Development of Methane-Utilizing Mixed Cultures for Sustainable Biopolymers Production from Wastewater Treatment Streams

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Abstract

Methane is classified as the second major greenhouse gas with a global warming potential 25 times higher than carbon dioxide. Wastewater treatment plants are considered as one of the main anthropogenic sources for global methane emissions. As many of the existing wastewater treatment facilities flare the biogas produced during anaerobic digestion to the atmosphere, methanotrophs can offer a promising solution for methane mitigation and play an important role in the paradigm shift of considering wastewater streams as a proactive energy and value-added material resource. Methanotrophs can convert methane into intracellular biopolymers mainly in the form of polyhydroxybutyrate (PHB) under specific environmental conditions. These biopolymers can be extracted and transformed into products which can replace conventional polymers in a wide range of applications due to their competitive physical and thermal properties. However, PHB accumulation is mainly limited to type II methanotrophs only. Moreover, enrichment of type II from mixed cultures is one of the challenges facing the scaling up of this process due to other non-producing PHB methanotrophs invasion. Accordingly, this study aims to discuss the factors enhancing the enrichment of type II methanotrophs in mixed cultures and the selection parameters that can halt the growth of type I without affecting type II. In addition, to discuss the PHB accumulation potential of the enriched cultures and the conditions maximizing their capacity as an input for designing sustainable biotechnology for biopolymer recovery. Moreover, to examine the capability of integrating the accumulated biopolymers in other environmental processes as methanol production. Finally, to develop novel phosphorus-based PHB accumulation strategies using the methanotrophic strain *Methylocystis hirsuta* which can be then used as a selective pressure for targeting PHB-accumulating methanotrophs in mixed cultures.

Dedication

"To my wife Nada, I'm glad you are thoughtful. I'm glad you are understanding. I'm glad you are so much fun to be around but most of all I'm glad you are my wife. No greater light illuminates my path than the love and change you have brought into my life. Thanks for everything"

Acknowledgment

"Allah will exalt in degree those of you who believe, and those who have been granted knowledge. And Allah is Well-Acquainted with what you do."

Surah Al-Mujaadila, Verse 11

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List of Acronyms

AD	Anaerobic digestion
AMO	Ammonia monooxygenase
AMS	Ammonium mineral salt
ATP	Adenosine Triphosphate
C/N	Carbon to nitrogen ratio
CBB	Calvin–Benson–Bassham
CHP	Combined heat and power
F/M	Food to microorganisms ratio
FDH	Formate dehydrogenase
FID	Flame ionization detector
FST	Final settling tank
GHG	Greenhouse gases
HB	Hydroxybutyrate
HV	Hydroxyvalerate
ICM	Intracytoplasmic membrane
M/O	Methane to oxygen ratio
MDH	Methanol dehydrogenase
MMO	Methane monooxygenase
MSM	Mineral salts medium
N/M_i	Initial nitrogen to microorganisms ratio
N/M_o	Observed nitrogen to microorganisms ratio
NADH	Nicotinamide Adenine Dinucleotide - Hydrogen
NMS	Nitrate mineral salts
O/M	Oxygen to methane consumption ratio
O.D	Optical density
OTU	Operational taxonomic units
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate

PHBV	Polyhydroxybutyrate-co-valerate
Poly-P	Poly phosphate
RuMP	Ribulose monophosphate
SRT	Solids retention time
TCA	Tricarboxylic acid
TCD	Thermnal conductivity detector
TCE	Trichloroethylene
TP	Total phosphorus
VFA	Volatile fatty acids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WRRF	Water resources recovery facilities
WWTP	Wastewater treatment plants

Chapter 1

Introduction and Research objectives

1.1 Biopolymers production from methane

Petroleum based plastics are used globally while consuming about 270 million tons of oil and gas annually [1]. Moreover, these fossil fuels are non-renewable and have negative effects on the environment. One of the green alternatives to overcome this problem is to produce degradable plastic raw materials (i.e. Bioplastic/Biopolymer), which can be recycled again at their end-life. Bioplastic are natural products synthesized by different type of microorganisms at competitive physical properties (i.e. melting temperature and mechanical strength) [2], [3]. Under specific nutrients and environmental conditions, wide range of bacteria, microalgae and yeast can accumulate biopolymers from different carbon sources including simple sugars, fatty acids and plant oils [4], [5]. Most of these bioplastics are found in the form of Polyhydroxyalkanoates (PHA). Until now, more than 150 PHAs with different monomer composition have been identified containing 100-30000 repeated units [6], [7].

Currently, bioplastics are mainly produced using various food crops and vegetable oils and can be linked to the global carbon dioxide cycle. During the first step, the cultivated crops fix the atmospheric CO_2 followed by the processing step in which these harvested crops are used as the feedstock for PHA production. At their end life, the bioplastics are disposed and degrade back to CO_2 producing a closed cycle [8]. However, production of PHAs using the aforementioned methodology is facing some challenges mainly related to the price of the employed feedstock and its conversion to an accessible substrate for the micro-organisms to grow on [9]. Hence, recent research is focusing on developing new reliable feedstocks with cheaper production processing as wastewater, the organic fraction of municipal solid waste, methane and carbon dioxide.

Biogas, the end-product of the anaerobic digestion process (AD), contains up to 70% methane and is considered as a potential candidate to supply the biopolymers industry with their desired cheap feedstock. Methane can be converted to a biodegradable polymer poly hydroxybutyrate (PHB) from the PHA family using methanotrophs. Therefore, these microorganisms can combine the need for a beneficial use of the biogas emissions remediation and a cheaper feedstock to produce bioplastics. Methanotrophs can convert methane aerobically to PHB under unbalanced growth conditions, deficiency or limitation of essential nutrients i.e., nitrogen and phosphorus prevent their cellular growth and force them to store PHB as intracellular granules [10]. Wastewater treatment facilities represent 4% of the yearly global methane production [11] which make them a valuable biogas source. In 1986, Corder and his team were the first to utilize methanotrophs enriched from anaerobic digester sludge confirming their potential of accumulating methanol from methane [12]. Recently, numerous studies isolated or reported the existence of different types of methanotrophs in sewage sludge from different locations such as waste activated sludge (WAS), and anaerobically digested sludge [11], [13]–[16]. Moreover, methanotrophs successfully dominated mixed cultures seeded from the aforementioned types of sewage sludge in different studies [16]–[19].

1.2 Methanotrophs

Methanotrophs are gram-negative proteobacteria descending from the methylotrophic family, characterized by their ability to utilize one-carbon compounds “methane” as their sole carbon and energy source [20]. Most of methanotrophs are considered to be obligate, however, some strains were capable of utilizing multi-carbon substrates and considered to be facultative [21], [22]. Methanotrophs were isolated from many environments including; landfills [23], rice paddies [24], lakes[25] , freshwater sediments [26], forests [27], manure digestate [28] , agricultural soils [29],

soda lakes [30], volcanos [31], oilsands tailing ponds [32], marine sediments [33], and wastewater treatment plants (WWTPs) sludge [34].

Methanotrophs can be classified into three main groups; Group I "Gamma-proteobacteria" which consist of type I and type X methanotrophs, Group II "Alpha-proteobacteria" consisting of type II methanotrophs and Group III "Verrucomicrobia". The major difference between these groups is their carbon assimilation pathway [35]. Group I assimilate methane using the Ribulose Monophosphate "RuMP" cycle, group II use the Serine cycle, while group III use Calvin-Benson "CBB" cycle as shown in **Figure 1.1.**

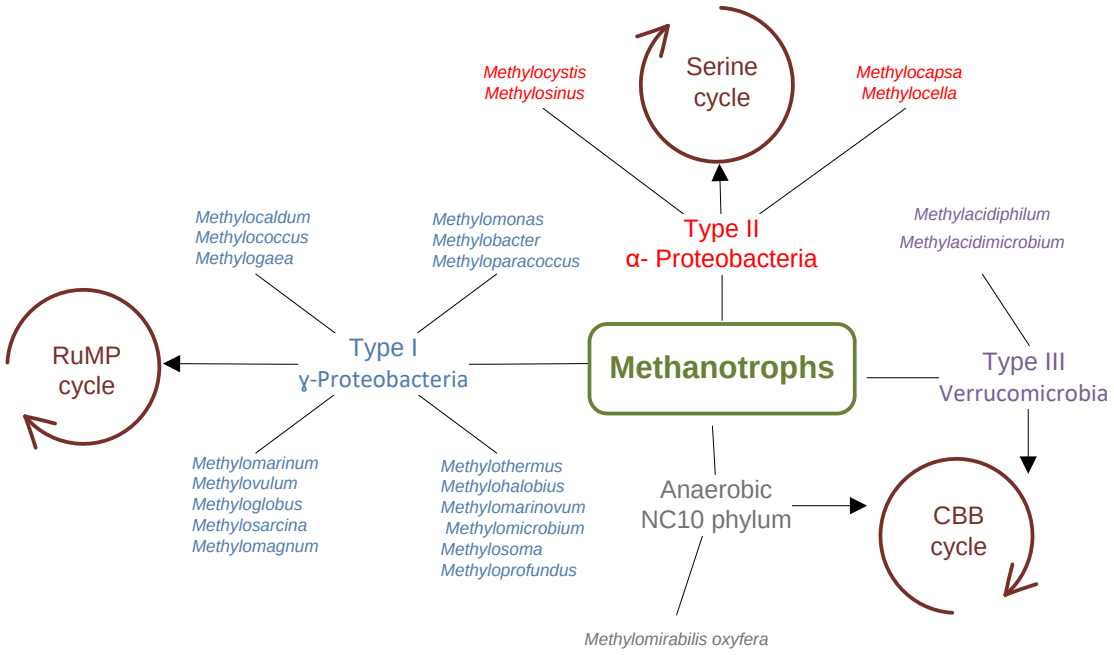

Figure 1.1: Methanotrophs taxonomy and carbon assimilation pathways [20]

1.3 Methane metabolism

All methanotrophs oxidize methane to carbon dioxide through a series of linked reactions for their energy requirements and cell replication. These reactions are completed with the aid of enzymes

secreted by methanotrophs. Firstly, methane is oxidized to methanol by the methane mono-oxygenase (MMO) in which a reducing equivalent break the O_2 bond and one oxygen atom is reduced to H_2O while the other is incorporated in the methanol conversion. MMO is found in two forms; the cytoplasmic soluble form "sMMO" and the copper containing particulate form "pMMO" located in the intracytoplasmic membrane (ICM) [36].

Secondly, methanol is oxidized to formaldehyde using the quinoprotein methanol dehydrogenase (MDH) located in the periplasm of the gram negative methanotrophs [3], [37]. Formaldehyde plays an important role in the metabolism of the methanotrophs, it is considered as the central intermediate. Part of the formed formaldehyde is oxidized to CO_2 for energy generation while the other part is incorporated in the carbon assimilation pathways for cell replication; RuMP pathway for type I and Serine pathway for type II. Formaldehyde is directed to either cycle rapidly due to its toxicity to methanotrophs.

Then formaldehyde is converted to formate mediated by the enzyme Formaldehyde dehydrogenase located in the cytoplasm. The last step is the production of CO_2 through the NAD-dependent enzyme formate dehydrogenase [38], [39]. The last two steps are considered to be the major source for the reducing equivalents required for the methane metabolism [20].

For the methane assimilation pathways, as previously mentioned, type I undergoes the RuMP pathway while type II use the serine pathway and type X can use both cycles. Each cycle has completely different reactions and enzymes involved for methane assimilation, energy production and cell replication as shown in **Figure 1.2.**

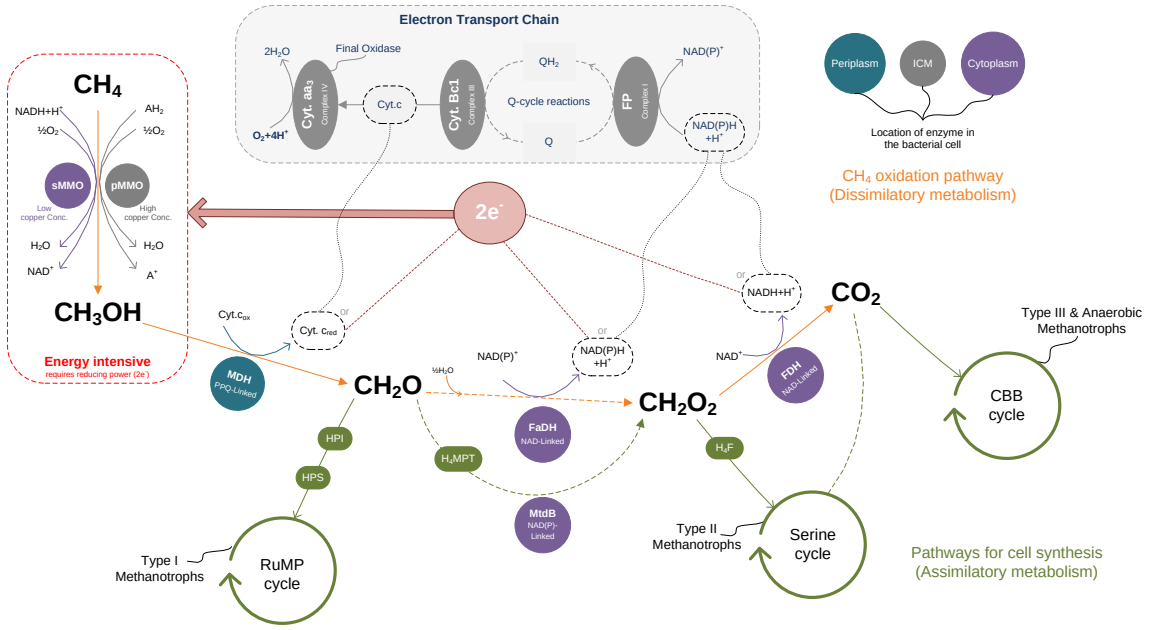

Figure 1.2: Different methane oxidation pathways in methanotrophs [20]

Type II methanotrophs are mainly the involved bacterial strain in PHB accumulation. Many studies were performed on pure cultures to maximize biomass density and PHB accumulation [40]–[43]. However, few studies were performed on mixed cultures or enrichments from activated sludge [3], [44]–[46]. In addition, studies that used activated sludge for enrichment of type II methanotrophs are not yet optimized. Many factors need to be assessed such as the choice of nitrogen source and its concentration, the solids retention time (SRT), the methane to oxygen ratio. Furthermore, the ratios between the methane, nitrogen and microorganisms to achieve a stable long-term strategy for biopolymer production while preserving the biomass density.

