POZNAN UNIVERSITY OF TECHNOLOGY

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Conversion of methane into selected polyhydroxyalkanoates with the use of methanotrophic microorganisms

Self-reference of PhD dissertation

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1. Introduction

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), 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. Current climate policies are aiming towards reducing GHGs emissions with the goal of achieving climate neutrality by 2050 (European Commission, 2023). 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 & 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 20^{th} 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 (L. 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 & 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 (Knief, 2015).

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, 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 & 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., 2016), 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.

2. Research aim

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.

3. Materials and methods

Conducted research was divided into 4 main tasks which results were disseminated in 3 research articles (one published, two in preparations). Figure 1. Shows the flowchart of the research presented in the PhD thesis. At each of the stage the results achieved were evaluated based on the GC and HPLC analysis of main products and metabolites and microbial community structure of mixed cultures changes under investigated conditions was observed through Oxford Nanopore sequencing.

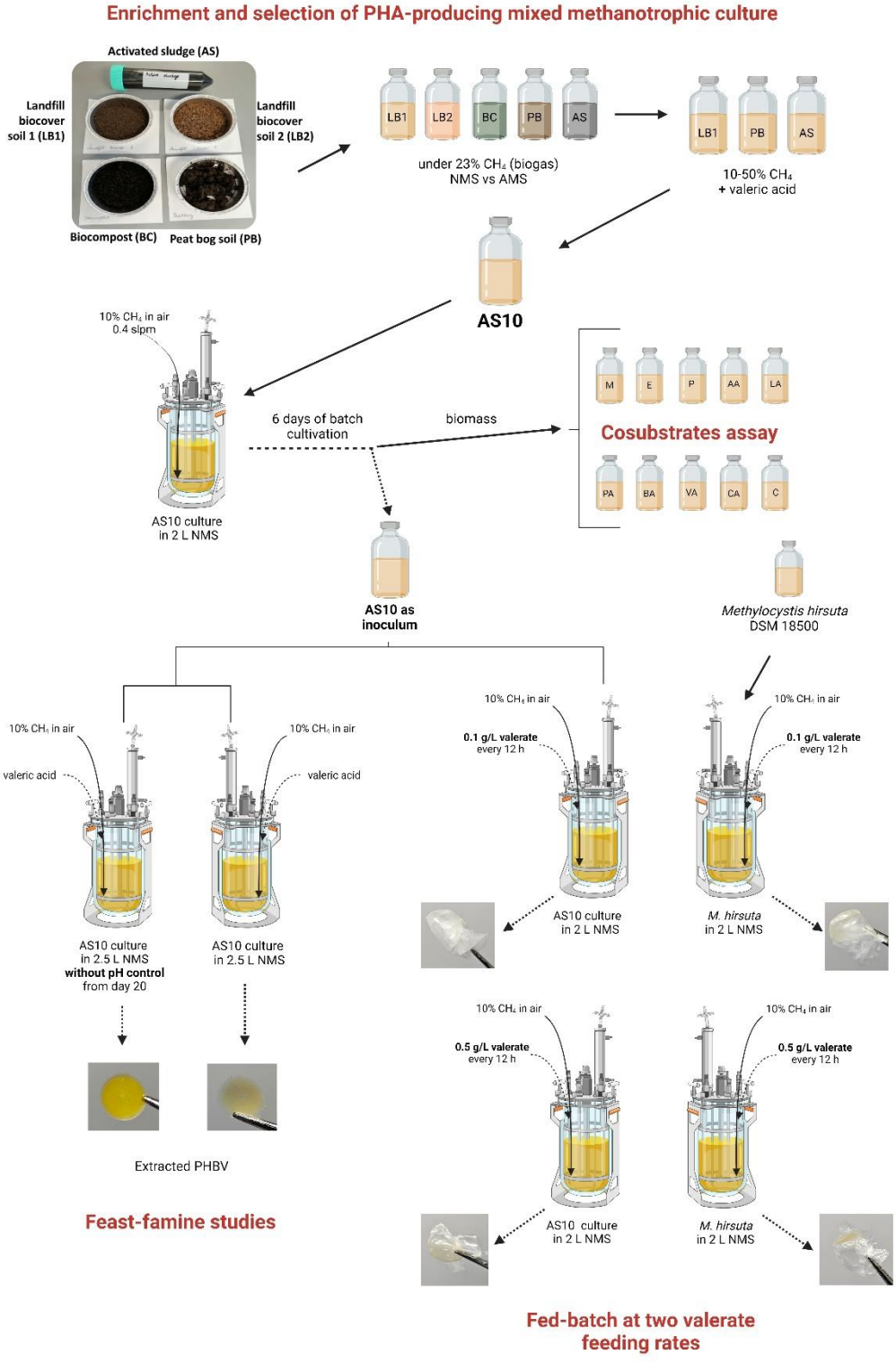

Figure 1 Scheme of the research conducted as a part of the PhD thesis

3.1. Culture medium

Methanotrophic cultures were grown on nitrate mineral salt (NMS) medium prepared as per on Whittenbury et al. (1970) with slight modifications. For the initial culture enrichments an ammonium mineral salt (AMS) medium was also used. It was prepared the same way as NMS with the only difference in the nitrogen source (1 g/L KNO_3 substituted with 0.5 g/L of NH_4Cl).

3.2. Culture enrichment on biogas

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.

Collected environmental samples were used as initial inoculum for enrichment cultivations under biogas with CH_4 content of 56% mixed with air as a sole carbon source (final CH_4 concentration of 23%) on NMS and AMS medium in parallel. All cultures were done in triplicates, 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. Cultures were incubated in an orbital shaker at 30^{o}C and 130 rpm. Enrichment was conducted as a series of six one-week culture transfers and after seven weeks enriched cultures biomass were collected and transferred to N-free NMS to induce PHB accumulation. THE PHB content in the cells after 2 days of accumulation was assessed. To compare the PHB-producing potential of enriched cultures with a pure culture of methanotrophic bacteria *Methylocystis. hirsuta* DSM 18500 (DSMZ, Braunschweig, Germany) was cultured for a week in both media in the same conditions and then induced for PHB accumulation.

