external gas mass flow controllers (MFC) (DPC, Aalborg, USA) at the level of 0.4 standard litter per minute (slpm) with 10% CH_4 in the air. At the gas outlet, a gas analyser (DP-28 BIO, Nanosens, Poland) was connected to periodically measure gas composition. The bioreactor setup is shown in Figure 5.1. Two reactors were run in parallel with the same culture type at a time with a working volume of 2 L. NMS medium was inoculated with 50 mL of pre-grown culture (2.5 % v/v) (*M. hirsuta* at OD_{600}=0.645 and AS10 at OD_{600}=0.553). When the nitrate concentration in the culture was less than 250 mg NO_3/L (day 4 for *M. hirsuta* and day 3 for AS10) the PHBV accumulation was initiated by adapting cosubstrate cyclic feeding at 0.1 and 0.5 g/L sodium valerate for reactor F1 and F2, respectively every 12 h for 4 consecutive days. A solution of sodium valerate of 50 g/L concentration was prepared as in Lee et al. (2023). In short, 42.64 ml of valeric acid and 15.5 g of NaOH were dissolved

in distilled H_2O to the volume of 600 mL. Part of the solution was diluted with distilled H_2O to make 10 g/L solution. After 3 days of PHBV accumulation 300 mL of culture was collected to extract biopolymer. The CH_4 flow to the culture was then switched off to observe the changes in PHBV accumulation and microbial community dynamics while the valerate was provided as the only carbon source. Only air was sparged into the culture at a rate of 0.4 slpm for an additional 24 hours before concluding the experiment and collecting biomass for polymer extraction. The samples for OD_{600}, dry cell weight (DCW), PHA, nitrate and valerate concentrations, as well as biomass for microbial analysis, were taken daily to monitor the culture growth and PHA accumulation. To keep the culture volume stable the nitrate-free NMS medium was added at the volume equivalent to the taken sample. The system was kept sterile and all solutions added to the culture were pre-sterilised. In addition, the purity of *M. hirsuta* culture was monitored by plating a sample of culture every 24 h on nutrient agar plates and incubating at 30°C. Nutrient agar was prepared as follows: 5 g peptone, 3 g meat extract and 15 g of agar were dissolved in distilled water to 1 L and sterilised in autoclave.

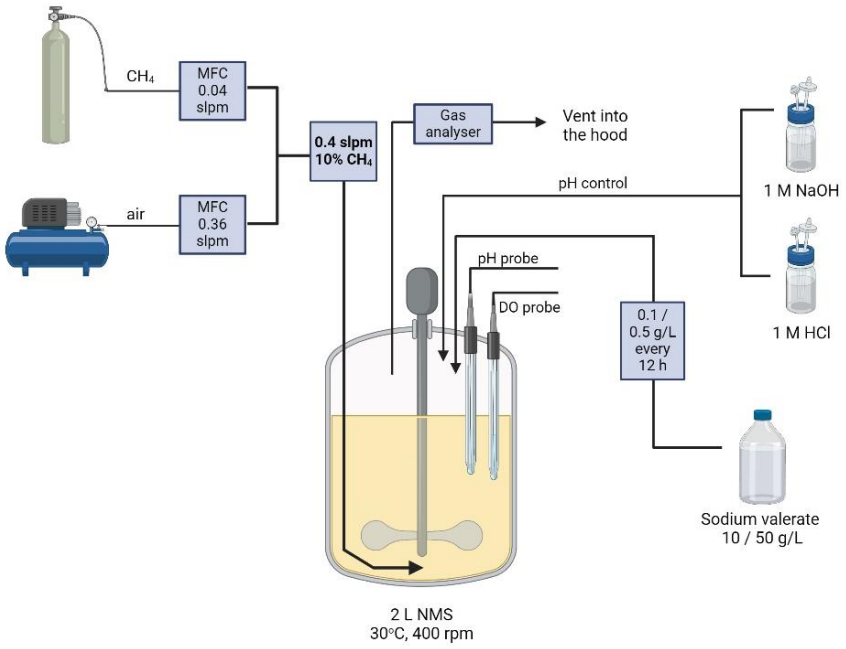


Figure 5.1. The bioreactor setup for fed-batch cultivation

5.2.3. Analytical methods

The biomass growth was monitored through the optical density (OD_{600}) measurements and the DCW analysis as described previously (Gęsicka et al., 2024). The nitrate concentration

was measured photometrically using Spectroquant® Nitrate Tests (1.09713 Supelco, Merck, Germany) according to the provided protocol. The sodium valerate concentration was measured with high-performance liquid chromatography (HPLC) (Shimadzu LC-20) equipped with Rezex ROA-Organic Acid column and RID. A 5 mM sulfuric acid was used for elution at the flow rate of 0.6 mL/min at 63^{o}C.

The PHA accumulation was measured using gas chromatography on GC-2014 Shimadzu with a capillary column Zebron ZB-FFAP and coupled with FID as described before (Gęsicka et al., 2024). In short, 2 mL of chloroform, 1 mL propanol with concentrated HCl (80:20% v/v), and 10 µL of internal standard (25 g/L benzoic acid in propanol) was added to a 5-10 mg of freeze-dried biomass. The samples were then heated for 3 h at 100^{o}C, 1 mL of distilled H_2O was added after, shaken and left for phases to separate. 1.5 mL of the bottom phase was filtered through a 0.45 µm syringe filter and analysed on GC. Biologically sourced PHBV copolymer with 8 mol% of PHV content (Sigma-Aldrich) was used for calibration. PHA accumulation was expressed as a PHA content in the DCW (% w/w), PHA concentration in culture (g/L) and 3HV fraction was reported on a molar basis (mol%).

5.2.4. Microbial composition analysis

Microbiome composition analysis was done by Nanopore sequencing of full rRNA operon amplicons. Biomass samples were collected by centrifugation and stored frozen at −20°C until processing. Total metagenomic DNA was isolated using ZymoBIOMICS DNA Microprep Kit (Zymoresearch, Poland) according to the manufacturer's recommendations. ZymoBIOMICS Microbial Community Standard D6300 (Zymoresearch, USA) was used as a mock microbial community to evaluate the analysis pipeline. Amplification of V3 and V4 region of 16S rRNA gene was carried out with Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/µL) and primers 16S-27F (5′- TTTCTGTTGGTGCTGATATTGC AGrGTTTGATyhTGGCTCAG) and 23S-2241R (5′-ACTTGCCTGTCGCTCTATCTTC ACCrCCCCAGThAAACT-3’). The resulting amplicons were indexed during 8 cycles of the second PCR reaction, carried out with LongAmp® Hot Start Taq DNA Polymerase (NEB, M0533S), 50 ng of amplicon and individual forward-reverse barcode primer pair for each sample. After pooling barcoded amplicons in equimolar concentration further library preparation was carried out using Oxford Nanopore Sqk-lsk114 Ligation Kit. Sequencing

was done on FLO-MIN114 (R10.4.1) flow cell utilizing MinION Mk1C sequencer (Nanopore Technologies), to the depth of ca. 10k full-length amplicons per sample.

Acquired raw data was subsequently basecalled using super-accurate dna_r10.4.1_e8.2_400bps_sup@v4.3.0 model with dorado v0.5.3 basecaller. The reads were run through Duplex Tools v0.2.9 (Oxford Nanopore Technologies, 2022) to split concatenated reads by Nanopore adapter. Reads were then filtered by average Phred ≥ 10 using NanoFilt v2.8.0 (De Coster et al., 2018) and those in size between 3000bp and 7000bp were retained for further analysis. Demultiplexing and removal of synthetic sequences were done with minibar (Krehenwinkel et al., 2019). The reads were then classified using the EMU v3.4.1 (Curry et al., 2022) program by mapping to rrn operon database, ncbi_202006 "NCBI RRN" (Kinoshita et al., 2021). Pseudo tax-table containing all samples and phyloseq object were created as described by (Petrone et al., 2023). Downstream analyses and visualisations were carried out using the Phyloseq package (Mcmurdie and Holmes, 2013). Raw sequences obtained in this study were submitted to the NCBI Sequence Read Archive (SRA) database and are available under BioProject ID PRJNA1111824.