1.4 Research contribution

1.4.1 Problem statement

Methanotrophs can offer a long-term reliable solution for two of the major problems causing environmental pollution. These microorganisms can convert methane i.e. second major GHG into polyhydroxybutyrate (PHB) a type of biodegradable polymers with competitive thermal and

physical properties to conventional ones [10]. Not all methanotrophs have the ability of PHB accumulation which is mainly limited to type II only [47]. Most of the previous studies considered pure cultures of type II to discuss the factors affecting their growth and PHB accumulation. However, mixed cultures as waste activated sludge and anaerobic digestion sludge may provide a better choice if the scaling up this process is considered. Mixed cultures eliminate the sterilization requirements for running a pure culture bioreactor and reduce the running cost. Additionally, mixed cultures can promote the growth conditions through the synchronization of the existing microorganisms together by removing excessive toxic metabolites or supplementation of essential vitamins [48]. Moreover, the seed for cultivating the PHB-producing methanotrophs is always available in any WWTP.

The main drawback associated with mixed culture enrichments is maintaining a stable community dominated by type II without type I invasion. Due to their higher growth rates, type I tends to invade in mixed cultures running under normal growth conditions when all the essential nutrients are available. Selection strategies based on copper, pH, methane and dilution were not reliable on long-term basis [47]. Moreover, the only reliable selection parameter, which is the nitrogen source, relies on halting the growth type I but at the expense of type II growth as well [16].

Type II methanotrophs can utilize different nitrogen sources to support their growth. While nitrate resulted in the highest growth rate during pure culture studies, nitrate results in the dominance of type I in mixed culture enrichments. Type II are known for their unique ability to fix nitrogen gas for growth requirements which can be used for type II selection [48]. However, growth on nitrogen gas results in the slowest growth rates. Although ammonium can be utilized by methanotrophs for cellular requirements, ammonium can inhibit their growth by competing with methane for MMO oxidation or formation of toxic hydroxylamine or nitrite [49]. Type II methanotrophs show higher

resistance to the inhibitory effect caused by ammonium and dominates in mixed cultures supplemented with ammonium as a selective pressure. Unfortunately, PHB results for type II mixed cultures are lower than pure ones due to the elevated concentrations of ammonium used to washout type I [16].

In addition, most of the conducted studies on PHB production using methanotrophs were employing synthetic feeds to support their growth or PHB accumulation and nutrient rich waste streams were barely discussed. Furthermore, pure methane was fed as the main carbon source while the effect of utilizing real biogas is also unknown especially on the methanotrophic mixed cultures. Finally, the effect of the inoculum source used for methanotrophs enrichment needs to be investigated.

1.4.2 Research objective

The main objective of this research to introduce a deep investigation on the effect of ammonium as a nitrogen source on type II methanotrophs enriched from the sludge generated in WWTPs (activated and anaerobic). Then, to explore the effect of other operational parameters as the food to microorganisms (F/M), nitrogen to microorganisms (N/M), carbon to nitrogen (C/N) ratios and the solids retention time (SRT) on the growth of the enriched culture. In addition, to apply different methodologies for maintain type II selection in mixed cultures using ammonium on long-term basis while deducing their kinetic and stoichiometric growth parameters. Also, to discuss the effect of these methodologies on the PHB productivity of the enriched cultures.

Afterwards, the development of a sustainable microbial culture and feedstock within the wastewater and waste stream process will be tested using the following strategies: (i) find alternative sources of type II methanotrophs inoculum (i.e. anaerobic digestion (AD) sludge and

waste activated sludge (WAS), (ii) identify a source for the nutrients-sufficient medium using AD sludge centrate to replace synthetic media in the growth stage and the effluent from final sedimentation tank (FST) with low nutrients concentrations to induce the PHB accumulation, and (iii) utilize and convert the real biogas into PHB.

Moreover, the incorporation of PHB production for another application (methanol production) will be examined on the methanotrophic strain *Methylocystis hirsuta*. Finally, the effect of another main nutrient limitation i.e. phosphorus on the same strain will be studied in an attempt to discover novel operational conditions that would yield high PHB accumulation.

1.5 Research approach and Dissertation layout

To achieve the previously mentioned goals a literature survey is presented in **Chapter 2.** Throughout **Chapter 2** the PHB production mechanism by methanotrophs is discussed, Afterwards, the factors affecting the PHB accumulation capacity of methanotrophic bacteria were elucidated. In addition, the capacity of methanotrophs to produce other types of biopolymers from the PHA family is illustrated. Thereafter, in **Chapter 3**, the effect of ammonium as a nitrogen source on methanotrophic mixed cultures enriched from waste activated sludge is elaborated. In addition, the single and combined effects of different parameters such as; ammonium concentration, copper concentration, F/M ratio, C/N ratio and N/M ratios on the growth rate, biomass yield and methane utilization rate are demonstrated. In **Chapter 4**, The long-term effects of the deduced parameters from the previous chapter on methane-utilizing mixed cultures are studied. Moreover, the capability of establishing methanotrophic mixed cultures with high PHB accumulation capacity on a long-term basis are investigated. Then, the effect of employing another inoculum source for establishing methanotrophic mixed cultures using AD sludge is presented in

Chapter 5. All the kinetic growth parameters as well as the PHB production capacity were also estimated. Moreover, the potential of the enriched culture to accumulate another type of biopolymer is explored and the factors affecting and controlling the biopolymer composition are investigated. The impact of substituting all the synthetic growth components for the enriched methanotrophic mixed cultures is illustrated in **Chapter 6**. Pure methane is substituted with real biogas as the carbon source while other essential nutrients are supplied from either AD centrate for growth or FST effluent to induce PHB accumulation. In **Chapter 7**, a novel concept for methanol production using methanotrophs is presented relying on the PHB accumulation capacity of the strain *Methylocystis hirsuta*. The conditions inducing the PHB consumption to support methanol production are set and the factors aiding the methanol production of the strain while minimizing the reliance on external carbon sources are established. In **Chapter 8**, the capability of *Methylocystis hirsuta* to accumulate polyphosphates and to consume it to support PHB production is discussed for the first time. In addition, the impact of the nitrogen source under aerobic/anaerobic conditions is presented. Then, novel strategies yielding high PHB accumulation depending on phosphorus limitation are established. Finally, conclusions and recommendations for future directions are presented in **Chapter 9**.

Chapter 2

Literature review[1]

2.1 PHB production by Methanotrophs

Biogas can be converted to a biodegradable polymer PHB from the PHA family using methanotrophs. PHAs are biodegradable polymers and used in the manufacturing of bottles, packaging materials, shopping bags, containers and paper coatings in the material industry sector. In addition, they were developed in fabricating biomedical implants material and drug delivery carriers in the medical sector and insecticides carriers in the agricultural sector [10]. Therefore, these microorganisms can combine the need for a beneficial use of the biogas emissions remediation and a cheaper feedstock to produce bioplastics to be used in different applications. Methanotrophs can convert methane aerobically to PHB under unbalanced growth conditions, deficiency or limitation of essential nutrients i.e., nitrogen and phosphorus prevent their cellular growth and force them to store PHB as intracellular granules [10]. **Figure 2.1** shows a suggested schematic diagram for the cycle of PHB production using biogas. After extracting the accumulated PHB inside the bacterial cell, it can be either used as it is or combined with other polymers to get a desired product.

As previously mentioned, food crops and some vegetable oils are mainly used to produce biodegradable plastics. The current annual production for industrial PHB manufacturers ranges from 50-50000 tons of PHB at a market price around $3/kg which is still higher than petroleum based plastics which is $1-2/kg [7], [10].

[1] This chapter has been published in Reviews in Environmental Science and Bio/Technology pp. 1–43, Mar. 2018 https://doi.org/10.1007/s11157-018-9464-3

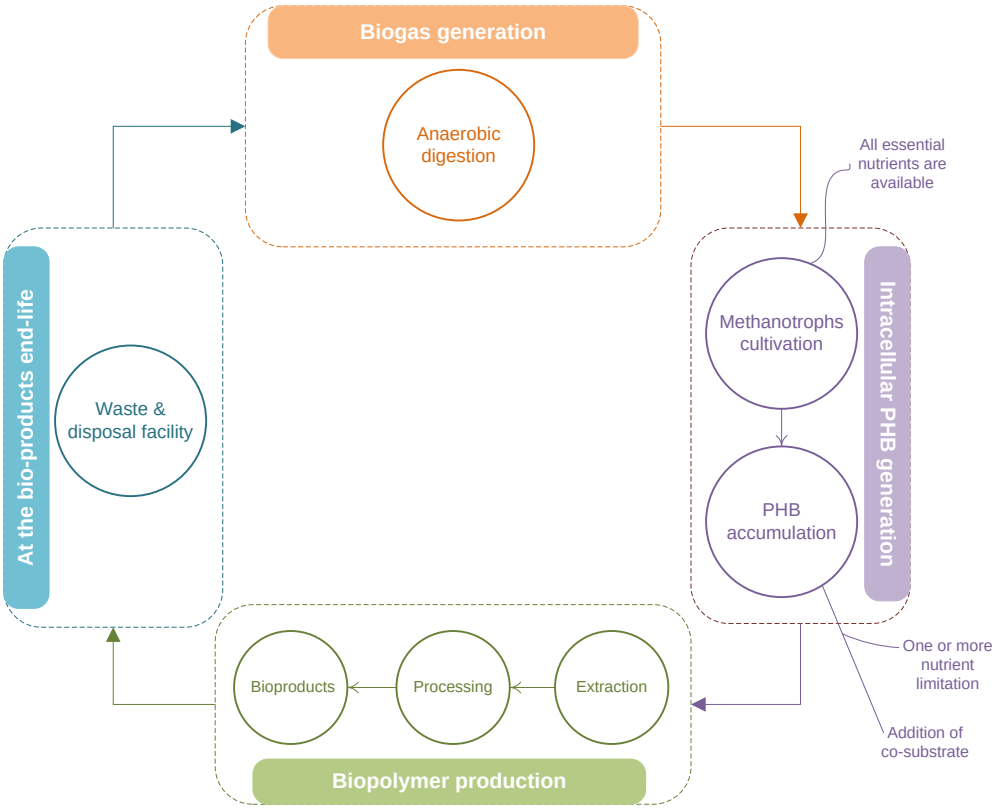

Figure 2.1: PHB production and degradation cycle using methane

Almost 30-50% of the production cost of PHAs are contributed to the feedstock price, thus, Production of those bioplastics using methanotrophs appears to be more efficient by combining bioplastics "PHB" production and biogas remediation. It was estimated that the cost of feedstock can be reduced from 1$/kg to 0.25$/kg-PHB if employed as feedstock. In addition, the revenue from biogas can be doubled if it is directed towards biopolymers production in comparison with other applications as combined heat and power (CHP) [8].

- **Mechanism of PHB production in methanotrophs**

Under balanced conditions when all the essential nutrients are available, methanotrophs proceed to TCA cycle to obtain their energy needs. While under unbalanced conditions when one or more nutrient is not available, they switch to the PHB cycle to provide the energy required for the cell

maintenance i.e., survival in this case. Acetyl-CoA plays the intermediate role under limited conditions; it is the first entry to the PHB cycle with the aid of some specific enzymes. Firstly, Acetyl-CoA is converted to Acetoacetyl-CoA through the enzyme β-ketothiolase encoded by PhaA gene that is then reduced to β-hydroxybutryl-CoA by the enzyme Acetoacetyl-CoA reductase encoded by PhaB gene. PHB synthetase enzyme encoded by PhaC gene, which is a polymerase, starts to form PHB from β-hydroxybutryl-CoA. The second part of the cycle include the enzyme PHB depolymerase encoded by PhaZ gene that depolymerizes PHB granules formed to hydroxybutyrate monomers. Secondly, acetoacetate is formed with the aid of β-hydroxybutyrate dehydrogenase which is then converted to Acetoacetyl-CoA by Acetoacetate succinyl-CoA transferase to complete the cycle [39], [50]. **Figure 2.2** illustrates the pathway for the PHB accumulation cycle in methanotrophs.

As described above, Acetyl-CoA is crucial for the PHB cycle and most probably can be produced only through the serine pathway [51]. Moreover, the RuMP pathway does not proceed a complete TCA cycle to obtain energy from Acetyl-CoA [10] which supports the hypothesis that PHB accumulation is exclusive to type II methanotrophs. Moreover, the consumption of the accumulated PHB generates the reducing equivalent $NAD(P)H^+$ which is utilized in methane oxidation as a survival mechanism. As previously mentioned, sMMO is mainly found in type II methanotrophs and only use the $NAD(P)H^+$ as reducing equivalent for the methane oxidation step. Accordingly, several strains from type I and type II methanotrophs were tested for the existence of PhaC “PHB polymerase”. Only type II strains possess this gene confirming the conclusion of their exclusiveness for PHB accumulation [47].

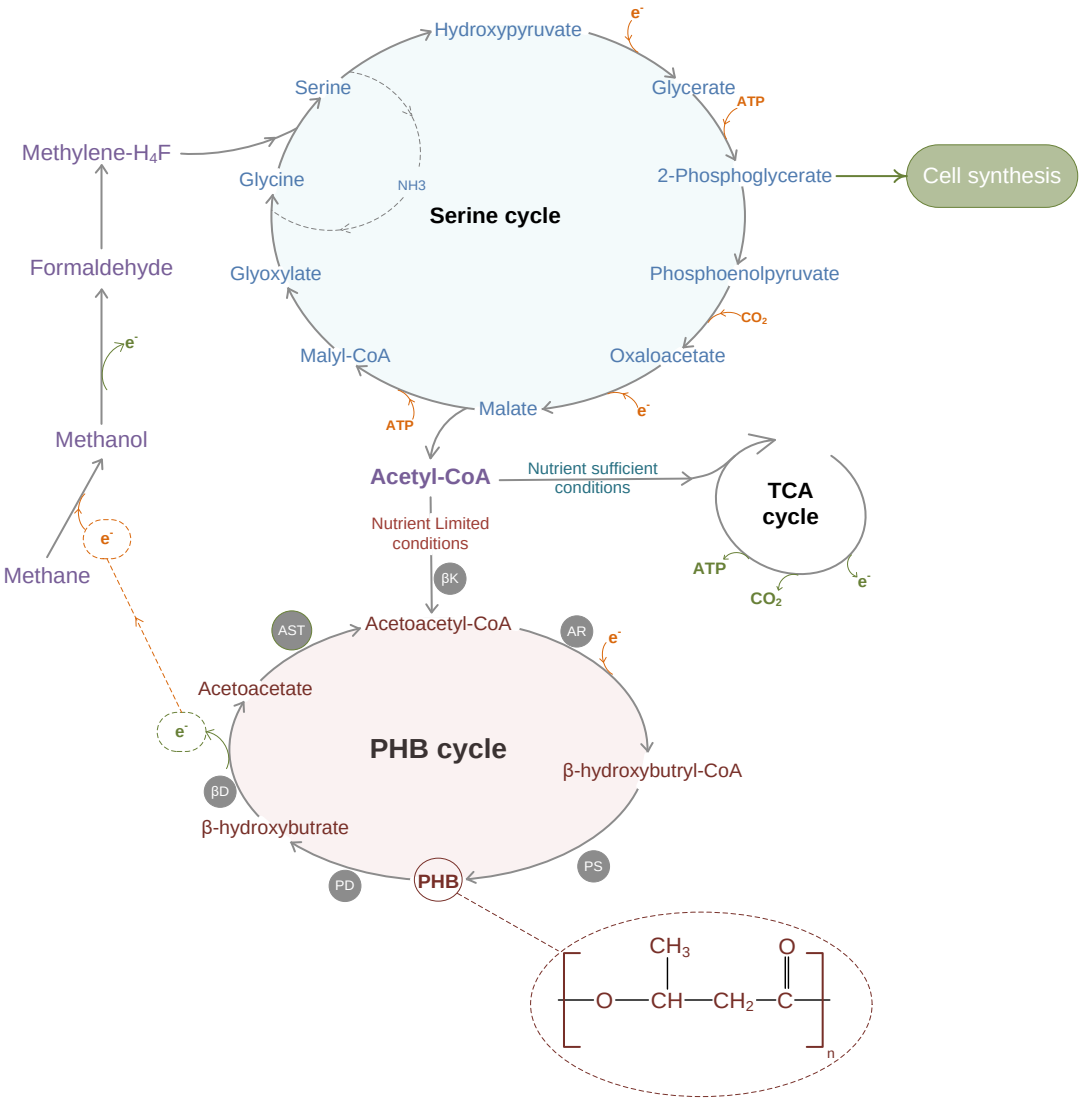

Figure 2.2: PHB cycle in methanotrophs showing the main involved enzymes; AST: Acetoacetate succinyl-CoA transferase, AR: Acetoacetyl-CoA reductase, βD: β-hydroxybutyrate dehydrogenase, βK: β-ketothiolase, PD: PHB depolymerase, PS: PHB synthetase.