3.3. Selection of PHAs producing mixed cultures

Three PHB-producing cultures grown on NMS: of LB2 (from here on named as LB), PB, or AS culture 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 2 weeks of cultivation (with transfer to a fresh medium after one week) culture biomass was transferred to N-free NMS supplemented with 100 mg/L of valeric acid to induce accumulation of PHBV. The PHA copolymer production was assessed after two days of accumulation based on PHA content in the cells and 3HV molar fraction of accumulated polymer.

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, in addition *M. hirsuta* DSM 18500 was cultured as a reference. Cultures were grown in a batch mode for 2 weeks in 200 mL of NMS in 580 mL serum bottles. PHBV accumulation under CH_4 and 100 mg/L valeric acid was studied. 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.

3.4. Batch cultivation of mixed culture

Selected AS10 culture was first grown in 200 mL of NMS and 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% CH4 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^{o}C, stirring at 400-450 rpm and pH was maintained at 6.8 with the use of 1 M HCl. 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.

3.5. Cosubstrate assays

The biomass recovered from batch reactor was used to study the effect of different alcohols and carboxylic acids as cosubstrates on culture growth and PHA accumulation under nitrogen-limited conditions. Cultures resuspended in N-free NMS and supplemented with either methanol (0.17 g/L), ethanol (0.12 g/L), propanol (0.12 g/L), acetic acid (0.16 g/L), lactic acid (0.17 g/L), propionic acid (0.13 g/L), butyric acid (0.11 g/L), valeric acid (0.1 g/L) and caproic acid (0.1 g/L) or none were cultured for 2 days under 10% CH_4 in air (renewed after 24 h). Biomass concentrations, alcohols and organic acids concentration, and PHA analysis were conducted as described.

3.6. PHA production under a feast-famine regime

Two F0-Baby bioreactors (Bionet, Spain) were upgraded to allow continuous system operation (as described in the PhD thesis) and were operated for over 30 days in a sequential feast-famine regime of 48 h:48 h cycles. AS10 culture was cultivated under continuous CH_4 supply to evaluate the effect of different carbon supply and pH control strategies on PHA accumulation and microbial community dynamics. The mixed culture in 2.5 L NMS was subjected to feast cycles under continuous operation 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, as detailed in Chapter 4 of PhD thesis. 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. Every 24 h samples for OD_{600}, DCW, PHA, nitrate, nitrite, valeric acid, and biomass for microbial analysis were collected.