5.2.5. Polymer characterisation

The culture collected after 3 and 4 days of PHBV accumulation was centrifuged and biomass was washed twice in distilled H_2O and freeze-dried (LyoQuest -85, Azbil Telstar Technologies, Spain). The accumulated polymer was then extracted using a modified chloroform extraction method (Vermeer et al., 2022). In short, 80-130 mg of freeze-dried biomass was suspended in 7 mL of chloroform and heated at 60^{o}C for 3 h with shaking every 30 min. The reaction mixture was centrifuged at 10000 rpm for 10 min at 20^{o}C and the liquid phase was filtered through 0.45 µm filters to glass test tubes to separate the extracted polymer from the cell debris. The extracted polymer was then left to dry at 60^{o}C for 2 days under the fume hood. The physicochemical properties of extracted polymers were determined using gel permeation chromatography (GPC), and nuclear magnetic resonance (NMR).

The number average molecular weight (Mn), weight average molecular weight (Mw), and polydispersity index (PDI=Mw/Mn) were determined on GPC. The polymer samples were dissolved in chloroform for 18 h at 35^{o}C, at the concentration of 2.5 mg/mL, filtered through a 0.22 µm PTFE membrane, and then analysed using Agilent 1200 HPLC series

with refraction index detector (RID) (Agilent Technologies, Santa Clara, CA, USA). The instrument was equipped with two PLgel 5µm MIXED-C (300x7.5mm) columns (Agilent Technologies). The temperature of analysis was maintained at 35ºC and chloroform was used as a mobile phase at the flow rate of 0.7 mL/min. For the calibration curves polystyrene standards with weight in the range of 474 g/mol-1800000 g/mol were used.

^{1}H NMR spectra were obtained on a Bruker NEO 400 MHz. ^{1}H-NMR chemical shifts were expressed in parts per million downfield from tetramethylsilane (TMS) as an internal standard ($\delta = 0$) in deuterated chloroform ($CDCl_3$).

5.3. Results and discussion

5.3.1. Cultures growth under a fed-batch

The pure *Methylocystis hirsuta* and mixed methanotrophic culture enriched in *M. hirsuta* (at 62% of relative abundance) exhibited different growth trends. At the initial growth stage the mixed culture had a shorter lag phase under 10% CH_4 in air sparging and entered exponential growth after 2 days, while *M. hirsuta* needed 3 days (Fig. 5.2A). However, the biomass increase of *M. hirsuta* exceeded that of AS10 culture, reaching a higher final biomass concentration (Fig. 5.2A). After nitrate in the culture was depleted to below 250 mg NO_3/L, on day 3 for AS10 and day 4 for *M. hirsuta* (Fig. 2B), the rectors were fed with sodium valerate at 0.1 or 0.5 g/L every 12 h, for F1 and F2 reactor, respectively. The biomass increase of *M. hirsuta* culture was consistent at both valerate feeding rates and reached maximum productivity of 0.29 and 0.30 g DCW/Ld on day 5 for the F1 and F2 reactors respectively. These results match the biomass productivity of *M. hirsuta* culture under batch in CSTR as previously reported by Rodríguez et al. (2023) (0.32 g/Ld by day 9).

Regardless of the valerate feeding rate *M. hirsuta* utilised approximately 0.5 g/L of sodium valerate during the 4 days of accumulation phase, which would explain the similar maximum biomass concentration in reactor F1 and F2 of 1.67 and 1.78 g DCW/L (Fig. 5.2A). In case of mixed culture, the higher concentration of additional carbon source resulted in an increase in the biomass concentration from a maximum of 1.06 g DCW/L for F1 to 1.45 g DCW/L for F2 (Fig. 5.2A). At the same time AS10 culture at higher feeding rate utilised more valerate, 0.75 g/L in F1 and 1 g/L in F2 culture. At lower valerate feeding the biomass productivity of AS10 culture was more uniform at around 0.18 g DCW/Ld

while for 0.5 g/L feeding, it reached a peak on the 5th day with 0.26 g DCW/Ld. The presence of consortia members other than *M. hirsuta* and the shifts in their relative abundances during the process operation under different valerate feeding rates (see section 5.3.4), were the causes for the different biomass growth profiles between the *M. hirsuta*-dominant mixed culture and *M. hirsuta* monoculture.

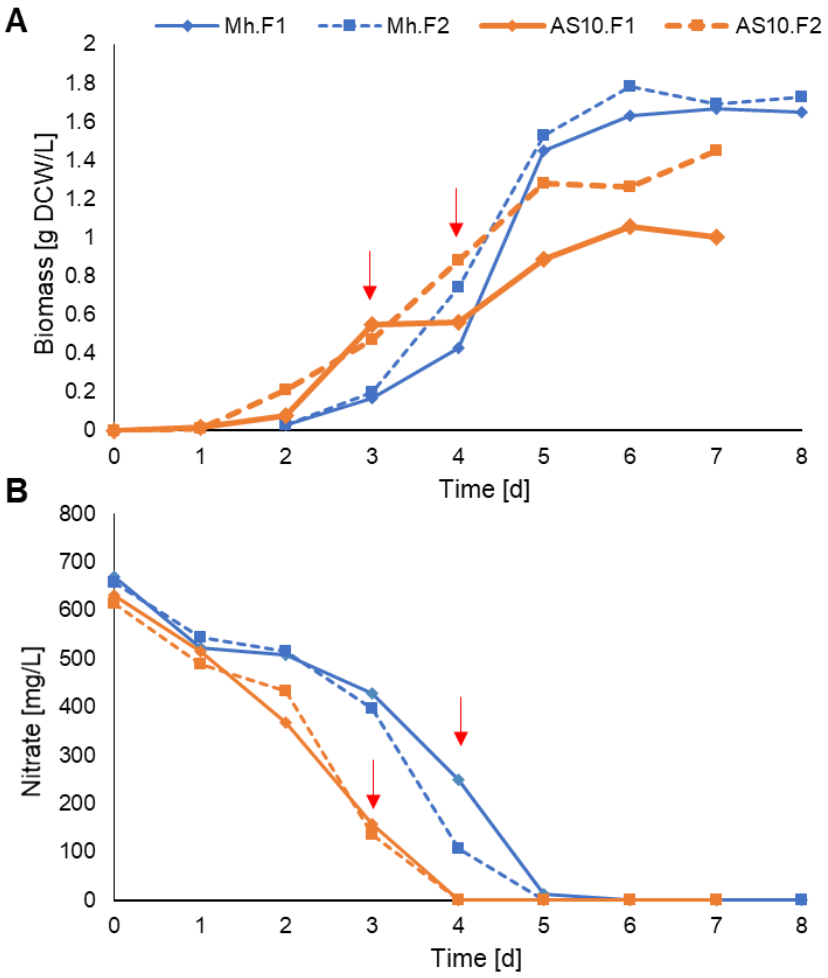


Figure 5.2. Time course of biomass concentration [g DCW/L] (A) and nitrate concentration [mg NO_3/L] (B) of *M. hirsuta* (Mh) and mixed culture (AS10) at 0.1 g/L (solid line) and 0.5 g/L (dotted line) valerate feeding, for reactor F1 and F2 respectively. Red arrows show the beginning of sodium valerate feeding.

It is important to note that the *M. hirsuta* culture in reactor F1 became contaminated with *Bacillus subtilis* and *Paenibacillus pasadenensis* on the last day of cultivation. This emphasizes the operational limitations of pure culture processes and highlights the advantageous use of mixed cultures, as it does not pose a risk of process disruption and product loss due to process contamination. Although the herein observed contamination did not notably impact factors such as biomass concentration and PHA accumulation

capacity since it was only active for one day, extended cultivation of a contaminated culture could lead to more noticeable shifts in process stability.