While other microbial strains can grow using the stored biopolymers in absence of carbon [52]–[54], PHB was assumed to function as a carbon source for methanotrophs growth under the limited conditions [55], [56]. However, experimental studies revealed that PHB is not consumed in the absence of methane, showing that PHB cannot be used as a sole carbon source. Thus, PHB can be used only as a source of reducing equivalents for the methane uptake and its conversion to methanol [57].

2.1.1 Selection parameters for type II Methanotrophs and PHB accumulation

Since PHB accumulation is likely limited to type II methanotrophs, previous studies focused mainly on two targets, the factors affecting type II growth and conditions enhancing their PHB accumulation capability. Accordingly, pure culture studies were conducted to understand the effect of different nutrient concentrations on the growth of these bacteria and their role in type II metabolism while fewer studies considered mixed cultures. In the following section, we will discuss some of the reported factors affecting the growth and PHB accumulation of different type II methanotrophic strains.

- **Nitrogen source**

Nitrogen limitation was one of the most discussed conditions for PHB accumulation by methanotrophs. Moreover, it was concluded that the nitrogen limitation choice can be the only reliable parameter for long-term productivity of PHB [16]. However, contradictive data are available for the optimum choice of nitrogen source to maximize the bacterial growth and enhance their PHB accumulation capacity.

Type II strains have different responses to the nitrogen source available in the growing medium where the type of nitrogen source employed for growth the PHB accumulation phase. The strain *Methylosinus trichosporium* OB3b accumulated 50% PHB after nitrogen depletion in a nitrate salt medium with an initial concentration of 20 mM [58]. In another study, the same strain accumulated 38% PHB in nitrogen limited conditions after growing in a medium with initial nitrate concentration of 10 mM [47]. Using ammonium as a nitrogen source with an initial concentration of 10 mM resulted in only 13% PHB accumulation while switching to nitrogen gas increased the PHB accumulation to 45% [59]. When both nitrate and ammonium were used with concentrations of 10 mM and 8 mM, respectively, PHB accumulation reached 30% [60].

On the other hand, *Methylocystis parvus* OBBP accumulated 60% PHB after growing on ammonium and transferred to nitrogen limited conditions [59] compared to 36% accumulation when it was growing using nitrate as nitrogen source [47]. The strain *Methylosinus trichosporium* IMV3011 accumulated 47% PHB after growing on both ammonium and nitrate as nitrogen sources with initial concentration of 16 mM & 10 mM, respectively [42].

After growing in a medium with an initial nitrate concentration of 10 mM, *Methylobacterium organophilum* CZ-2 accumulated 38% PHB in limited conditions [46]. On the other hand, *Methylocystis hirsuta* accumulated up to 51% PHB after growing on 13 mM ammonium then transferred to deficiency conditions [40].

In mixed culture consortium, an enrichment dominated by the *Methlocystis* GB25 strain accumulated 51% PHB under ammonium deficiency conditions [61]. Another enrichment growing on nitrogen gas prior to nitrogen limitation accumulated 43% PHB and this enrichment was dominated by the *Methylocystis* & *Methylosinus* genus [47]. Also, an enrichment dominated by *Methylocystis* genera & growing on 13.5 mM ammonium accumulated 39% PHB in nitrogen limitation conditions [16]. Recently, a new nitrogen source was introduced to a mixed consortium of PHB producing methanotrophs, which is Urea. Urea is gradually converted to ammonium decreasing its adverse effect and then can be utilized by methanotrophs resulting in 39% PHB accumulation under deficiency conditions [62].

All of the previous studies especially on mixed cultures revealed that when nitrate was chosen as the nitrogen source biomass density was higher during the growth stage. However, the PHB production was less due to the invasion of non-producing PHB type I methanotrophs. Since nitrate does not have an inhibitory effect i.e. selective pressure on methanotrophs, type I dominates. Contrarily, growth on ammonium produce higher level of PHB at the expense of the biomass

density [63]. Ammonium can decrease methanotrophs' activity either by competing with methane for oxidation by MMO enzyme or by forming hydroxylamine if ammonium is oxidized which have an inhibitory effect on methanotrophs. Interestingly, type II methanotrophs shows higher resistance to the ammonium inhibition. Based on these data, a new strategy was developed on a mixed culture from an activated sludge where the mixed culture firstly grew on ammonium as a selection for the PHB producing microorganisms and then transferred to grow on nitrate to increase their biomass density. These microorganisms mainly composed of type II methanotrophs accumulated about 40% PHB. This strategy appears to be successful, however, some modification can be applied to increase the PHB productivity including the optimization of nitrogen concentrations [64].

- **Phosphorus**

Phosphorus concentration affects both type II growth and their PHB accumulation. A concentration of 2-25 mM is needed to maintain the sMMO activity [65] while concentrations above 40 mM completely inhibited the growth of *Methylosinus trichosporium* OB3b [66]. On the other hand, phosphorus deficiency resulted in an increased PHB accumulation values. *Methylocystis parvus* OBBP accumulated 31% PHB in a phosphorus deficient modified medium containing 0.12 mM compared to 18% PHB in a control medium with phosphorus concentration of 2.9 mM [62]. In addition, under phosphorus deficiency conditions, PHB accumulation reached 46% in an enrichment dominated by the *Methlocystis* GB25 strain [61]. Moreover, decreasing phosphorus concentrations from 7.3 mM to 5.7 mM increased PHB accumulation from 16% to 26.5% by *Methylosinus trichosporium* IMV3011 strain [67].

- **Copper**

Similar to nitrogen contradictive data is reported for the effect of copper on the PHB accumulation capacity of different strains. In a study on *Methylosinus trichosporium* OB3b, bacteria accumulated 42% PHB in a copper free medium while accumulating 50% PHB in a medium supplemented with 10 µm copper [58]. On the other hand, PHB accumulation for *Methylocystis parvus* OBBP increased from 18% to 49% when copper concentration was decreased from 15 µm to 5 [62]. In a study on the combined effect of nitrogen source and copper on the growth rates and PHB accumulation of the *Methylosinus trichosporium* OB3b strain, it was found that the highest growth rate was achieved while the bacteria was growing on ammonium in a copper free medium and accumulated 25% PHB under limited conditions. On the other hand, switching to nitrate with 5 µm copper relatively decreased the growth rate but the PHB accumulation reached 51% [68].

- **Other nutrients**

Most of the used medium for the cultivation of methanotrophs had iron concentration ranging from 6 to 20 µm [60]–[62], [67], [69]. However, it was found that concentrations ranging from 40 to 80 µm supports sMMO activity [66]. Moreover, iron deficiency didn't result in high PHB accumulation (11%) in an enrichment dominated by the *Methlocystis* GB25 strain while Sulphur deficiency resulted in 33% PHB accumulation [44].

A study showed that sodium, potassium and magnesium had inhibitory effects at high concentrations on *Methylobacterium* sp. On the other hand, magnesium & potassium deficiency resulted in a PHB accumulation of 28% & 34%, respectively [70]. Decreasing potassium concentration from 0.58 mM to 0.09 mM increased PHB accumulation from 18% to 28% in *Methylocystis parvus* OBBP strain. In addition, when calcium concentration was decreased to 7.2 µm PHB accumulation was doubled to reach 39% in *Methylocystis parvus* OBBP strain [62].

Moreover, sMMO activity was significantly affected when 1 mM of zinc was added to the medium and completely stopped by 0.01 mM of mercury [71].

- **Temperature**

Most of the studies were carried out in temperature ranging from 20 °C to 40 °C and rarely discussed the effect of temperature on biomass yields and PHB accumulation. However, pMMO activity declined sharply after 45°C in a study on the *Methylosinus trichosporium* OB3b strain [72] while sMMO activity decreased at temperatures higher than 30°C [66].

- **pH**

Most of the studies used culturing medium having a pH ranging from 6 to 7. However, increasing the medium acidity and decreasing the pH to 5 can be used as a selection parameter for type II methanotrophs. The acidic medium increased the dissolution of CO_2 in the culturing medium which can be used by type II as an input for the serine cycle [47].

- **Methane and oxygen**

In a study on the effect of oxygen on the reactions rates of a type II methanotrophic strain (MTS) it was found that, at high oxygen levels in the headspace (35%), the rate of methanol to formaldehyde increase leading to the inhibition of the whole metabolic reaction [73]. Moreover, in a study on the effect of oxygen partial pressure on biomass growth & PHB accumulation, the maximum PHB accumulation for *Methylosinus trichosporium* OB3b was 45% at 0.2 atm while *Methylocystis parvus* OBBP accumulated 60% PHB at 0.3 atm. The maximum biomass yield was observed at 0.4 atm [59].

Methane-limitation can be used as a selective pressure for PHB producing methanotrophs when ammonium is chosen as nitrogen source. Low methane levels (methane to ammonium ratio) favors

ammonium co-oxidation, then hydroxylamine production and forces methanotrophs to consume intracellular PHB -if present- to support methane uptake. Then, an increased PHB production overtime is expected. Methanotrophs accumulating high levels of PHB can use their stored reducing power to survive through those severe conditions, while bacteria lacking appropriate PHB levels will be unable to survive. Afterwards, the selected organisms producing PHB can grow on nitrate with normal methane concentrations to increase their biomass density [64]. Based on the information presented in this section, the suggested growth conditions for targeting type II methanotrophs are illustrated in **Table 2.1.**

Table 2.1: Suggested conditions for targeting type II methanotrophs in mixed cultures

Nitrogen source	Nitrogen gas	Ammonium (mM)	Ammonium + Nitrate*(mM)	Nitrogen + Nitrate*(mM)
Nitrogen source concentration (mM)	-	5-10	5-10 for NH_4 stage 10-20 for NO_3 stage	10-20 for NO_3 stage
CH_4:O_2	Oxygen partial pressure below 0.2 atm	1:1-1.5		
Copper (µmol)	Zero	Zero	Zero for NH_4 stage 5-15 for NO_3 stage	Zero for N_2 stage 5-15 for NO_3 stage
Phosphorus (mM)	2-25 to support sMMO activity during NH_4 or N_2 stages			
Iron (µmol)	40-80 µmol to support sMMO activity during NH_4 or N_2 stages			
pH	5-6 to increase CO_2 dissolution (favorable to the serine cycle)			
Temperature (°C)	20-30			

**Growth stage is divided into two phases; a nitrogen gas or ammonium growth phase for type II selection followed by a nitrate growth phase to increase the final biomass density.*

2.1.2 Molecular weight of extracted biopolymers

The molecular weight of the PHB accumulated in type II methanotrophs is mainly regulated by the combined effect of the PHB synthetase and depolymerase enzymes activity [42]. In addition, under nutrient deficiency conditions the activity of these enzymes increases while increasing the molecular weight of the PHB accumulated [43], [61]. Addition of inhibitors to the TCA cycle

induced the PHB cycle and resulted in higher PHB accumulation with higher molecular weight [42], [67] during the nutrient deficiency conditions. **Table 2.2** shows molecular weight of accumulated PHB corresponding to different nutrient deficiency conditions or addition of some organic acids (inhibitors for the TCA cycle).

Table 2.2: Observed molecular weight of the extracted biopolymer under different conditions

Condition	PHB%	MW (Da)*10^6	Reference
Addition of 0.3 g/l citric acid	50.7	1.79	[42]
Addition of 0.2 g/l malic acid	55.6	1.83	
Addition of 0.3 g/l sodium acetate	55.1	1.81	
Addition of 0.3 g/l citric acid	40	1.5	[67]
Ammonium deficiency	3	1	[43]
Magnesium deficiency	27	1.1	
Nitrate deficiency	24	1.3	
Phosphorus deficiency	18	1.2	
Phosphorus deficiency	51	2.51	[70]
Sulfur deficiency	32.6	2.46	[44]
Potassium deficiency	33.6	3.1	
Iron deficiency	10.4	1.81	
Ammonium deficiency	51.3	2.5	[61]

2.1.3 PHB production in bioreactors

Most of the experiments performed on methanotrophs studied the effect of different parameters on the growth of type II parameters and their PHB accumulation potential in small scale serum bottles [16], [59], [67]. Accordingly, results obtained from small-scale studies can be used as the input design parameters for methanotrophic bioreactors to scale up the process. These studies provided the microbial growth rates, biomass yield and inhibitory effects on both growth rates and PHB accumulation. In the following section, we will discuss the input design parameters reported in

previous studies which can help in estimating the optimum design parameters required for PHB production from methanotrophs in a larger scale.

- **Biomass and PHB yields**

According to the recorded values of PHB yields, an estimation for methane, oxygen and nitrogen requirements and yields can be determined for designing a bioreactor. Most of the studies reported the PHB yields under Nitrogen limitation condition only. However, other studies reported biomass yields and PHB yields under different nutrient deficiency conditions rather than nitrogen. **Table 2.3** show different values for the PHB yield reported in different studies for some type II strains. On the other hand, fewer studies reported the biomass yields for their experiments, generally, biomass yield ranged from 0.4-0.8 g-biomass/g-CH_4 [16], [74]–[76].