3.7. One-step PHBV production in a fed-batch system

The PHBV accumulation by AS10 and pure *M. hirsuta* culture was studied in 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. Two F0-Baby reactors were run in parallel with the same culture type at a time with a working volume of 2 L. When nitrate was depleted below than 250 mg NO_3/L 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. After 3 days of PHBV accumulation 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. The samples for OD600, 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.

3.8. Analytical methods

Bacterial growth was monitored by observing the optical density at 600 nm (OD_{600}) and the gravimetrically measuring the dry cell weight (DCW). The gas composition (CH_4 and CO_2) in the bottles headspace was analysed on Shimadzu GC-2014 gas chromatograph equipped with the Porapak N packed column and the TCD. 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. Methanol and lactic acid concentrations were measured with high-performance liquid chromatography (HPLC) (Shimadzu 20AT) equipped with Rezex ROA-Organic Acid column and RID. 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. All GC and HPLC analysis were conducted following the specific methodology described in the PhD thesis. Nitrate and nitrite concentration during bioreactor trials were measured photometrically using appropriate Supelco, Merck test kits.

In Chapter 4 and 5 accumulated polymer was extracted from the dried biomass through chloroform extraction at 60^{o}C. Samples of extracted polymer were analysed to determine the molecular weight of the polymer by gel permeation chromatography as described in the thesis.

3.9. Microbial composition analysis

Biomass samples collected for microbial composition analysis were stored in -20^{o}C until further processing. Total metagenomic DNA isolation was performed and 16S rRNA gene amplicon library was prepared for sequencing using MinION Mk1C sequencer (Nanopore Technologies). This methodology was applied and described in Chapter 3. For chapter 4 and 5 a modified method was applied targeting full rRNA operon amplicons as described in PhD thesis.

4. Selected Results and Discussion

4.1. Enrichment and selection of PHA-producing mixed methanotrophic cultures

Cultures enriched for methanotrophic bacteria using a biogas-air mixture as a carbon source after seven weeks of cultivation showed different microbial compositions and potential for PHB production. Growth in AMS media seemed to favour biopolymer accumulation in nitrogen-limited conditions, but restricted biomass growth. 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), while the highest PHB accumulation observed in NMS cultures ranged between 19% and 27% PHB (LB2, PB, AS, and reference strain). 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 & Klotz, 2011). Additionally, the toxicity of the end-products of ammonium oxidation, i.e., hydroxylamine or nitrite, adds to the bacterial growth inhibition (R. He et al., 2017). Studies have also suggested that physiological responses and environmental adaptations of methanotrophs to the nitrogen source are organism-specific (Nyerges et al., 2010). Microbial community analysis of the samples at the final stage of enrichment showed that all cultures were enriched in various methanotrophic bacteria, mainly *Methylocystis* and *Methylobacter* sp.. AMS cultures were also enriched in *Thiomonas* sp., *Bordetella* sp., *Dyella* sp., and *Pandoraea* sp., while NMS cultures were more diverse in their microbial community, and some of their other major genera included *Alicycliphilus* sp., *Massilia* sp. and *Thermomonas* sp.. Cultures with substantial PHB-producing potential had a high presence of *Methylocystis* sp., a known PHB-producing methanotroph genus (Rodríguez et al., 2020; Sundstrom & Criddle, 2015).

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. 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. AS cultures showed the highest accumulation of 37 ± 2 % of DCW PHA (at 30% CH_4) and the PHA productivity of 19 ± 2 mg/L·d (at 10% CH_4). 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, and provide a better C_1 oxidation at O_2-sufficient conditions in supporting a higher conversion rate of CH_4 to CO_2 and biomass (Karthikeyan, Chidambarampadmavathy, Nadarajan, et al., 2015; Wei et al., 2016). 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., 2018). AS culture seemed to adapt well

to the different CH_4:O_2 ratios, as it did not have much of an impact on their microbial composition or ability to produce PHAs. The observed high PHA accumulation in all AS cultures, despite their relatively low abundance of *Methylocystis* sp., could be attributed to the presence of other PHB-accumulating bacterial strains that are utilising CH_4-derived carbon, such as *Alicycliphilus* sp. (Oosterkamp et al., 2015).