5.3.2. PHBV production at 0.1 g/L valerate

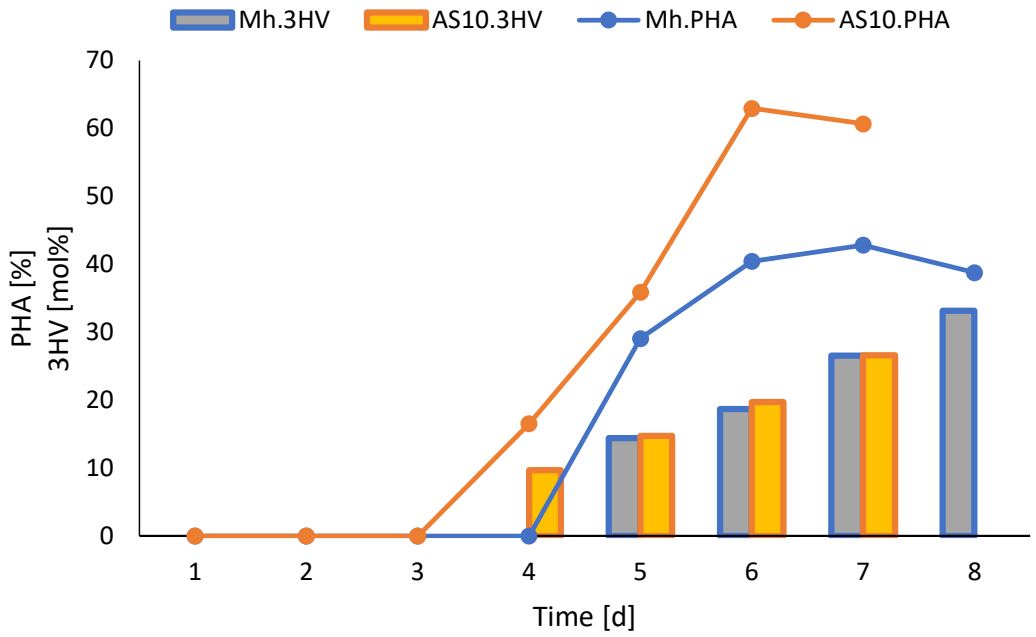


Figure 5.3. Comparison of PHA accumulation [% of DCW] and 3HV molar fraction [mol%] of *M. hirsuta* and AS10 culture at 0.1 g/L sodium valerate feeding.

The impact of low valerate feeding (0.1 g/L every 12 h) on the PHBV accumulation by pure and mixed culture was evaluated over 4 days. For the first 3 days, the cultures were under 10% CH_4 as the main carbon source, and on the 4th day, valerate was the sole carbon source. Despite different PHA accumulation capacities, with AS10 reaching up to 63% PHA in DCW and *M. hirsuta* reaching up to 43% (Fig. 5.3), both cultures showed comparable PHA and 3HV production trends based on the overall concentration (Fig. S5.1). Both cultures achieved similar maximum PHA concentrations: 0.67 g/L for AS10 and 0.71 g/L for *M. hirsuta* (Fig. S5.1). The highest PHA productivity for *M. hirsuta* was observed after the first 24h of PHA accumulation at the level of 0.42 g PHA/Ld, after which the productivity started to decrease to 0.24 g/Ld at the end of the CH_4 supplied phase. A similar PHA productivity of 0.22 g PHA/Ld after 3 days of accumulation was achieved by AS10 culture. The achieved PHA productivities were generally higher than that of *Methylocystis parvus* in a CSTR under different nitrogen feeding strategies (Rodríguez et al., 2022), but lower than that of *Methylocystis* sp. MJC1 in a CSTR or *M. hirsuta* in a bubble column

bioreactor (BCB) (García-Pérez et al., 2018). While *Methylocystis* sp. MJC1 was shown to be a very effective strain for PHA production from CH_4, able to produce 8.9 g/L of PHBV copolymer (Lee et al., 2023) and 34.5 g/L of PHB under less than a week of cultivation (Hong et al., 2024), the use of BCB with gas circulations allows for better CH_4 utilisation than in CSTR bioreactor design which results in the higher volumetric PHB productivities in those studies.

When sodium valerate was introduced to the culture as a co-substrate at 0.1 g/L every 12 h the mixed culture showed good utilisation of additional carbon source while for *M. hirsuta* culture partial accumulation of valerate in the culture was observed (Fig. S5.2). Both cultures were able to synthesise PHBV at given conditions (Fig. S5.3). The 3HV fraction of PHBV increased with time in both cultures reaching 20 mol% and 27 mol% at the end of CH_4 fed accumulation phase, for AS10 and *M. hirsuta* respectively (Fig. 3). The produced copolymers had a 3HV fraction similar to that of pure cultures in small bottles during a 2-day batch with 0.1 g/L of valerate added (López et al., 2018; Myung et al., 2016a). On the other hand, AS10 enriched culture in the previous study showed a 2 times higher 3HV fraction of PHBV in batch culture (Gęsicka et al., 2024). The difference in the microbial consortia composition and the operating conditions, especially the herein applied continuous sparging with 10% CH_4 in air, resulted in a higher synthesis of 3HB monomer and lower 3HV fraction.

When valerate was the only available carbon source, the biomass concentrations decreased which coincided with the drop in the accumulated PHA (Fig. 5.2 and 5.3). In the conditions of CH_4 limitations, PHA can be catabolised into acetyl-CoA, providing reducing equivalents to restore biomass activity (Karthikeyan et al., 2015a), which could explain the decrease in PHA concentration (Fig. S5.1). However, supplied valerate was consumed and incorporated into the 3HV monomer as seen by the increase in 3HV concentration in the culture (Fig. S5.1 and S5.2). The reducing power generated from the partial depolymerisation of accumulated PHA could have been used for the assimilation of organic acids and incorporation of valerate into 3HV inclusions (Luangthongkam et al., 2019b). The valerate assimilation for 3HV synthesis in the absence of CH_4 was previously shown for *M. hirsuta* and *Methylosinus*-dominated culture (López et al., 2018; Luangthongkam et al., 2019b). During the CH_4 limited stage, the 3HV fraction increased for both pure and mixed culture reaching 27 mol% for AS10 and 33 mol% for *M. hirsuta* at the end of the fed-batch process (Fig. 5.3). After 4 days of accumulation, mixed culture utilised almost

all of the supplied valerate (94%) (Fig. S5.2) from which 30% of utilised carbon was distributed for 3HV synthesis and achieved a yield of 0.24 g-3HV/g-valerate. On the other hand, *M. hirsuta* culture achieved a higher 3HV yield of 0.46 g-3HV/g-valerate, while utilising 64% of provided valerate. Pure culture had higher valerate flux for PHBV synthesis with 57% of utilised carbon distributed for 3HV inclusions. Valerate supplied to the culture is oxidized to valeryl-CoA that can be further converted to 3-hydroxyvaleryl-CoA and together with 3-hydroxybutyryl-CoA used for the synthesis of PHBV in reaction catalysed by PHA synthase (Fig. 5.4). In mixed cultures the broader spectrum of metabolic pathways may create a higher flux of the valeryl-CoA to other consortium members and results in lower distribution of utilised valerate into a PHBV synthesis pathway than in pure cultures.