Table 2.3: PHB yield for different strains of type II methanotrophs

Strain	PHB yield (g PHB/g CH_4)	Accumulation condition	Reference
***Methlocystis* GB25**	0.4	Sulfur deficiency	[44]
	0.45	Potassium deficiency	
	0.22	Iron deficiency	
	0.52	Ammonium deficiency	[61]
	0.55	Phosphorus deficiency	
	0.37	Magnesium deficiency	
***Methylocystis parvus* OBBP**	0.34	Nitrogen limitation	[57]
***Methylobacterium Organophilum* CZ-2**	0.43	Nitrogen limitation	[46]
Mixed culture dominated by type II	0.4	Nitrogen limitation	[16]
***Methylocystis* sp. WRRC1**	0.67	Nitrogen & copper limitation	[77]

- **Specific Growth rate**

As all other parameters, methanotrophs growth rate is affected by the cultivation conditions as pH, copper and nitrogen source. When the pH of the copper free growing medium was increased from 6 to 8.5 the specific growth rate (μ) of *Methylosinus trichosporium* OB3b decreased from 0.087 h^-

^{1} to 0.039 h^{-1} [66]. On the other hand, the specific growth rate for the same strain was 0.07, 0.095, 0.0083 h^{-1} at copper concentrations of 0.21, 1.25, 20 µmol, respectively [72]. For the strain *Methylocystis parvus* OBBP reducing copper concentration from 15 µmol to 5 µmol increased the specific growth rate from 0.065 h^{-1} to 0.08 h^{-1} [62]. Compared to other nitrogen sources, nitrogen gas resulted in the slowest growth rates for type II methanotrophs. In a study on the *Methylosinus trichosporium* OB3b strain, the growth rate was 0.015 h^{-1} during nitrogen gas growth compared to 0.11 h^{-1} while growing on ammonium under the same conditions.

However, in terms of PHB accumulation, the bacteria grown on both nitrogen sources could accumulate up to 40% PHB under nutrient limited conditions [68]. This slow growth rate can be contributed to the oxygen sensitivity of the nitrogen fixing genes for type II methanotrophs as increasing the oxygen partial pressure from 0.1 atm to 0.2 atm decreased the growth rate of *Methylosinus trichosporium* OB3b from 0.24 h^{-1} to 0.06 h^{-1} [59]. Thus, to maintain high growth rates for type II methanotrophs the combined effects of the discussed parameters should be taken into consideration for designing a methanotrophic bioreactor. **Table 2.4** illustrates some of the recorded growth rates for different type II strains in correspondence to the nitrogen source.

Table 2.4: reported values for specific growth rate of type II methanotrophs using different nitrogen sources

Strain	**Nitrogen source**	**Specific growth rate (µ) h^{-1}**	**Reference**
***Methylosinus trichosporium* OB3b**	NO_3	0.087	[66]
	NO_3	0.125	[78]
	NO_3	0.11	[58]
	NH_4 - NO_3	0.16 – 0.19	[59]
	NO_3	0.11	[57]
	NH_4 - NO_3	0.11 – 0.17	[59]
	N_2	0.02	[68]

***Methylocystis parvus* OBBP**	NH_4	0.06	[79]
	NH_4	0.053	[80]
	NH_4 - NO_3	0.06 -0.08	[62]
Mixed culture dominated by type II	NH_4	0.04	[16]

- **Bioreactor configuration**

Production of PHB using methanotrophs occurs in two stages, a growth phase and a PHB accumulation phase. These two phases can take place in the same reactor or separately. In the combined mode, PHB accumulation is expected to take place at the end of the logarithmic growth phase and the beginning of the stationary phase. Accordingly, nutrient sufficient conditions were applied for the first 144 hours until no significant increase in the growth was observed followed by application of limiting conditions to induce the PHB accumulation [62], [67]. *Methylosinus trichosporium* OB3b accumulated 45% PHB after 160 hours in a copper free medium while a PHB content of 50% was achieved in a medium supplemented with 10 µmol copper after 120 hours when the growth and PHB accumulation phases were simultaneously occurring in the same bioreactor [58]. On the other hand, after separating the growth phase from the PHB accumulation phase, an enrichment dominated by type II accumulated 39% PHB after a 48 hours nitrogen limitation period [16]. In addition, PHB accumulation reached 51% in the first 24 hours of the experiment under deficiency conditions in another separate mode [61].

An effective bioreactor configuration for scaling up PHB production from methanotrophs have some challenges to overcome. First, mixing two flammable gases oxygen and methane in a safe method without affecting the overall performance of the bioreactor. Secondly, provide techniques to increase methane delivery to the microorganisms due to the low methane solubility. Thirdly,

minimizing the power requirements and operational costs. Lastly, achieving a sustainable capability of maintaining the selected type II methanotrophs with a stable PHB accumulation. Different type of bioreactors with different configurations were tested to improve methane delivery by testing different types of diffusers, testing mixing speeds, include gas recirculation or using pressurized reactors. **Table 2.5** summarizes the results obtained for bioreactors studies under different conditions.

Table 2.5: Comparison between different bioreactors used for PHB accumulation studies

Bioreactor configuration	**pH**	**Temp .(°C)**	**CH_4:O_2 or Air**	**Nitrogen source**	**Biomass density (g/l)**	**PHB (%)**	**Ref.**
5-L batch fermenter	6.8 - 7.2	30	1:3	Nitrate	18.6	50	[58]
2-L batch fermenter	7	30	1:3	Ammonium & Nitrate	20	30	[60]
5-L fed batch fermenter	7	34	1:1	Ammonium & Nitrate	2.7	47	[42]
70-L Pressure bioreactors	5.7	38	*p*CH4 = 30%, *p*O2 = 15%	Ammonium	25-65	51	[70]
3-L Two-phase partitioning bioreactors	-	30	Gas flow 0.42 l/min CH_4 loading 80 $gm^{-3}\ h^{-1}$	Nitrate	0.23	38	[46]
1-L Bubble column bioreactor	7	30	1:1	Ammonium	2.9	42.5	[40]
1.4-L Vertical loop bioreactor	7	30	1:1	Ammonium	1	51.6	[40]
0.5-L Jacketed stirred tank reactors	7.2	25	Gas flow 0.4 l/min CH_4 conc. $2g/m^3$	Nitrate	2.1	12.6	[69]
4-L Completely mixed batch reactor	-	-	1:1	Cyclic between Ammonium and Nitrate	3	39	[64]
15.2-L Fluidized bed reactor	6.5 - 6.9	20-23	1:2.3	Nitrogen gas	-	10	[74]

2.1.4 Co-polymers production by methanotrophs

While scaling up the biopolymers production process using methanotrophs is still under research, thermal and mechanical properties of the accumulated biopolymers are taken into consideration for a wider application. Unfortunately, using PHB can be limited due to its low thermal stability as the melting temperature for PHB (≈180°C) is close to the degradation temperature (≈200°C) making it harder for processing [77]. Moreover, the stiffness, brittleness and high crystallinity are considered from the main drawbacks of PHB. PHB co-polymers can provide a possible solution for the aforementioned drawbacks. The incorporation of hydroxyvalerate HV units to PHB results in the formation of the co-polymer poly hydroxybutyrate-co-valerate PHBV which has a lower melting temperature, crystallinity, water permeability and enhanced mechanical properties [81]. Properties of the produced co-polymer PHBV mainly depends on the HV fraction. For example, increasing the HV fraction from 3 mol% to 25 mol% decreased the melting temperature from 170°C to 137°C [82].

During the PHB accumulation phase, under nutrient limited condition, a co-substrate is introduced to the culturing medium such as citrate, propionate or valerate yielding ketovaleryl-CoA which is then converted into hydroxyvaleryl-CoA. The formed hydroxyvaleryl-CoA combines with hydroxybutyrate to form the PHBV polymers as illustrated in **Figure 2.3**. The accumulation of PHBV is linked to the methane oxidation rate, moreover, it is prohibited in absence of methane. Methane oxidation rate increased when valerate is added to the growing medium and increased the energy requirements for methane oxidation. The electron equivalent conversion fraction for energy (f_e) increased from 0.25 without valerate to 0.35 and 0.45 after addition of 100 mg/l and 400mg/l valerate, respectively [16], which can be linked to the ATP required for the valerate uptake [79] as shown in **Figure 2.3**.

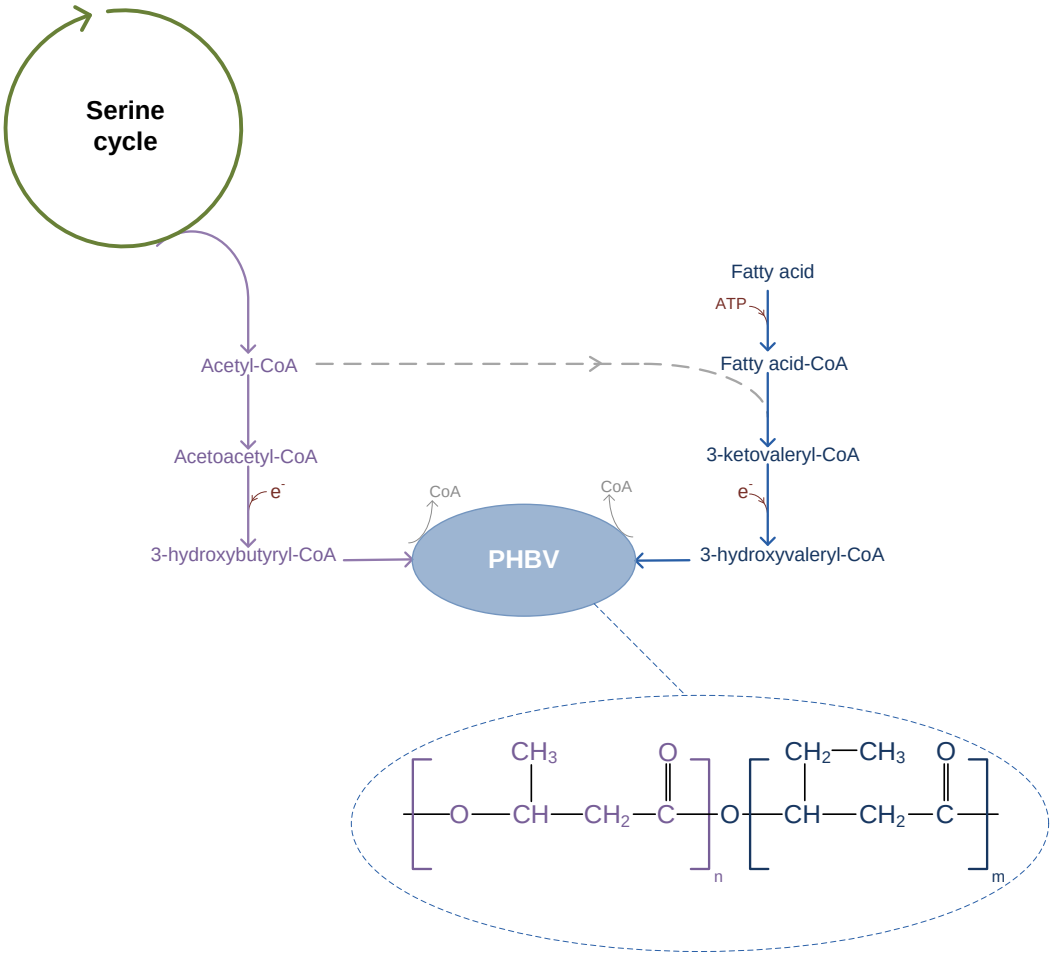

Figure 2.3: Co-polymer production pathway in type II methanotrophs; the reaction between acetyl-CoA and fatty acid-CoA represented by the dashed line occurs in case of propionate only.

Different co-substrates were tested for PHBV production. Valerate achieved highest results, on the other hand, pentanol resulted in 80% reduction in the methane uptake and 44% in the PHBV yield [77]. In addition, valerate outcompeted propionate in the incorporation of HV monomers and total co-polymer production [79].

Valerate concentrations above 0.7% (v/v) (2.17 g/l) showed an inhibitory effect on HV incorporation and on PHBV accumulation in general for the strain *Methylocystis* sp. WRRC1. At high valerate concentrations only PHB was accumulated with 15% content only compared to 30% PHB in absence of valerate. On the other hand, at 0.34% v/v valerate the PHBV accumulated was

60% with 50% incorporated HV monomers. Furthermore, eliminating copper from the PHBV accumulation phase resulted in increasing biopolymer accumulation to 78% with an HV content of 58% [77]. However, the inhibitory level of the added co-substrate seems to be strain specific where the amount the biopolymer accumulated decreased after concentration of 100 mg/l for both the strains *Methylocystis parvus* OBBP and *Methylocystis hirsuta* [79], [83]. **Table 2.6** shows results for co-polymer production using different methanotrophic strains.

Table 2.6: Results for PHBV accumulation under different substrates and co-substrates

Methanotrophs	**Substrate**	**Co-substrate and concentration**	**PHBV %**	**HV content%**	**Reference**
Mixed culture dominated by *Methylocystis* sp.	Methane	Valerate 100mg/l	44	20	[16]
	Methane	Valerate 400mg/l	30	39	
	Methane	Propionate 100mg/l	32	8	
***Methylocystis parvus* OBBP**	Methane	Valerate 100mg/l	54	22	[79]
	Methanol	Valerate 100mg/l	52	22	
	Formate	Valerate 100mg/l	58	15	
***M.trichosporium* OB3b**	Methane	Valerate 100mg/l	50	20	
Methylocystis hirsuta	Biogas	Valeric acid (130 mg/l)	54	25	[83]
***Methylocystis* sp. WRRC1**	Methane	Valerate 0.34%	60	50	[77]
	Methane	Valerate 0.34%*****	78	58	

*******biopolymer accumulation phase was conducted in a copper free medium*

Chapter 3

Behavior of Type II methanotrophic bacteria enriched from activated sludge process while utilizing ammonium as a nitrogen source[2]

3.1 Introduction

Recently, methane emissions mitigation is receiving global attention. Atmospheric methane concentration has already reached 1.75 ppm with an anticipated concentration of 4 ppm in 2050 if no actions are taken to address this problem [84]. The main problem associated with methane emissions is the ability of methane molecules to absorb 20 times more heat than the carbon dioxide therefore; minimizing methane emissions will have 20-60 times impact on the global warming phenomena [38]. Anthropogenic methane sources resulting from human activities represent about 70% of global methane emissions and wastewater treatment facilities represents 20% of these anthropogenic emissions [85]. Methane produced from waste treatment facilities i.e. biogas can be employed in various biotechnological applications rather than being flared to the atmosphere.