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. 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%). However, there was a significant difference in the PHA productivity and polymer accumulation in the cells ($p \leq 0.05$) as they ranged from 12 to 39 mg/L·d and from 9% to 27% of DCW, respectively. 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). 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. 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. 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.

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.

4.2. 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. 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. The use of acetic and lactic acid as cosubstrates proved unfeasible as they resulted in the lowest increase in PHA accumulation. 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. However, the acetic acid supplementation caused a pH drop below 5 creating limiting conditions for mixed culture activity and growth. 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. 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., 2017a). 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. 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). 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. 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. 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. Different feeding time-based cosubstrate supply strategies were applied to evaluate their effect on the PHA accumulation. During the first famine stage (days 3-5), PHB was not accumulated because nitrate was still readily available above 300 mg NO_3/L. 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. 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. 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. 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. To overcome the possible existing limitations for the efficient PHA accumulation the carbon supply to the reactor was doubled after which the PHA accumulation returned to 20-30% PHA in DCW, and the PHA production reached 281 and 465 mg PHA/L for F1 and F2, respectively. 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. 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., 2023). Under low CH_4 supply, regardless of the feeding strategy used, the 3HV fraction reached approximately 60 mol% and when the gas supply was increased, the 3HV fraction stabilised at around 40 mol%. 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). 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 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).

4.4. 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. After deactivating the pH control in reactor F1 (day 20), the pH rose to 8.2 and then stabilised at around 8.0 for the rest of the process. The pH increase did not affect the PHA accumulation capacity, 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. 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). 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 number-average molecular weight of polymers extracted at the end of feast-famine process operation varied slightly between the reactors and were generally lower than PHA produced from CH_4 in previous studies but comparable to the PHBV of natural origin from Sigma-Aldrich. 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 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.

4.5. One-step PHBV production in a fed-batch system

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. However, the biomass increase of *M. hirsuta* exceeded that of AS10 culture, reaching a higher final biomass concentration. 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. 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. 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. 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 5^{th} day with 0.26 g DCW/Ld.

Despite different PHA accumulation capacities, with AS10 reaching up to 63% PHA in DCW and *M. hirsuta* reaching up to 43%, both cultures showed comparable PHA and 3HV production trends based on the overall concentration with similar maximum PHA concentrations: 0.67 g/L for AS10 and 0.71 g/L for *M. hirsuta*. 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. 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. When valerate was the only available carbon source, the biomass concentrations decreased which coincided with the drop in the accumulated PHA. In the conditions of CH_4 limitations, PHA can be catabolised into acetyl-CoA, providing reducing equivalents to restore biomass activity (Karthikeyan, Chidambarampadmavathy, Cirés, et al., 2015), which could explain the decrease in PHA concentration. 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, Strong, et al., 2019). 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. 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.

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. *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. 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%. The higher valerate concentration in the medium, although resulted in higher 3HV fraction, was detrimental to the PHA accumulation was lower by 10-20% and overall PHA production was also slightly lower than at 0.1 g/L feeding rate. 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., 2018; 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). 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.

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., 2017b). 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

The microbial community of the AS10 culture underwent changes depending on the operational conditions. 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. When valerate feeding was initialized and PHA started to be accumulated, the culture favoured the growth of *Acidovorax delafieldii* at the expense of methylotrophs (*Methylobacillus flagellates* which was abundant at the growth stage). 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 *Novosphingobium subterraneum*. The accumulation of valerate in the medium at a higher feeding rate negatively affected the *M. hirsuta* abundance, which decreased from 62% to 39% at the end of the process. In addition to *A. delafieldii* and *N. subterraneum*, 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. 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.

5. Outlook

5.1. 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.

5.2. 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.

5.3. 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 & 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., 2020). 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.

5.4. 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.

5.5. 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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