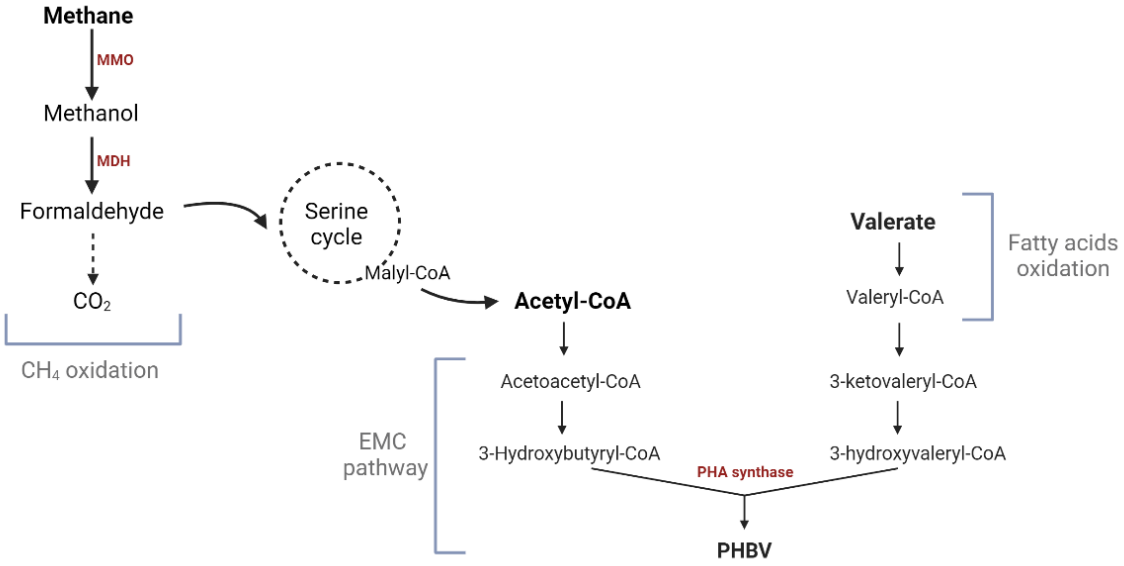


Figure 5.4. Simplified scheme of PHBV synthesis from CH_4 and valerate by type II methanotrophs, based on Myung et al. (2016). Key enzymes are written in red. Abbreviations: MMO – methane monooxygenase, MHD – methanol dehydrogenase.

While both cultures supported similar PHA production, the *M. hirsuta* culture used valerate more effectively for 3HV synthesis, resulting in a higher 3HV fraction. However, the mixed culture proved to be just as effective for PHA production as the pure culture. This, along with the elimination of contamination issues, could reduce the final product costs by saving on operational expenses typically associated with maintaining sterile conditions.

5.3.3. PHBV production at 0.5 g/L valerate

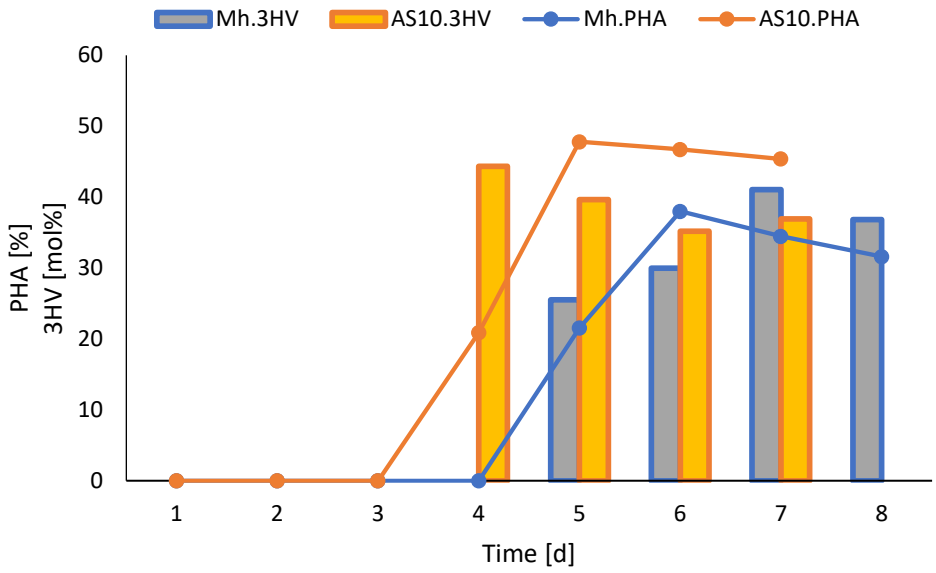


Figure 5.5. Comparison of PHA accumulation [% of DCW] and 3HV molar fraction [mol%] of *M. hirsuta* and AS10 culture at 0.5 g/L sodium valerate feeding.

The effect of higher valerate concentration on pure and mixed methanotrophic culture potential for PHA accumulation was studied by applying 0.5 g/L sodium valerate cyclic feeding every 12 h (Fig. 5.5). *M. hirsuta* and AS10 cultures showed similar trends in PHA accumulation with the highest PHA increase during the first 48h from the initial valerate feeding, at the end of which maximum PHA accumulation and productivity of 38% and 0.34 g PHA/Ld for *M. hirsuta* and 48% and 0.31 g PHA/Ld for AS10 was achieved (Fig. 5.5). The subsequent decrease in PHA concentration could have resulted from partial PHA depolymerisation under conditions of high valerate concentration in the medium. Since the biomass concentration of *M. hirsuta* did not decrease notably while the PHA concentration did, it can be inferred that the accumulated PHA, was being catabolised for energy to sustain bacterial activity (Fig. 5.2 and S5.4). The exact mechanism behind this is not clear. It could be a result of the accumulated valerate in the culture at more than 1.5 g/L that could potentially negatively affect the CH_4 oxidation by methanotrophic bacteria and create a need to acquire energy from the intracellularly stored sources. A better understanding of this process could be obtained from studying CH_4 oxidation capacity at different valerate concentrations could help to test this hypothesis.

In the case of AS10 culture, PHA production plateaued after 48 h but resumed under CH_4 starvation when valerate was the only carbon source (Fig. S5.4). The provided valerate was

partially incorporated into the biopolymer resulting in the PHBV synthesis (Fig. S3) with a high 3HV fraction of 40-44 mol% (Fig. 5.3). The 3HV molar fraction of accumulated polymer decreased in time for mixed culture and increased for pure culture, while the final copolymer composition at the end of the process was the same for both cultures at the level of 37 mol% (Fig. 5.3). The higher valerate concentration in the medium, although resulted in higher 3HV fraction, was detrimental to the PHA accumulation was lower by 10-20% (Table 5.1) and overall PHA production was also slightly lower than at 0.1 g/L feeding rate (Fig. S5.1 and S5.4). A similar effect of valerate concertation higher than 0.1 g/L on PHA accumulation decrease while the 3HV fraction was either stable or higher was previously observed in mixed cultures studies (Fergala et al., 2018c; Myung et al., 2015), while 0.7% v/v valerate concertation and higher become inhibitory to both PHA accumulation and 3HV incorporation (Cal et al., 2016). On the other hand, when *Methylocystis* sp. MJC1 was being supplied with 0.2 g/L valerate every 3 h in a fed-batch CSTR system the 3HV fraction was increasing up to 28 mol% without affecting the general PHBV accumulation of around 40 % of DCW (Lee et al., 2023). In this case, possibly due to higher biomass concentration the provided valerate of a total of 2.4 g/L was quickly utilised and not accumulated in the medium with 99% of supplied valerate being incorporated into the PHBV (Lee et al., 2023).

Over the 4 days of accumulation phase, the cultures were supplied with 4 g/L of valerate but used only a small part of it. *M. hirsuta* utilised 12% of fed valerate while AS10 twice as much at 26% of supplied valerate (Fig. S5.4). Interestingly the distribution of utilised valerate was similar at both cosubstrate feeding conditions, the same with 3HV yield which was 0.46 and 0.26 g_{3HV}/g-valerate for *M. hirsuta* and AS10 culture, respectively. A previous study on the feast-famine regime with the same culture showed a higher utilisation rate of provided valerate, with CH_4 and O_2 supply being lower than in the present study (Chapter 4). It was previously observed that the CH_4 in air flow rate to the reactor affected the effectiveness of valeric acid utilisation with 0.2 slpm providing good conditions for the metabolism of 0.5 g/L valeric acid during 24 h. Herein applied twice higher flow rate (0.4 slpm) didn't follow the same trend, as the 1 g/L valerate provided during 24 h was only partially consumed.

For both pure and mixed cultures, higher valerate concentration provided resulted in a lower PHA production as compared with 0.1 g/L valerate feeding. The additional accumulation of a high concentration of unused valerate showed these conditions to be less optimal for PHBV accumulation in a fed-batch system. This was especially evident for *M.*

hirsuta which did not show much difference in the produced biomass at both valerate feedings (Fig. 5.2).