Many wastewater treatment plants use the produced biogas during anaerobic treatment processes for heat and electricity generation. However, the cost and efficiency of converting the biogas into heat and electricity is high and the regenerating process is low. Nevertheless, the recent discoveries in the biological utilization of biogas using methanotrophs gave the wastewater treatment plant a new perspective in methane mitigation while recovering value added products such as biopolymers, methanol and lipids or participation in other processes as bioremediation and denitrification [3], [38], [86].

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Methanotrophic bacteria have the ability to utilize single-carbon (C1) compounds (i.e. methanol or methane) as a carbon and energy source [20]. Methanotrophs oxidize methane through a series of interlinked reactions into carbon dioxide with methanol, formaldehyde and formate as intermediates to fulfil their energetic and cellular requirements. As shown in **Figure 3.1a**, The first step of this reaction is the oxidation of methane into methanol using their unique methane monooxygenase (MMO) enzyme in which a reducing equivalent break the O_2 bond and one oxygen atom is reduced to H_2O while the other is incorporated in the methanol conversion. MMO is found in two forms; the soluble form (sMMO) found in the cell cytoplasm and the particulate one (pMMO) located in their intracytoplasmic membrane (ICM) [55]. The expression of either enzyme is mainly regulated by the copper concentration in the growing medium. The sMMO is expressed in copper free medium or at very low concentration (0.3μM) while the pMMO requires copper in order to be activated [36].

Secondly, methanol is oxidized to formaldehyde using the quinoprotein methanol dehydrogenase (MDH) located in the periplasm of the gram negative methanotrophs [87]. Due to its toxicity, formaldehyde is rapidly converted to formate while the last step is the production of CO_2 through the NAD-dependent enzyme formate dehydrogenase (FDH) [38], [39].

Aerobic methanotrophs are mainly classified into two main types according to their carbon assimilation pathway. Type I methanotrophs undergo the ribulose monophosphate (RuMP) pathway while type II methanotrophs use the Serine pathway [20]. Type I methanotrophs are characterized by their higher growth rates, higher efficiency in methane oxidation and can incorporate 5-15% of the generated CO_2 into cellular biomass. On the other hand, Type II methanotrophs can form a slower but more stable communities [88], [89], incorporate up to 50%

of CO_2 in cellular biomass (as an input to the Serine cycle for methane assimilation) and their unique ability to accumulate PHB under unbalanced conditions [109] [10]. These biopolymers can be considered a green alternative for resources-consuming plastics due to their similarity in physical and chemical properties [92], [93] with the ability of different microorganisms to degrade them at their end-life minimizing their impact to the environment compared to petroleum based plastics [94], [95].

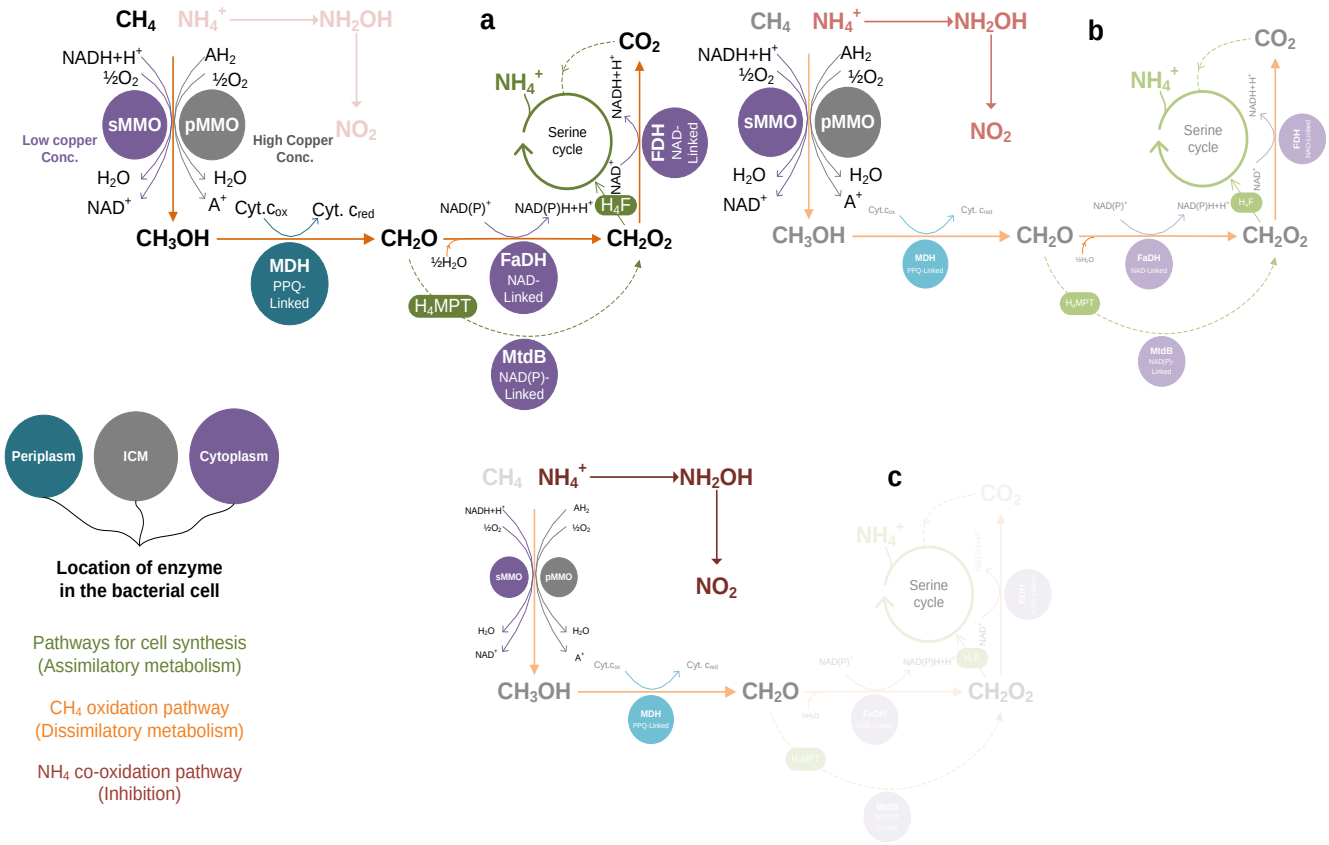

Figure 3.1: Methane oxidation pathway for type II methanotrophs and the effect of ammonium co-oxidation on methane metabolism a) ammonium is used for cell synthesis b) competition between methane and ammonium for MMO c) inhibition by the by-products resulting from ammonium co-oxidation.

Due to the ability of type II methanotrophs in biopolymers accumulation and possession of sMMO enzyme, cultivation of type II methanotrophs gained a great interest in the last few years. However,

the majority of the conducted research studies were mainly focused on pure cultures experiments to study the factors affecting different type II strains growth including nitrogen source type and concentration, phosphorus and copper concentration, pH, temperature and methane to oxygen ratio [59], [65], [71], [72].

In order to scale-up the use of type II methanotrophs and integrate it into wastewater treatment plant, enrichments of type II methanotrophs from mixed cultures such as activated sludge may be beneficial in eliminating the sterilization requirements for running pure cultures bioreactors. Moreover, the accompanying microorganisms could help in eliminating some toxic metabolites leading to an improvement in the growth conditions [50]. However, maintaining a stable community dominated by type II methanotrophs remains one of the major challenges towards the sustainability of a methanotrophic bioreactor [64]. In addition, methods for targeting type II using pH, dilution, methane limitation and copper concentration were not reliable on long-term basis. On the other hand, applying nitrogen gas as the sole nitrogen source for type II selection resulted in the slowest growth rate among all other nitrogen sources i.e. ammonium and nitrate [59] due to the sensitivity of the nitrogen fixing enzymes towards oxygen which are mainly found in type II methanotrophs. It is noteworthy to mention that the elimination of copper was assumed to select type II by activating the sMMO enzyme, which is found only in type II methanotrophs, however, it was recently discovered that type I methanotrophs can express this enzyme [19], [96]. Moreover, some sMMO lacking methanotrophs (i.e. *Methylomicrobium* and *Methylobacter*) can survive and grow under very low copper concentration [36], [97].

Ammonium as a nitrogen source can be used as a main selection parameter for type II methanotrophs on a long-term basis [16]. Although ammonia is mainly used by methanotrophs as

a nutrient, ammonia oxidation by methanotrophs can inhibit their growth in two ways. Firstly, through the competition with methane for pMMO due to the similarity between the MMO and the ammonia monooxygenase enzyme (AMO) in the reducing equivalent, inhibitor and active sites, though ammonia can compete with methane for the pMMO oxidation step which decrease the rate of methane to methanol conversion and accordingly the overall metabolism [98] as illustrated in **Figure 3.1b**. Secondly, through the accumulation of the toxic hydroxylamine and nitrite which inhibits the formate dehydrogenase and by consequences affect both the assimilatory and dissimilatory pathways for methane oxidation [99] as shown in **Figure 3.1c**. Type II methanotrophs shows high resistance to the ammonium inhibition and tends to dominate in cultures supplemented with high ammonium concentrations [100].

However, studies where ammonium was used as a selection parameter halted the growth of type I methanotrophs at the expense of type II growth as well [16]. Accordingly, more studies are needed to investigate the effects of ammonium on type II methanotrophs enriched from mixed culture to deduce the operational conditions that will result in a stable dominated type II enrichments without affecting their growth rates. In this study, different ammonium and copper concentrations were applied on type II methanotrophs mixed culture enriched from an activated sludge process to investigate their effect on the ammonium co-oxidation and consequently on the growth conditions of the enriched cultures. Moreover, the effect of methane to oxygen ratio, initial nitrogen to microorganisms ratio (N/M_i), and food to microorganisms ratio (F/M) on the growth of the enriched type II methanotrophs was studied in an attempt to manipulate other operational conditions that would support the ammonium based selection for type II methanotrophs in mixed cultures without affecting their growth.

3.2 Materials and methods

3.2.1 Cultivation conditions

All enrichments were cultivated in the mineral salts medium (MSM) described by [90] and had the following concentrations of chemicals (mg/l):1000 $MgSO_4.7H_2O$, 200 $CaCl_2.H_2O$, 272 KH_2PO_4, 610 K_2HPO_4, 4 Fe-EDTA and 1 ml trace metal solution. The trace metal solution had the following concentrations of chemicals (mg/l): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.4H_2O$, 30 H_3BO_3, 3 $Na_2MoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$ and 20 $CoCl_2.6H_2O$. Ammonium chloride (NH_4Cl) was added as the sole Nitrogen source to the MSM medium according to the desired final concentration. Five NH_4Cl concentrations (270, 535, 1070, 2140, 4280 mg-NH_4Cl/l) were used throughout the experiments to give an ammonium concentration of (5, 10, 20, 40, 80 mM), respectively. These concentrations were chosen to manipulate the effect of ammonium concentration, the carbon to nitrogen (C/N) and the initial nitrogen to microorganisms (N/M_i) ratio on the growth of the enriched culture. Copper was added from a stock solution having a concentration of 500 mg $CuSO_4.5H_2O$/l according to the desired final copper concentration.

Unless otherwise specified, all the experiments for studying the effect of different parameters on the growth of type II methanotrophs using ammonium were running in a batch mode. All enrichments were incubated in 250 ml serum bottles capped with butyl rubber stoppers. The liquid volume was 50 ml and the headspace was filled with a methane and oxygen (>99.9% purity, Praxair) mixture of volumetric ratio 1:1. All the experiments were running at a temperature of 25°C using table orbital shakers and a speed of 160 rpm. pH was kept between 6-7 using 10% NaOH prepared solution. Samples were taken from the headspace periodically to monitor the gases consumption. The growth of the enriched culture and ammonium consumption were measured at the beginning and the end of the experiment by measuring the optical density (O.D) and

ammonium concentration as mg-N/l in the culturing medium. During the experiments, the specific growth rate, biomass yield, methane utilization rate, oxygen to methane consumption ratio (O/M) and the nitrogen consumed to biomass generation ratio N/M_o were used as performance indicators of the enriched culture.

3.2.2 Type II cultivation

Activated sludge was collected from Humber wastewater treatment plant located in Toronto, Canada and used as a seed for methanotrophs cultivation. The activated sludge was filtered through a 100 µm cell strainer to remove large bodies. After centrifuging, the filtered sludge was added to 50 ml of the MSM having initial ammonium and copper concentrations of 20 mM and 5 µmol, respectively to give an initial optical density of 0.2±0.05 (95±20 mg-VSS/l). Air was removed from the headspace using a vacuum pump and replaced with 100ml of O_2 and 100 ml of CH_4 to give a volumetric ratio of 1:1. Every 48 hours the cultures were centrifuged (4000x) for 20 min then the collected biomass pellets were resuspended in 50 ml of fresh medium and headspace was refilled with CH_4 and O_2. After several transfers, when the turbidity and the increase in O.D were noticed, 50% of the biomass was transferred to fresh medium and the rest of the enriched culture was harvested and used as a seed for the next experiments.

3.2.3 Effect of ammonium concentration in copper supplemented medium

The aim of this experiment was to investigate the effect of different ammonium concentrations on the growth rate, biomass yield and methane utilization rate of the enriched culture. In addition, to investigate the effect of ammonium concentration on the methane uptake and if ammonium co-oxidation is affecting the growth of the enriched culture. At the beginning of the experiment, 250 ml serum bottles were filled with 50 ml of liquid medium and 200 ml of CH_4 and O_2 in the headspace (1:1). The liquid medium in each bottle contained a different concentration of

ammonium chloride to give a final concentration of 5, 10, 20, 40, 80 mM. Then, 0.125 ml of copper stock solution was added in all bottles to give a final copper concentration of 5µmol. 1 ml of gas was withdrawn from the headspace periodically using a gas tight syringe to monitor methane and oxygen consumption. The O.D was measured at the beginning and the end of the experiment to monitor the growth. Samples were taken from each bottle at the beginning and end of the experiment to calculate the ammonium consumption. All the bottles had an initial O.D of 0.44±0.09 and the experiments lasted for 40 to 44 hours.

3.2.4 Effect of F/M on ammonium inhibition

This experiment aimed to explore the effect of biomass density on the growth of the enriched culture at different ammonium concentrations while maintaining methane concentration like the previous experiment. Accordingly, the initial O.D was increased from 0.44±0.09 to 0.66±0.09 and the food to microorganisms ratio (F/M) decreased from 5.29±0.49 to 2.75±0.15 mg-CH_4/mg-VSS. Three ammonium concentrations were introduced to the incubation bottles; 5, 10 and 20 mM. The running operational conditions were similar to the previous runs.