5.3.4. The influence of valerate addition on microbial community structure

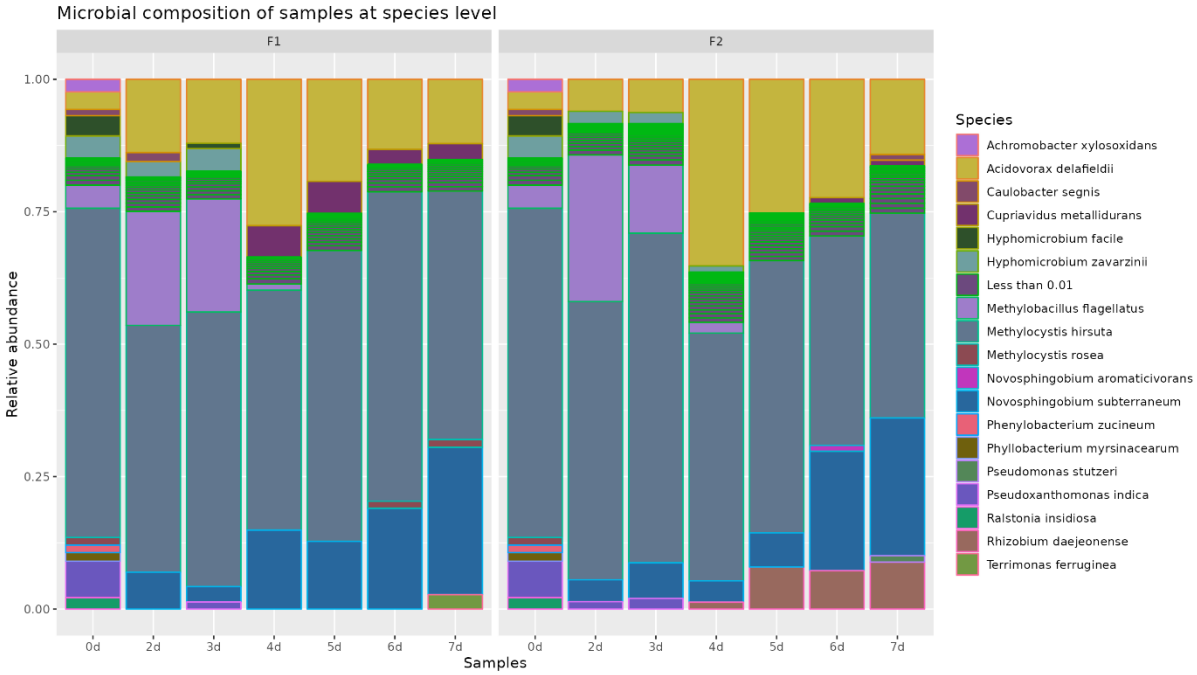


Figure 5.6. Microbial community structure changes of AS10 culture in reactor F1 with 0.1 g/L valerate feeding from day 3 and in reactor F2 with 0.5 g/L valerate feeding.

The microbial community of the AS10 culture underwent changes depending on the operational conditions (Fig. 5.6). Initially, the reactors were inoculated with a mixed culture, which constituted 62% of *M. hirsuta*. This species remained a major consortium member throughout the entire process. During the initial growth stage, the CH_4 oxidation by-products excreted to the culture, such as methanol, promoted the growth of methylotrophic bacteria *Methylobacillus flagellates*. This bacterium became another major species in the culture during the first 3 days of operation. Two other species, *Acidovorax delafieldii* and *Novosphingobium subterraneum* grew in abundance during the PHA accumulation stage. When valerate feeding was initialized and PHA started to be accumulated, the culture favoured the growth of *A. delafieldii* at the expense of methylotrophs becoming another major species in the culture at the abundance of 28-35%. The introduction of another readily available carbon source to the culture led to a more diverse bacterial growth, including potential PHA accumulating species such as *Acidovorax* sp. (Grzesiak et al., 2023) or *Novosphingobium* sp. (Strabala et al., 2012). In

addition, *Acidovorax* sp. is also known as PHB degrading bacteria and its growth could be also associated with acquiring energy from PHB depolymerisation (Schloe et al., 2000; Sugiyama et al., 2004; Vigneswari et al., 2015). At low valerate feeding of 0.1 g/L the abundance of *M. hirsuta* remained relatively stable, comprising half of the culture, while the *A. delafieldii* was decreasing in time in favour of *N. subterraneum*. Moreover, a small presence of *Cupriavidus metallidurans*, a species that was abundant in the AS10 culture at higher pH, was also observed (Chapter 4). The accumulation of valerate in the medium at a higher feeding rate (Fig. S4) negatively affected the *M. hirsuta* abundance, which decreased from 62% to 39% at the end of the process. Similarly, at a 0.1 g/L feeding rate, *A. delafieldii* and *N. subterraneum* become other major species. Moreover, the high valerate concentration resulted in the increase in *Rhizobium daejeonense* abundance to 9%, another potential PHA-producing species (Ratcliff et al., 2008). The observed effect of valerate concentration on the abundance of *M. hirsuta* and other major species in the AS10 culture emphasizes the influence of operational conditions and substrate availability on the microbial community structure and PHA production. Further research in this area could provide valuable insights into optimizing PHA production processes through targeted manipulation of microbial communities. By determining which consortium members are actively participating in PHA accumulation in given conditions it would be possible to determine how the microbial community structure corresponds with the PHA monomer composition. This will in turn allow for the development of a process in which through adjusting the operating conditions, the copolymer composition can be controlled, allowing for the tuning of polymer properties for specific applications.

5.3.5. Polymer characterisation

The molecular weight distribution of polymers, extracted from biomass after 3 and 4 days of the accumulation, was measured using GPC (Table 5.1). Except for *M. hirsuta* at a higher valerate feeding rate, all polymers showed an increase in molecular weight over time. After 4 days, they reached 4.4-4.7 x 10^5 Da which is similar to the weight distribution of PHBV of natural origin from Sigma-Aldrich (Myung et al., 2017a). Despite having the highest 3HV fraction of all samples after 3 days of accumulation, the *M. hirsuta* culture in reactor F2 produced a polymer with the lowest molecular weight distribution among all reported PHBV produced from CH_4 (Table 4.2). At tested conditions, mixed culture generally resulted in higher molecular weight which could result from possible cooperative PHBV

production by different bacterial species in the consortium. The increase in molecular weight of the polymers over time, reaching a range similar to that of commercial PHBV, suggests the potential for producing high-quality PHBV using this approach. Moreover, the observed differences in molecular weight distribution and 3HV fraction among the cultures highlight the importance of understanding the microbial community dynamics for optimizing PHBV production.

Table 4.1. PHA accumulation [% DCW], 3HV fraction [mol%] and molecular weight distribution of extracted polymers

Culture	Valerate feeding (g/L every 12h)	Day of accumulation	PHA (% DCW)	3HV fraction (mol%)	Molecular weights M_n [x 10^5 Da]	PDI
AS10	0.1	3	63.0	19.7	4.21	2.89
		4	60.7	26.6	4.42	2.85
	0.5	3	46.7	35.2	4.63	2.63
		4	45.4	37.0	4.69	2.48
M. hirsuta DSM 18500	0.1	3	42.8	26.6	3.48	3.00
		4	38.8	33.2	4.44	2.50
	0.5	3	34.5	41.1	3.09	2.75
		4	31.6	36.8	2.72	2.93

5.4. Conclusions

PHBV accumulation by pure and mixed methanotrophic culture in a fed-batch system was compared at two cosubstrate feeding rates. Mixed culture enriched in *M. hirsuta* and other potential PHA-producing strains was capable of achieving similar PHA production as pure *M. hirsuta* culture. However, *M. hirsuta* culture utilized valerate more effectively for 3HV synthesis. High valerate concentration resulted in decreased PHA production for both cultures, although with a higher 3HV fraction. This study for the first time directly showed the mixed culture to be equally effective for PHBV production as the pure methanotrophic culture. In addition, polymer extracted from mixed culture generally had higher and more uniform molecular weight distribution showing its potential for producing high-quality PHBV using this approach. Further development of the mixed culture PHBV production process should aim to test the possible strategies for higher cell density cultivation that would increase the overall PHA production.