3.2.5 Effect of methane to oxygen ratio

This experiment aimed to discuss the effect of the initial methane to oxygen ratio on the growth of the enriched culture under ammonium as nitrogen source. In addition, to study if this ratio would have an impact on the ammonium co-oxidation. Four ratios were tested by changing the gases composition in the headspace of each serum bottle as shown in **Table 3.1**. Helium was added to complete the headspace to 200 ml. All the bottles had an initial ammonium and copper concentration of 5 mM and 10 µmol, respectively. All the bottles had an initial O.D of 0.6±0.05. All other conditions were similar to the previous experiments. The methane and oxygen

consumption were monitored periodically and the final O.D and nitrogen were measured once the methane or oxygen were depleted from the headspace.

Table 3.1: Headspace composition throughout the methane to oxygen ratio experiments

Experiment	Methane volume added (ml)	Oxygen volume added (ml)	Helium volume added (ml)
A	100	50	50
B	100	100	0
C	50	100	50
D	25	100	75

3.2.6 Effect of maintaining the initial nitrogen to microorganism (N/M_i) ratio and increasing F/M

The aim of this experiment was to study the effect of maintaining the same ammonium to microorganisms ratio (N/M_i) on the growth (performance indicators) of the enriched methanotrophs. The same N/M_i ratio used in the previous experiment was kept during these experiments (0.24±0.02 mg-N/mg-VSS). The O.D was 0.60 in one setup then decreased to 0.3 in another setup and the required amount of ammonium was added to each bottle to maintain the aforementioned initial N/M ratio. The headspace was evacuated using a vacuum pump before the start of the experiment and refilled with methane and oxygen with volumetric ratio of 1:1. The gases consumption, growth and nitrogen consumption was periodically monitored as previously described.

3.2.7 Effect of ammonium concentration in copper free medium

During this experiment, two setups were prepared using the prescribed methodology to monitor if ammonium would result in an inhibitory effect in absence of copper. The first was supplemented

with a medium having ammonium chloride concentration of 5 mM while the other used a medium having 20 mM ammonium chloride. No copper was added during this experiment to induce the expression of the sMMO enzyme. The initial O.D of both setups was adjusted to 0.4±0.06. The gases consumption, growth and nitrogen consumption were periodically monitored as previously described.

3.2.8 Effect of copper concentration

Four copper concentrations were tested to study their effect on the growth of Type II methanotrophs. 0.250, 0.5, 1 and 2 ml of copper stock solution were added to the 250 ml serum bottles containing 50 ml of MSM to give a final copper concentration of 10, 20, 40 and 80 µmol, respectively. All the bottles had an initial O.D of 0.44±0.06 and the cultivation conditions were similar to the previous experiments.

3.2.9 Analytical methods

Samples withdrawn using gas tight syringe from bottles headspace were injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD) and Molecular sieve column (Restek, Bellefonte, PA.) to monitor methane, oxygen and nitrogen concentrations. The temperature program was as following: injector, 60^{o}C; Oven, 80^{o}C; TCD, 80^{o}C and helium gas was used as carrier gas with flowrate of 15 ml/min. external calibration curves were constructed using a known mixture of gases and used to convert the measured peak areas into gases concentrations and masses. For bacterial growth monitoring, optical density O.D was measured using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). A correlation curve between O.D and VSS was developed where VSS was measured using standard methods. Liquid samples were collected from the supernatant after the cultures centrifuge (4200 g) for 20 min. Then, HACH methods and testing

kits were used to measure inorganic nitrogen (NH_3-N, NO_2-N, and NO_3-N). The effect of each tested parameter on the performance indicators was statistically tested using spearman correlation test on GraphPad Prism software package where ρ represents Spearman's correlation coefficient and p represents the significance. Parameters are considered significant for $p \leq 0.05$. The p-values and correlation coefficients of those parameters are summarized in **Table A.1** in the appendix section.

3.2.10 Molecular biology analysis

Two Samples were taken from the enrichment during the cultivation to ensure the dominance of Type II in the mixed culture. The first sample was taken after 14 days of cultivation while the second sample taken after 35 days. DNA extraction and the amplification of the V4 region of the 16S SSU rRNA were carried using the previously described method (Stearns et al., 2015; Thevaranjan et al., 2016). DNA was extracted using MoBio PowerMag Soil DNA Isolation Kit as per the manufacturer protocol. Primers 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'- GGACTACNVGGGTWTCTAAT-3') were used and the barcodes incorporated into the forward primer enabled the usage of various reverse primer constructs to obtain longer amplicons and removal of biases.

Briefly, each 25µl PCR mixture contained the following in order to amplify V4 of the 16S rRNA gene by PCR: 13µl of PCR-grade water (Sigma), 10 µl of Platinum Hot Start PCR Master Mix (2x) (ThermoFisher), 1 µl of Template DNA, 0.5 ml of 515FB primer (10 µM), 0.5 ml of 806RB primer (10 µM). The reaction then was run for 35 cycles (94°C for 2 min, 94°C for 30 s, 50°C for 30°C, 72°C for 30 s), with a final polymerization step at 72°C for 10 min. The products were separated by electrophoresis in a 2% agarose gel and visualized under a UV transilluminator light, and the products corresponding to the amplified V4 (300-350 bp) were excised and purified using

standard gel extraction kits (Qiagen). Products were then quantified with Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher). Equal amounts of products from each sample (240 ng) were combined into a single, sterile tube then cleaned using MoBio UltraClean PCR Clean-Up Kit. The final concentration was measured using nanodrop spectrophotometer and the product quality was checked by ensuring that the absorbance ratio at 260/280 nm is ranging from 1.8-2.

Samples were sent to Farncombe Metagenomics Facility at McMaster University DNA Sequencing Facility and sequenced using an Illumina MiSeq per the manufacturer's instructions. The completed run was demultiplexed with Illumina's Casava software. The resulting sequenced data were used by Cutadapt to trim the forward and reverse paired-end reads at the opposing primers for input into DADA2 for processing. Chimeras were removed and sequences were organized into operational taxonomic units (OTUs) with a clustering threshold of 97% using Abundant OTU+. Output from this tool was then formatted where taxonomy was assigned using the Silva database.

3.3 Results and discussions

3.3.1 Type II methanotrophs enrichment from activated sludge

In order to incubate type II methanotrophs, three bottles of 250 mL were seeded with activated sludge and running with all essential nutrients required to induce their enrichment. During the first phase of this process (i.e. 5 days of cultivation), turbidity started in all bottles and the O.D was increasing as an indicator for methanotrophs growth. The color of the medium shifted towards the tan color known for type II methanotrophs [74]. However, a change in the microbial structure was noticed during the second phase of cultivation when 50 % (from day 7 to day 26) and the color of the enrichments was shifting towards the pink color known for type I methanotrophs [74] and the relative abundance of the genus *Methylomonas* (type I) was 6 times higher than the genus

Methylocystis (type II). Afterwards, type II dominated without any observed invasion from type I for 14 days of operation and the relative abundance of *Methylocystis* reached 26% which was the highest in the mixed culture while *Methylomonas* genus completely disappeared from the mixed culture as shown in **Table 3.2**. At this point, the dominance of type II methanotrophs in the mixed culture was confirmed and the biomass was used to perform the upcoming experiments. Although the initial ammonium concentration was relatively high during the enrichment phase (20 mM) and the transfers ensured that the ammonium concentration is always high in the cultivation medium, type I invasion was noticed during the cultivation period. Moreover, it was reported in the literature that type II methanotrophs has higher resistance to the elevated ammonium concentration [64] which resulted in their dominance at the latter phase of the enrichment.

The existence of type I may have occurred due to the development of a resistance to the inhibition resulting from the elevated ammonium concentration but after a long adaptation period or at a certain point when type II growth initiated first and started consuming ammonium then type I growth started when the ammonium concentration became non-toxic for them. Moreover, none of type I invasion was noticed during the rest of the experiments at any ammonium concentrations. However, further studies are required to figure out the optimum solids retention time (SRT) that should be maintained for type II methanotrophs and determine the required time to transfer of the biomass enriched without giving any chance for type I invasion.

Table 3.2: Taxonomic classification and relative abundance of the bacterial community resulting from 16S rRNA gene sequences at genus level with relative abundance above 1% at days 14 and 35.

Phylum	Class	Family	Genus	Day 14(%)	Day 35(%)
Acidobacteria	Acidobacteria	Acidobacteriaceae	Acidobacterium	0.0	1.1
Actinobacteria	Actinobacteria	Cellulomonadaceae	Cellulomonas	0.0	0.1
		Microbacteriaceae	Humibacter	0.0	3.3
			Leifsonia	0.0	3.1
		Nocardiaceae	Rhodococcus	0.0	1.9
Bacteroidetes	Flavobacteriia	Flavobacteriaceae	Chryseobacterium	2.2	1.7
			Cloacibacterium	10.6	0.0
			Flavobacterium	16.1	0.0
	Sphingobacteriia	Sphingobacteriaceae	Pedobacter	2.3	0.0
		Chitinophagaceae	Terrimonas	1.9	0.0
Proteobacteria	Alphaproteobacteria	Acetobacteraceae	Acidisoma	0.0	8.1
		Caulobacteraceae	Brevundimonas	5.8	0.4
		Methylocystaceae	**Methylocystis**	**2.0**	**26.1**
		Hyphomicrobiaceae	Devosia	1.4	0.0
			Hyphomicrobium	0.2	3.3
		Rhodobiaceae	Parvibaculum	0.0	1.0
		Rhizobiaceae	Rhizobium	3.3	0.0
		Rhodobacteraceae	Rhodobacter	5.4	8.3
		Sphingomonadaceae	Sphingomonas	0.1	20.8
			Sphingopyxis	5.1	0.0
		Acetobacteraceae	Rhodovastum	0.0	1.8
	Betaproteobacteria	Methylophilaceae	Methylophilus	1.3	7.2
	Gammaproteobacteria	Xanthomonadaceae	Dyella	0.1	6.3
		Methylococcaceae	**Methylomonas**	**12.4**	**0.0**
		Shewanellaceae	Shewanella	1.4	0.0
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiaceae	Prosthecobacter	9.6	0.0
Unclassified				2.2	7.7

3.3.2 Effect of ammonium concentration

As shown in **Figure 3.2a**, increasing ammonium concentration significantly affected the specific growth rate of type II methanotrophs and the growth rate dropped from $0.048\pm0.006 hr^{-1}$ to $0.01\pm0.002 hr^{-1}$ while growing on 5 and 80 mM, respectively ($p= 0.0124$, $\rho= -0.952$). Additionally,

the observed biomass yield decreased from 0.6±0.011 to 0.26±0.006 mg-VSS/mg-CH_4 (p= 0.0137, ρ= -0.949), whereas, the methane utilization rate followed the same trend with the increase in ammonium concentration (p= 0.0018, ρ= -0.987). However, the results shows that the negative impact of ammonium was more evident at concentrations above 20 mM where the drop observed in the growth rate was around 25% when ammonium concentration elevated from 5 to 20 mM but further increase to 80 mM led to a 75% decline in the specific growth rate. Similar observations were found for the observed biomass yield where the drops were 13% and 50% before and after 20 mM, respectively. The methane utilization rate was almost constant until 20 mM at a value of 0.076±0.008 mg-CH_4/mg-VSS.hr followed by a significant decrease to 0.03±0.004 mg-CH_4/mg-VSS.hr at 80 mM.

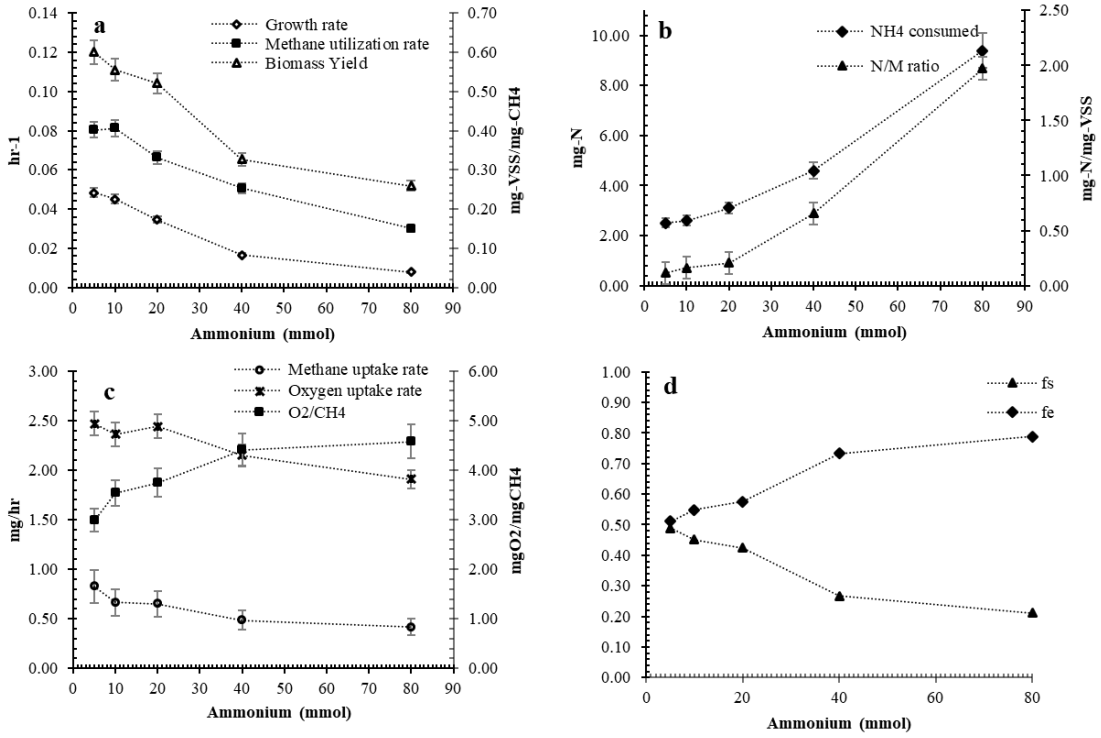

Figure 3.2: The effect of increasing ammonium concentration on the **a)** growth rate, Biomass yield and methane utilization rate **b)** The nitrogen consumption and observed N/M ratio for different ammonium concentrations **c)** The methane uptake rate, oxygen uptake rate and observed consumption ratio between oxygen and methane **d)** The electron fraction equivalent for energy (f_e) and cell synthesis (f_s).

The decline in the growth rate and biomass yield accompanied with elevating the ammonium concentration can be referred to the inhibitory effect resulting from either the completion between ammonium and methane for oxidation by the MMO enzyme or toxicants in the form of hydroxylamine (NH_2OH) or Nitrite (NO_2) that result from ammonium oxidation. Considering the chemical formula for biomass is $C_5H_7O_2N$, the expected ratio between the nitrogen utilized and the increase in biomass N/M_o (observed) should be around 0.12 mg-N/mg-VSS. As shown in **Figure 3.2b**, despite the rise in the ammonium consumption with the increase in the concentration, the N/M_o ratio was following the same behavior ($p= 0.0033$, $\rho= 0.980$). The N/M_o ratio was 0.12 mg-N/mg-VSS at 5 mM ammonium concentration confirming that 100% of the utilized ammonium was converted into biomass compared to 1.97 mg-N/mg-VSS with only 6% of the utilized ammonium converted into biomass. Moreover, **Figure 3.2c** shows that the elevation in the ammonium concertation reduced the methane uptake to half, compared to around 20% inhibition on oxygen uptake results. On the other hand, a significant increase in the consumption ratio between the oxygen and methane ($p= 0.0396$, $\rho= 0.896$) especially after 20mM ammonium following the same pattern of the growth rate and biomass yield.

These results confirm that the inhibition resulting for the ammonium is caused by the competition between methane and ammonium for MMO oxidation. Moreover, if ammonium oxidation occurs, the formation of hydroxylamine or nitrite is expected and even some studies shows the capability of some type II methanotrophs to convert these toxic compounds into nitrous oxide or even nitrogen gas [98]. The existence of the latter activity can be excluded since all the experiments were running under aerobic conditions and this conversion requires anoxic conditions to be performed. In addition, the nitrite was measured for all the enrichments and the concentration was 0.19 ± 0.02 mg-NO_2/l that is lower than the inhibitory levels recorded in previous studies [100].

Accordingly, the main inhibitor for type II growth could be the formation of NH_2OH resulting from the ammonium that outcompeted methane for MMO oxidation.

Another effect was observed for increasing the ammonium concentration on the electron fractions equivalent for energy (f_e) and cell synthesis (f_s). Both fractions were calculated according to the stoichiometry developed by [59] for the growth of methanotrophs or methanotrophic mixed cultures [16] on methane using ammonium as their nitrogen source. The electron donor reaction (R_d), electron acceptor reaction (R_a), cell synthesis reaction (R_c) and the overall reaction (R) are summarized in ***Eq.1-5***.

R_d: ¼ CO_2 + H^+ + e^- → ¼ CH_4 + ¼ O_2 **(1)**

R_a: ¼ O_2 + H^+ + e^- → ½ H_2O **(2)**

R_c: 1/5 CO_2 +1/20 HCO_3^-+ 1/20 NH_4^++ H^+ + e^- → 1/20 $C_5H_7O_2N$+9/20 H_2O **(3)**

R: $f_eR_a + f_sRc - R_d$ **(4)**

R: 1/4 CH_4 +(1/4+f_e/4) O_2+(f_s/23) HCO_3^-+ (f_s/23) NH_4^++(f_e+ 20f_s/23 -1) H^+ →(1/4- 4f_s/23) CO_2+ (f_e/2 + 9f_s/23) H2O+ (f_s/23) $C_5H_7O_2N$+9/20 H_2O **(5)**

Accordingly, f_e and f_s can be estimated where the biomass yield is equal to 113 f_s/ (23/4) and f_e+f_s=1. It is noteworthy to mention that usually the molar consumption ratio between oxygen and methane is used to calculate f_e and f_s based on the mentioned stoichiometry [59]. However, based on the results during this experiment, ammonium co-metabolism contributed to a significant increase in the oxygen to methane (O/M) consumption ratio then the observed yield was used to estimate their values. As shown in **Figure 3.2d**, elevating the ammonium concentration from 5 mM to 80 mM led to an increase in the f_e from 0.51 to 0.79 and consequently dropped the f_s from

0.49 to 0.21 ($p= 0.0125$, $\rho= \pm 0.952$). These changes in the f_e and f_s also confirms the negative effect of ammonium on the enrichments where they had to force their metabolism towards the energy production to survive at the expense of the replication when the methane oxidation was decreased at high ammonium concentrations.

3.3.3 Effect of F/M on ammonium inhibition

The previous experiment explored the effect of different ammonium concentration without considering the initial amount of biomass introduced in each bottle. During this experiment, the initial O.D was adjusted to 0.66±0.09 compared to 0.44±0.09 in the previous experiments resulting in a change in the F/M ratio from 5.29±0.49 to 2.75±0.15 mg-CH_4/mg-VSS. In addition, the results from the previous experiment showed that the negative effect of ammonium effect was more obvious at concentrations above 20mM. Accordingly, this experiment explored the effect of lowering the F/M ratio at ammonium concentrations up to 20mM. When the experiments were running at a lower F/M ratio, elevating the ammonium concentration from 5 to 20 mM did not have a significant effect on the growth rate or the biomass yield as shown in **Table 3.3.** However, it is important to mention that the reason for lowering the F/M ratio is to study the lowest ratio that type II methanotrophs can grow on with a minimum effect on the growth rate. This F/M (with the choice of ammonium as nitrogen source) can be consequently tested on type I in further experiments to see if they can be inhibitory on them as a selective pressure for type II on a long-term basis . Nonetheless, the specific growth rate was 0.018±0.001hr^{-1} while the observed biomass yield was 0.49±0.09 mg-VSS/mg-CH_4. However, decreasing the F/M ratio resulted in an average decrease of 56.3% in the growth rates ($p= 0.0079$, $\rho= 0.677$) compared to the previous experiments as shown in **Table 3.3** and a lower effect on the biomass yield which declined by an average of 19.8% ($p= 0.1533$, $\rho= 0.403$).

Table 3.3: Observed biomass yields and growth rates for different F/M ratios under different ammonium concentrations

NH_4 / F/M	5 mM		10 mM		20 mM		% Decrease	
	μ (hr^{-1})	Y(g-VSS/g-CH4)	μ (hr^{-1})	Y(g-VSS/g-CH4)	μ (hr^{-1})	Y(g-VSS/g-CH4)	μ	Y
5.29±0.49	0.048±0.006	0.6±0.011	0.045±0.007	0.55±0.013	0.035±0.006	0.52±0.02	**27**	**13**
2.75±0.15	0.018±0.001	0.49±0.09	0.019±0.003	0.43±0.07	0.018±0.005	0.42±0.08	**5**	**12**
% Decrease	**62**	**18**	**58**	**22**	**49**	**19**		

While the decrease in the specific growth rate can be mainly due to the decrease in the F/M ratio, the stability in the specific growth rate can be referred to higher initial biomass density used for these experiments. Increasing the initial O.D decreased the initial ratio between the ammonium and biomass (N/M_i) which decreased the inhibitory effect of the ammonium co-oxidation. That can be also confirmed by the stable average methane uptake rate noticed during the experiments 0.65±0.044 g-CH_4/hr. In addition, unlike the Higher F/M, the f_s and f_e were not changing with values of 0.36±0.03 and 0.64±0.03, respectively ($p= 0.1487$, $\rho= \pm 0.481$).

Ammonium co-oxidation was still occurring especially during the period when the methane concentration decreased in the headspace and ammonium could outcompete methane for pMMO oxidation. The N/M_o ratio doubled from 0.13 to 0.24 mg-N/mg-VSS when the ammonium concentration rose from 5 to 20 mM, respectively.

3.3.4 Effect of methane to oxygen ratio

When different methane to oxygen ratios were tested to determine their effect on the growth of Type II methanotrophs, decreasing the methane to oxygen ratio from 1:1 to 1:2 resulted in a drop

in the growth rate from 0.025±0.007hr^{-1} to 0.021±0.006hr^{-1} and a further decrease to 1:4 showed a growth rate of 0.011±0.003hr^{-1}. On the other hand, when the methane to oxygen ratio was 1:1, ammonia had no inhibitory effect on the growth rate and the N/M_o was 0.12±0.03 mg-N/mg-VSS compared to 0.24±0.06 mg-N/mg-VSS at lower methane to oxygen ratio indicating the existence of ammonium co-oxidation. Further decrease in the methane to oxygen ratio to 1:4 allowed the N/M_o ratio to reach 0.3±0.05g-N/g-VSS which is one of the main reasons for the decrease noticed in the growth rate ($p= 0.032$, $\rho= -0.469$). This can be also confirmed with the increase in the molar consumption ratio between methane and oxygen that inclined from 1.37 to 1.66 when the methane: oxygen ratio was decreased. In addition, lowering the methane: oxygen ratio resulted in a decline in the F/M. As shown in the previous experiments, decreasing the F/M ratio contributes to the decrease in the growth rate that was also observed in this experiment. However, it is noteworthy to mention that maintaining a high F/M ratio for a low soluble gas such as methane is one of the major challenges facing the up scaling of methanotrophic bioreactors.

Despite the inhibition to the growth rate caused by ammonium when the methane concentration was decreased, type II methanotrophs were able to maintain a constant biomass yield of 0.41±0.07 mg-VSS/mg-CH_4 which confirms their tolerance to the inhibition caused by ammonia. In other words, they were able to convert the methane oxidized into biomass equally regardless of the amount of inhibition caused by ammonium. As this behavior was not noticed in the ammonium experiments where both the growth rates and biomass yields were negatively affected by increasing the ammonium concentrations, another factor should be taken into consideration, which is the initial nitrogen to biomass ratio. In this experiment, the main cause of the inhibition for type II methanotrophs is the decrease in the methane (carbon) to ammonium (Nitrogen) ratio (C/N) while the N/M_i ratio was constant for all the bottles. Whereas in the previous experiment the

decrease in the growth rate was caused by the combined, effect of the C/N ratio and the initial N/M_i ratio and consequently decreased the biomass yield as shown in **Table 3.4**.

Table 3.4: Effect of methane to oxygen ratio on the growth rate, biomass yield and N/M_o

Methane: Oxygen	**C/N (g-CH_4/g-N)**	**N/M_i (g-N/g-VSS)**	**F/M (g-CH_4/g-VSS)**	**Growth rate (hr^{-1})**	**Biomass Yield (g-VSS/g-CH_4)**	**Observed N/M (g-N/g-VSS)**
2:1	10.95±0.95	0.21±0.043	2.68±0.28	0.037±0.005	0.82±0.11	0.12±0.04
1:1	11.42±0.82	0.25±0.013	3.61±0.36	0.025±0.007	0.42±0.09	0.12±0.03
1:1*	1.82±0.23	2.90±0.27	1.41±0.22	0.017±0.002	0.33±0.06	0.65±0.08
1:2	6.22±0.74	0.23±0.03	1.43±0.29	0.021±0.006	0.42±0.12	0.24±0.06
1:4	2.70±0.19	0.27±0.05	0.72±0.16	0.011±0.003	0.41±0.07	0.30±0.05

*Results are from the previous F/M experiment

On the other hand, when the methane to oxygen ratio was increased to 2:1, the growth rate was $0.37\pm0.005 hr^{-1}$ and the biomass yield was 0.82±0.11mg-VSS/g-CH_4. Increasing the methane to oxygen ratio in the headspace released any competition between methane and ammonium which can be also confirmed by the N/M_o ratio which was 0.12±0.04mg-N/g-VSS. Interestingly, not only did increasing the methane to oxygen ratio increased the growth rate but also the biomass yield was doubled compared to the 1:1 ratio, despite both experiments had almost the same F/M and C/N ratios. Previous studies reported similar behavior for type II methanotrophs and mentioned that increasing the methane to oxygen ratio promotes the growth of type II [101]–[103]. In addition, the f_s increased from 0.34 to 0.67 in the higher methane: oxygen ratio confirming the favorability of the enrichment to this condition.

3.3.5 Effect of maintaining the N/M_i ratio and increasing F/M

To discuss the effect of the initial ratio between ammonium and the enriched culture and confirm its effect based on the results of the previous experiments two different setups were tested having

different O.D but the same ratio with ammonium (N/M_i). As shown in **Figure 3.3**, the cultures with higher initial biomass density had higher methane and oxygen uptake as expected. However, decreasing the initial biomass density while maintaining the same N/M_i ratio resulted in a significant improvement in the growth rate ($p= 0.0630$, $\rho= -0.413$). When the initial biomass density (O.D) decreased from 0.6 to 0.3, the F/M ratio increased from 3.61±0.36 to 4.77±0.44 mg-CH_4/mg-VSS and the C/N ratio rose from 11.42±0.82 to 14.5±1.3 mg-CH_4/mg-N. This increase in both the F/M and C/N ratios resulted in a significant improvement in the growth rate, biomass yield and methane utilization rate. The specific growth rate almost doubled from 0.025±0.007 hr^{-1} to reach 0.05±0.011 hr^{-1} and the observed biomass yield increased from 0.42±0.09 to 0.70±0.15 mg-VSS/mg-CH_4.

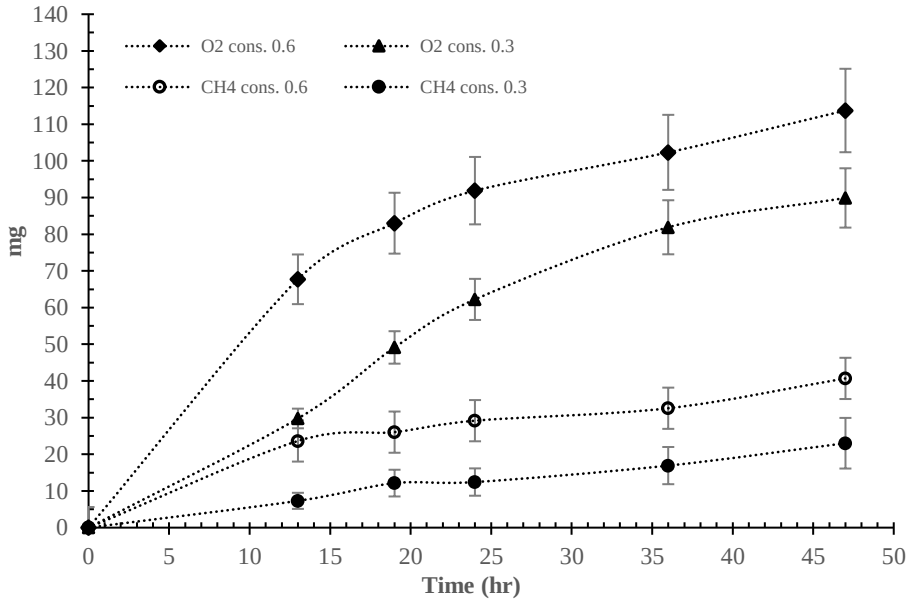

Figure 3.3: The effect of initial biomass density on the methane and oxygen uptake.

In addition, all the experiments during this phase showed that all the ammonium utilized was converted into biomass and the N/Mo consumption ratio was around 0.12 mg-N/mg-VSS which confirms the elimination of any co-oxidation from ammonium as shown in **Table 3.5**.