5.5. Acknowledgments

This work was financed by the National Science Centre, Poland under grant agreement no. 2019/35/D/ST8/03530. Figure 5.1 was created with BioRender.com.

5.6. Supplementary materials

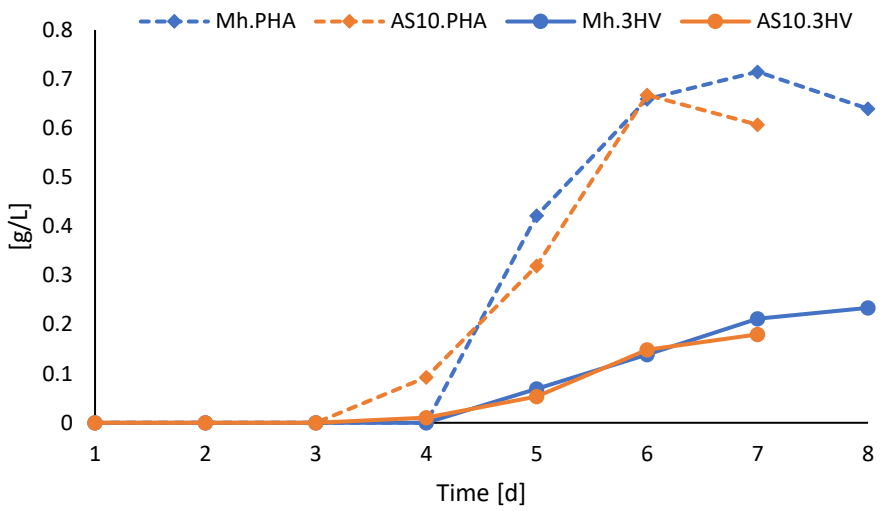


Figure S5.1 PHA (dotted line) and 3HV (solid line) concentration [g/L] in *M. hirsuta* (Mh) and AS10 culture at 0.1 g/L sodium valerate cyclic feeding.

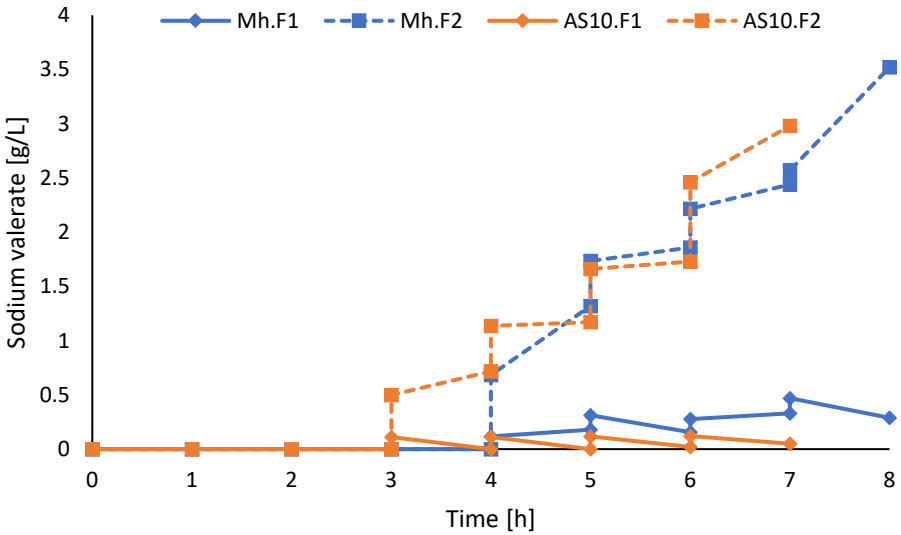


Figure S5.2. Sodium valerate concentration [g/L] during the cyclic valerate feeding of 0.1 g/L (solid line) and 0.5 g/L (dotted line) every 12h in *M. hirsuta* (Mh) and AS10 culture

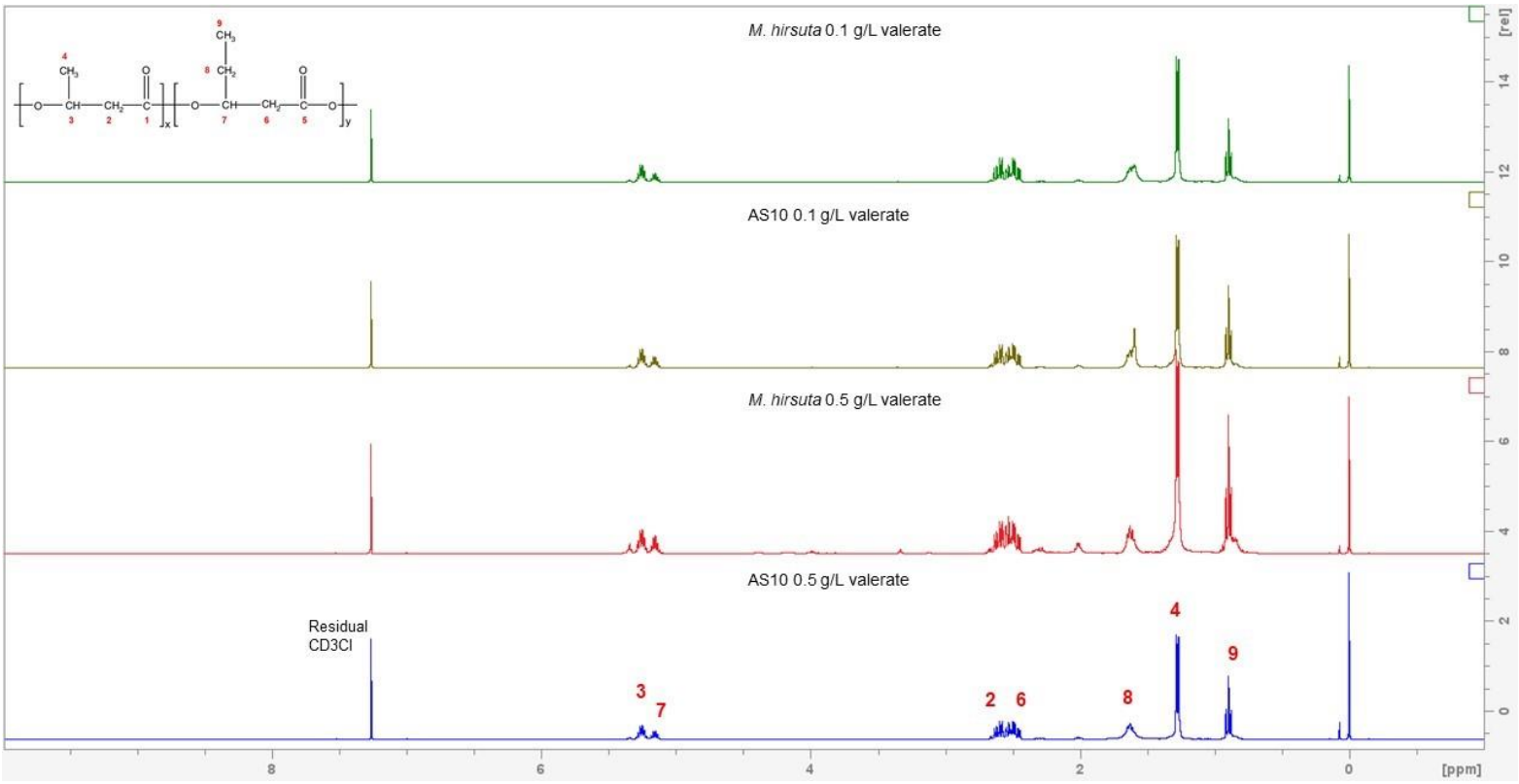


Figure S5.3. 400 MHz ^{1}H-NMR spectra of extracted polymers produced by *M. hirsuta* and AS10 cultures

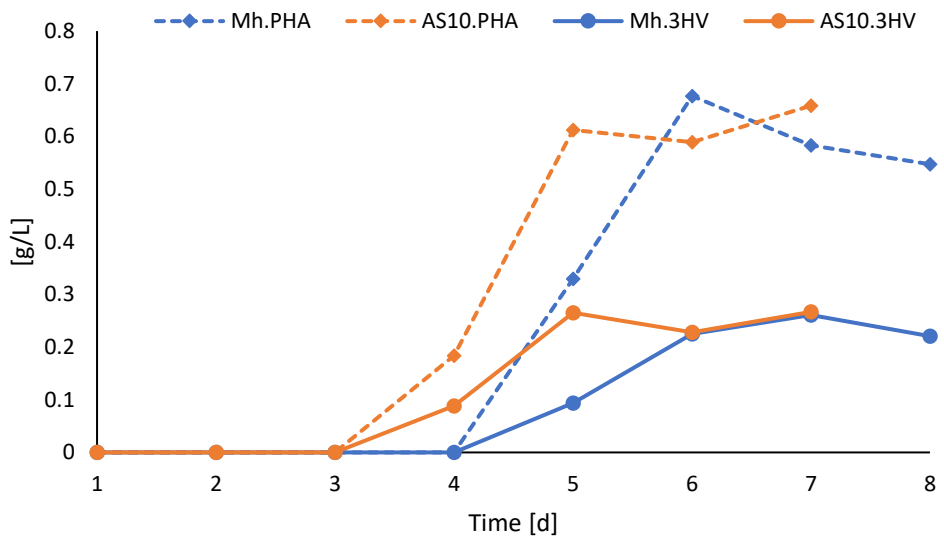


Figure S5.4. PHA (dotted line) and 3HV (solid line) concentration [g/L] in *M. hirsuta* (Mh) and AS10 culture at 0.5 g/L sodium valerate cyclic feeding.

Chapter 6

Outlook

6.1. Conclusions

This thesis encompasses an extensive study on the production of PHAs by a mixed methanotrophic culture using CH_4 as the primary carbon source. The main findings of the presented research are as follows:

- The efficiency of PHA accumulation is influenced by the microbial composition of cultures enriched from different environmental sources.
- 10% CH_4 in air (1:2 CH_4:O_2 ratio) is the most optimal for biomass growth and PHA production in mixed methanotrophic cultures among tested CH_4:O_2 ratios.
- Culture sampled from waste-activated sludge and enriched in *Methylocystis* sp. and other PHB-producing heterotrophs demonstrates the highest PHA productivity and yield among the tested enrichments, showing promise for application in CH_4 to biopolymer conversion technologies.
- When odd-carbon alcohols and acids (propanol, propionic and valeric acid) are added as cosubstrate the 3HV is synthesised and incorporated into a PHBV copolymer, with valeric acid resulting in the highest 3HV fractions.
- PHBV can be produced from CH_4 and valeric acid under a feast-famine regime in long-term CSTR cultivation.
- Adjusting the monomer composition of PHA produced in a CSTR under a feast-famine regime proves to be unachievable via changing the cosubstrate feeding strategy.
- Increasing the gas flow to the reactor enhances the availability of CH_4 and O_2 in the culture improving C1 oxidation, biomass and PHA production, and cosubstrate utilisation, which affects the polymer composition, with a higher 3HV fraction at lower gas flow rates.
- When the CSTR culture continuously fed with CH_4 operates without pH control, the pH increases to around 8, causing a shift in the microbial community without affecting PHA accumulation capacity.
- *Methylocystis hirsuta*-dominant mixed culture fed with valerate in a fed-batch mode can achieve similar PHA production as pure *Methylocystis hirsuta* culture
- A higher valerate concentration results in a higher 3HV fraction while limiting overall PHA accumulation in both pure and mixed cultures.

- PHAs with a molecular weight of 4.4-5.0 x 10^5 Da can be obtained from a mixed methanotrophic culture fed with CH_4 and valerate as cosubstrate in a bioreactor.

6.2. Targeted PHBV production

Methanotrophs and their cultures are good PHA producers although the capacity for PHA accumulation varies and is generally strain-specific. Predicting the PHA copolymer composition in response to the applied cultivation conditions depends heavily on the bacterial strain used. In the case of mixed culture enriched for CH_4 bioconversion, the presence and activity of other heterotrophic bacteria can significantly impact PHA accumulation. In research presented in **Chapters 3-5,** it was observed that conditions such as type of nitrogen source, CH_4 supply, cosubstrate concentration and pH operating strategies had an impact on the microbial composition of the mixed methanotrophic culture. This often affected PHA accumulation or 3HV fraction of the produced biopolymer. Further study into this phenomenon could enable the tuning of the PHBV accumulation with specific 3HV fractions and properties by adjusting the active culture composition through the cultivation conditions. The mutual interaction among members of the microbial consortium substantially influences culture growth and production. The conducted studies suggest that besides methanotrophs, other consortium members take part in the PHA accumulation while utilizing CH_4-derived carbon. Since PHA synthesised by different bacterial strains may differ in structure and properties, it is important to understand the distribution of PHA accumulation in the culture. Techniques such as fluorescence in situ hybridisation targeted for methanotrophic bacteria and other potential PHA-producers in the culture (Sruamsiri et al., 2020; Wolińska et al., 2013), flow cytometry (González et al., 2023) or metatranscriptomics analysis (Zhang et al., 2020) could help identify strains actively accumulating PHA in the mixed culture fed with CH_4 and valerate. In addition, combining specific bacterial strains in coculture studies on the mechanism of carbon metabolism and PHA accumulation can provide insights into optimal cultivation conditions and specific culture members interactions resulting in enhanced PHA production. This knowledge could contribute to improved process design for the production of PHA with desired properties using mixed methanotrophic cultures or synthetic cocultures.

6.3. Optimization of PHA yield

Although the biological production of PHA is environmentally friendly, its high cost remains the major barrier to large-scale application of these biobased technologies. The cost reduction can be achieved by using cheap and renewable carbon sources and by achieving high biopolymer productivity in possibly the shortest time. Optimising cultivation conditions for maximum biomass growth and accumulation yields is critical, yet it can be quite time-consuming. The application of high-throughput condition screening could significantly reduce the workload and time needed (Sundstrom and Criddle, 2015). Low culture volumes in such an approach make traditional quantitative PHA analysis on GC not applicable, while the development of quick analysis not requiring high biomass concentrations, like the fluorescence PHA staining (Lazic et al., 2021), could help with preliminary screening of the process conditions effect on PHA accumulation. While optimising fundamental process parameters and adapting culture with the best synergistic interaction between microorganisms are significant determinants of process efficiency, addressing the limitation of low gas-to-liquid mass transfer is a key factor affecting the successful implementation of CH_4-based processes. To increase production yield it is crucial to enhance the carbon availability for culture biomass growth and concurrent PHA increase. Several strategies have been considered to increase mass transfer, such as the use of CH_4 vectors or the adoption of different bioreactor designs. Adding paraffin or silicon oil to the culture as CH_4 vectors increases gas transfer due to higher CH_4 solubility in oil than in the medium (Han et al., 2009; Patel et al., 2020d). While different types of reactors, such as bubble column or airlift reactors (García-Pérez et al., 2018; Ghaz-Jahanian et al., 2018), fluidized or trickle bed reactors (Pfluger et al., 2011; Sheets et al., 2017), Taylor flow bioreactor (Cattaneo et al., 2022) or membrane reactors (Valverde-Pérez et al., 2020) can improve CH_4 delivery to the medium and enhance methanotrophic growth for CH_4-based processes. Each proposed bioreactor design has unique features and would require evaluation of culture performance in various configurations to optimize it for the highest PHA production.

The thesis results demonstrated a high PHA accumulation capacity of the mixed methanotrophic culture under applied carbon supply and pH conditions cultivated in a CSTR (30, 50 or 60% PHA in DCW depending on the culture). Additionally, a high 3HV fraction of accumulated PHBV was observed (40, 60 mol% depending on the culture),

indicating the good potential of the studied processes for further implementation. Although the highest achieved PHBV production was below 1 g/L; with around 0.5 g/L during the feast-famine process and up to 0.7 g/L in fed-batch, these processes still showed promising accumulation efficiency. The herein applied conditions were based on available literature and preliminary studies in small bottles cultivated batch-wise. Optimisation of biomass growth and PHA production in a broader spectrum of conditions in a controlled bioreactor system could lead to further yield improvements. Moreover, achieving a high yield of 3HV-rich PHBV production may be possible by applying the studied processes in a bioreactor designed for higher mass transfer.

6.4. Perspective for CH_4-based PHA production

The environmental concerns and the United Nations Sustainable Development Goals to fight plastic pollution drive the increase in global bioplastic production, while the Current developments in PHA production technologies are projected to triple their market share in the bioplastic market by 2028 (European Bioplastics, 2023). The use of mixed methanotrophic cultures for the production of high-performance PHBV from CH_4 and valeric acid shows a significant promise to contribute to this anticipated increase in biopolymers' market presence. The potential for industrial-scale PHBV production from CH_4 and valeric acid using mixed methanotrophic culture was recently evaluated through a techno-economic analysis (Amabile et al., 2024b). In their work, Amabile et al. (2024b) found that the selling price of PHBV could be reduced to 8.6 €/kg if the biomass concentration is increased to 30 g/L. These findings underscore the importance of establishing conditions for high-density mixed culture cultivation to make the process more cost-effective. In addition to the PHA production, product recovery and environmental impact should considered when developing PHA production technology. While product recovery was not a focus of this thesis, to analyse the properties of accumulated PHBV, a commonly used chloroform extraction was applied. However, due to the solvent's cost, its toxicity, and harmful environmental impact, this extraction method is not ideal. Alternative downstream product recovery methods are being evaluated, especially those using different green solvents as sustainable, eco-friendly replacements for halogenated solvents (Abate et al., 2024). The techno-economic analysis assumed the use of green solvent and solution recovery to reduce the downstream process costs (Amabile et al., 2024b). Developing more affordable product recovery methods could further decrease the final product's selling

price. Overall the industrial potential of CH_4 conversion to biopolymer was demonstrated, promoting its advancement for producing PHBV polymer with properties relevant to high-value industrial applications, especially in the biomedical sector.

6.5. Final remarks

This thesis focused on exploring the possible strategies for converting CH_4 into PHAs with a specific composition using a mixed methanotrophic culture. The study deepened the understanding of PHA synthesis mechanisms in mixed cultures by investigating PHA accumulation under various operating conditions. The competitiveness of mixed culture to a pure culture system was demonstrated, highlighting the potential use of mixed methanotrophic culture for future technology development. While this research advances the field of CH_4 valorisation into biopolymers, further analysis and process optimization are required to develop a mature technology that would allow for the tuning of the process for producing PHA with a specific monomer composition.

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Acknowledgement

The time comes and goes, but the memories remain. Throughout my four-year journey pursuing a PhD, I've met numerous people and had many wonderful experiences. Though it wasn't always easy, I'm thankful for the support I received along the way. I express my deepest gratitude for it.

First, I would like to thank my supervisors. **Piotr**, thank you for your guidance, mentorship and support. Your advice and feedback have been instrumental in shaping this thesis and I am grateful for the opportunity to work in your research group. **Mateusz**, thank you for granting me the opportunity to work on your project, the guidance through the laboratory work and data analysis. I appreciate your help in pursuing my interests during this PhD journey.

I am thankful to all PHA project team members for their collaboration and scientific contribution. **Siva**, thank you for all the help with DNA isolation, your insight and for always staying positive and showing how to go with the flow even when the flow is against you. **Natalia**, big thanks for all the help with chemical aspects of my work, all the time spend on the GC and HPLC analysis and your insight in all the technical aspects. For all those hours spent on helping me find the solutions to the analytical issues I was facing.

To the entire **Biorefinery Research Group**, thank you for the shared conferences and team outings that made this journey so much more enjoyable. **Anna**, thank you for sharing your experience navigating through the PhD process and for all the encouraging talks. **Hania**, your companionship during the late hours in the lab that turned into ABBA karaoke sessions, the shared frustrations we worked through, and the memes we exchanged made all the difference. **Filip**, I am grateful for all the advice you offered on work and life.

To my irreplaceable "depressed phd" companions, **Agata** and **Martyna**, thank you for your immense support and help in reaching the finish line. Your motivation and strength have been invaluable as we pushed through this journey together. University brought us together, and the PhD strengthened our bonds. Let's maintain this bond into our old age. Best of luck with your thesis - I know it will be fantastic.

I am deeply grateful to my long-time friends, **Dominika** and **Weronika**, for helping me take breaks to explore the world. Their efforts to adapt to my unpredictable research

schedule enabled us to embark on numerous trips across Europe, even if it was just for a weekend.

A special thanks to my friend, **Paulina**, without whose helpful message this thesis may have not come to be.

An endless gratitude is owed to my **family**, who supported my scientific career from the start. Thank you for believing in me, being understanding, and helping me relax when work seemed overwhelming.

And lastly, I am grateful to **The One Above** for guiding me on this path.

Curriculum Vitae

Aleksandra Gęsicka was born on 14th December 1996 in Połczyn-Zdrój, Poland. In 2015, she obtained her engineer's degree from the Poznan University of Life Sciences, majoring in Biotechnology. She then continued on with studying industrial applications of Biotechnology during her Masters at the same university. In 2020 she received a MSc title after completing a master thesis on the ethanol production from starch in simultaneous saccharification and fermentation process using a novel amylolytic preparation and thermotolerant yeasts. After graduating she pursued further opportunities to conduct research in the field of biotechnology and fermentation technology. The same year she began her PhD project on the methane conversion to polyhydroxyalkanoates by mixed methanotrophic cultures at the Biorefinery Research Group, Poznan University of Technology under a supervision of Prof. Piotr Oleskowicz-Popiel and auxiliary supervisor Mateusz Łężyk, PhD.

List of publications

Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M. Enrichment of mixed methanotrophic cultures producing polyhydroxyalkanoates (PHAs) from various environmental sources, 2024, Science of The Total Environment 912, 168844

Gęsicka A., Oleskowicz-Popiel P., Łężyk M., Recent trends in methane to bioproduct conversion by methanotrophs, 2021, Biotechnology Advances, 53, 107861

In preparation:

Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M. (2024). Sequential feast-famine process for polyhydroxyalkanoates production by mixed methanotrophic culture under different carbon supply and pH control strategies.

Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M. (2024). Influence of valerate addition during fed-batch process for PHBV synthesis by pure and mixed methanotrophic cultures.

Poznan 18.06.2023

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Gęsicka, A., Oleskowicz-Popiel, P., Łężyk, M., 2021. Recent trends in methane to bioproduct conversion by methanotrophs. Biotechnol Adv 53, 107861. https://doi.org/10.1016/j.biotechadv.2021.107861

Aleksandra Gęsicka, primary author: conceptualised and collected data on the reviewed topic, conducted formal analysis and visualisation of literature data and wrote the original manuscript.

Piotr Oleskowicz-Popiel: supervised the work and was responsible for the review and editing of the manuscript.

Mateusz Łężyk: conceptualised and supervised the work, and was responsible for review and editing of the manuscript, and funding acquisition.

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Gęsicka, A., Gutowska, N., Palaniappan, S., Oleskowicz-Popiel, P., Łężyk, M., 2024. Enrichment of mixed methanotrophic cultures producing polyhydroxyalkanoates (PHAs) from various environmental sources. Science of the Total Environment 912. https://doi.org/10.1016/j.scitotenv.2023.168844

Aleksandra Gęsicka, primary author: conceptualised and conducted the research, developed the appropriate methodology, collected data and performed formal analysis, visualised presented data and wrote the manuscript.

Natalia Gutowska: developed the appropriate methodology, and reviewed and edited the manuscript.

Sivasankar Palaniappan: reviewed and edited the manuscript.

Piotr Oleskowicz-Popiel: supervised the work and was responsible for the review and editing of the manuscript.

Mateusz Łężyk: conceptualised the research, developed the appropriate methodology, conducted formal analysis, and was responsible for supervision, manuscript review and editing, and funding acquisition.

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