Table 3.5: Effect of initial biomass density on the growth rate, biomass yield and N/Mo

Initial O.D	**0.6**	**0.3**
N/M_i (g-N/g-VSS)	0.25±0.013	0.26±0.05
C/N (g-CH_4/g-N)	11.42±0.82	14.49±1.3
F/M (g-CH_4/g-VSS)	3.61±0.36	4.77±0.44
Growth rate (hr^{-1})	0.025±0.007	0.050±0.011
Biomass Yield (g-VSS/g-CH_4)	0.42±0.09	0.70±0.15
Observed N/M (g-N/g-VSS)	0.12±0.04	0.12±0.03
f_s	0.34±0.03	0.57±0.04
f_e	0.66±0.03	0.43±0.04

3.3.6 Effect of ammonium concentration in copper free medium

Previous studies showed that the enzyme sharing similarities with AMO is mainly the pMMO and because the previous experiments were running in a copper supplemented medium pMMO was assumed to be the mainly the expressed enzyme [98]. Accordingly, elimination of copper from the growing medium can eliminate the competition between ammonium and methane on sMMO enzyme and decrease the inhibitory effect of ammonium on Type II growth. As expected, elimination of copper from the growing medium decreased the inhibitory effect of ammonium. The growth rate for the enrichment having 5 mM ammonium chloride was 0.066±0.07 hr^{-1} compared to 0.059±0.005 hr^{-1} in the medium having 20 mM. The inhibition of ammonium on the growth rate was approximately 10% when the ammonium concentration increased from 5 to 20 mM compared to an inhibition of 27% when copper was supplemented in the growth medium. Similar trends were also found for the yield, N/M ratio and methane to oxygen consumption ratio. Both enrichments had almost the same observed biomass yield of 0.96±0.1 mg-VSS/mg-CH_4 and the gases consumption ratio increased from 3.14 to 3.59 mg-O_2/mg-CH_4 in the 5 and 20 mM

enrichments respectively. A slight increase in the N/M_o ratio was also observed, as it was 0.11 and 0.14 mg-N/mg-VSS in the 5 and 20 mM cultures. In addition, the electron equivalent fractions f_s and f_e were almost the same for both concentrations at 0.78 and 0.22, respectively. Although copper free medium cultivations showed higher resistance to the ammonium, further studies are needed to explore this resistance at higher ammonium concentrations.

3.3.7 Effect of copper concentration

As shown in **Figure 3.4**, the elevation in the copper concentrations mainly affected the growth rate of type II methanotrophs ($p= 0.005$, $\rho= -0.798$). More precisely, increasing copper concentration affected the activity of the pMMO enzyme and consequently decreased the growth rate. The specific growth rate dropped from 0.072 ± 0.006 hr^{-1} to 0.037 ± 0.004 hr^{-1} when the copper concentration inclined from 10 µmol to 40 µmol. Further increase in copper concentration to 80 µmol had a minor effect on the growth rate which slightly decreased to $0.031\pm0.005 hr^{-1}$. Similar behavior was reported [78], [104] where the sharp decrease in the growth rate and pMMO activity was noticed after increasing copper concentration between 20-40 µmol followed by minor decrease in both of them when the copper concentration was elevated up to 120 µmol. However, the effect of increasing copper concentration was not as inhibitory as increasing the ammonium concentration.

In addition, although the decline in the growth rate accompanied by elevating the copper concentration the methane utilization rate was almost constant at 0.052 ± 0.01 mg-CH_4/g-VSS.hr. The effect of copper concentration on the biomass yield was noticed after 20 µmol with an observed 30% drop at 80 µmol $CuSO_4$/l ($p= 0.036$, $\rho= -0.284$). The results show that increasing the copper concentration had a minor effect on the N/M_o ratio and it was 0.13 ± 0.03 mg-N/mg-VSS indicating that the ammonium co-oxidation was low despite the pMMO activity as long as

the methane concentration was high enough to outcompete the ammonium [98]. In terms of f_s and f_e , the effect of copper concentration was not significant up to 20 µmol. the values for f_s and f_e were 0.8 and 0.2, respectively. Afterwards, due to the decrease in the pMMO activity and the methane oxidation, a drop in the fs to 0.57 was observed at copper concentration of 80 µmol.

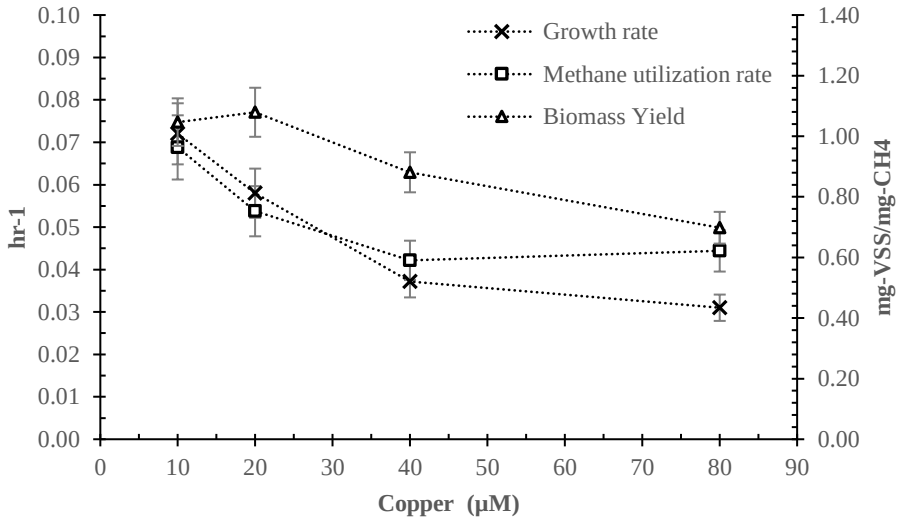

Figure 3.4: Effect of copper concentration on the growth rate, biomass yield and methane utilization rate

It is noteworthy to mention that the results for these experiments in general were better than the ammonium experiments in terms of growth rates, biomass yields and methane utilization rates. In addition, the results approached the ones for pure cultures [104], [105] in terms of specific growth rates, observed yields and methane utilization rates despite they were running under the same conditions of the previous experiments. The better growth conditions can be contributed to the conditions employed throughout the copper experiments that were the favorable conditions for Type II methanotrophs as shown in the previous experiments in terms of F/M, C/N and N/M_i . The values of F/M, C/N and N/M_i used were 4.36±0.22 mg-CH_4/mg-VSS, 13.98±0.88 mg-CH_4/mg-N and 0.30±0.04 mg-N/mg-VSS, respectively.

3.4 Conclusions

The conducted batch studies showed the behavior of Type II methanotrophs enriched from activated sludge while using ammonium as their nitrogen source under different operational conditions and deduced the factors that would increase their growth rates, biomass yields and methane utilization rates. Increasing ammonium concentration had a negative effect on both the growth rate and yields of Type II methanotrophs. The F/M ratio mainly affected the specific growth rate of type II methanotrophs while the effect on the observed biomass yield was lower. Both the C/N and the N/M_i affected the growth rate and biomass yield as well as the competition between methane and ammonium for MMO oxidation. The main effect of copper was obvious on the growth rate and copper elimination decreased the inhibitory effect resulting from ammonium co-oxidation.

Chapter 4

Factors affecting the selection of PHB accumulating methanotrophs from waste activated sludge while utilizing ammonium as their nitrogen source[3]

4.1 Introduction

Bioplastics have attained the focus of many researchers in the last years as a green alternative product with their ability to substitute petroleum based plastics in various applications [7], [106]. A wide range of microorganisms have been explored for their ability to accumulate biopolymers under unbalanced growth conditions. Most of these bacteria showed great potential to produce different types of polyhydroxyalkanoates (PHAs) and more than 150 PHAs with different monomer composition have been identified containing 100-30000 repeated units including but not limited to polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-valerate (PHBV) [81].

Methane has been explored as a cheap feedstock for biopolymers production using methanotrophic bacteria[61]. Hence, Methanotrophs can be considered as an ideal candidate for combining the need for a reliable feedstock for biopolymers production with an efficient sink for the second major greenhouse gas contributing to the global warming phenomena. Under balanced conditions when all the essential nutrients are available, methanotrophs proceed to tricarboxylic acid (TCA) cycle to obtain their energy needs. While under unbalanced conditions when one or more nutrient is not available they switch to the PHB cycle to provide the energy required for the cell maintenance "survival" in this case [57].

[3] This chapter has been published in Journal of chemical technology and biotechnology., vol. 93, no. 5, pp. 1359–1369, 2018

Generally, Aerobic methanotrophs are classified into three main types according to their carbon assimilation pathways; type I utilizing the ribulose monophosphate (RumP) cycle, Type II using the serine cycle and type III undergoing the Calvin-Benson-Bassham (CBB) cycle [20]. PHB accumulation is limited to type II only and all the tested strains from other types were not able to accumulate PHB under nitrogen-limited conditions [47]. Accordingly, pure culture studies considered different type II strains to discuss the factors promoting both their growth conditions and their PHB accumulation capacity [59].

The PHB production process is mainly divided into two main phases; a growth phase for the bacteria where all essential nutrients are available in order to increase the biomass density followed by deficiency conditions to induce PHB accumulation as intracellular inclusions [61]. The effect of many factors on both stages were studied including but not limited to the nitrogen source, copper, iron, phosphorus concentrations and methane to oxygen ratios [62], [107]. These studies provided the effect of these parameters on enzymes activities, growth kinetics, stoichiometry and inhibitory levels for type II methanotrophs during both phases.

However, few studies considered operational parameters as the food to microorganisms ratio (F/M), carbon to nitrogen ratio (C/N), nitrogen (ammonium) to microorganisms ratio (N/M) and solids retention time (SRT) to scale up this process into real life application. In addition, most of the previous studies were running using pure cultures conditions, which is –from an economic point of view- less feasible. Mixed cultures as waste activated sludge (WAS) can have the advantage of sterilization requirements elimination, promoted growth conditions through the cooperation of different types of bacteria in adjusting the levels of intermediate toxic metabolites. In addition, WAS can be considered as a readily available source for bacteria in any existing

wastewater treatment facility which collectively can lead to an effective natural selection of cultures with high PHB accumulation capacity [108].

However, maintaining a stable community of mixed culture enrichments dominated by type II methanotrophs is challenging for long-term operation without type I invasion. Due to their higher growth rates and methane affinity, type I tends to invade in mixed cultures running under normal growth conditions [63]. Selection strategies based on copper, pH, methane and dilution were not reliable on long-term basis [47]. Moreover, the only reliable selection parameter i.e. nitrogen source relies on halting the growth type I but at the expense of type II growth as well [16].

Type II methanotrophs can utilize different nitrogen sources to support their cellular requirements. While nitrate resulted in the highest growth rate during pure culture studies [59], nitrate results in the dominance of type I in mixed culture enrichments [74]. Type II are known for their unique ability to fix nitrogen gas to support growth which accordingly can be used for type II selection [74]. However, growth on nitrogen gas results in the slowest growth rates. Although ammonium can be utilized by methanotrophs for cellular requirements, ammonium can inhibit their growth by competing with methane to be oxidized by the methane monooxygenase (MMO) enzyme or by formation of toxic hydroxylamine or nitrite [49]. Type II methanotrophs show higher resistance to the inhibitory effect caused by ammonium and dominates in mixed cultures supplemented with high ammonium concentration.

Although an efficient method for type II selection from activated sludge using the inhibitory effect of ammonium and most importantly through the continuous recycling of part of the enriched type II culture was demonstrated [16]. Results for growth rates and PHB accumulation were lower than pure cultures due to the elevated ammonium concentration used to washout type I.

Accordingly, the main objective of this study is to determine the effect of the amount of biomass recycled and the SRT on attaining a mixed culture dominated by type II methanotrophs enriched from WAS. Moreover, this study aims to determine the influence of the F/M ratio and the N/M ratio on type II dominance in the enriched culture. In addition, this study targets the determination influence of copper elimination and switching the nitrogen source to nitrate on the behavior of the enriched culture and type II dominance. Finally, to determine the PHB accumulation capacity for the enriched culture under the aforementioned conditions to facilitate the scale up of the PHB production process at wastewater treatment facilities.

4.2 Materials and methods

4.2.1 Cultivation conditions

All enrichments were cultivated in the mineral salts medium (MSM) [90] and had the following concentrations of chemicals (mg/l):1000 $MgSO_4.7H_2O$, 200 $CaCl_2.H20$, 272 KH_2PO_4, 610 K_2HPO_4, 4 Fe-EDTA and 1 ml Trace metal solution. The trace metal solution had the following concentrations of chemicals (mg/l): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.4H_2O$, 30 H_3BO_3, 3 $Na_2MoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$ and 20 $CoCl_2.6H_2O$. Ammonium chloride (NH_4Cl) was added to the MSM medium with a concentration of 5mM. Copper was added from a stock solution having a concentration of 500 mg $CuSO_4.5H_2O$/l to give a 10µm final copper concentration.

Unless otherwise specified, all the experiments for studying the effect of different parameters on the growth of type II methanotrophs were running in a fed-batch mode and utilized methane and ammonium as their sole carbon and nitrogen sources respectively. All enrichments were incubated in 250 ml serum bottles capped with butyl rubber stoppers. The liquid volume was 50 ml and the headspace was filled with 200 ml of methane and oxygen (>99.9% purity, Praxair) having

volumetric ratio 1:1. All the experiments were running at a temperature of 25°C using table orbital shakers at a speed of 160 rpm and pH was kept between 6-7 using 10% NaOH prepared solution. Samples were taken from the headspace periodically to monitor the gases consumption. The growth and ammonium consumption were measured at the beginning and the end of the experiment by measuring the optical density (O.D) and ammonium concentration as mg-N/l in the culturing medium.

4.2.2 Fed batch cycles operation description

Activated sludge was collected from Humber wastewater treatment plant located in Toronto, Canada and used as a seed for methanotrophs cultivation. The main characteristics of the collected WAS are shown in **Table A.2** in the Appendix section. The activated sludge was filtered through a 100 µm cell strainer. After centrifuging, the filtered sludge was added to 50 ml of the MSM having an initial ammonium and copper concentrations of 5mM and 10µmol, respectively. Air was removed from the headspace using a vacuum pump and replaced with 100ml of O_2 and 100ml of CH_4 to give a volumetric ratio of 1:1 every 24 hours. After 48 hours of cultivation, part of the biomass was centrifuged (4000x) for 20 min then the collected biomass was used as a seed for the proceeding cycle under the mentioned conditions while the other part was resuspended in ammonium free medium to induce PHB accumulation as shown in **Figure 4.1**.

Three sets were employed during the experiments and each of them was running in triplicate and in a cyclic mode. At the beginning of each cycle, a portion of the enriched culture from the previous cycle was used as a seed. The main difference between the experiments was the control of the biomass returned from each cycle and accordingly different initial O.D in each set. During the first set, half of the enriched biomass during the growth phase was used for the proceeding cycle regardless the final biomass density while the initial O.D was adjusted to 0.69±0.07 and 0.34±0.05

at the beginning of each cycle for Set-2 and Set-3, respectively. This difference resulted in the change of various parameters through the three sets including the F/M ratio, N/M ratio and the SRT while the C/N ratio was slightly affected.

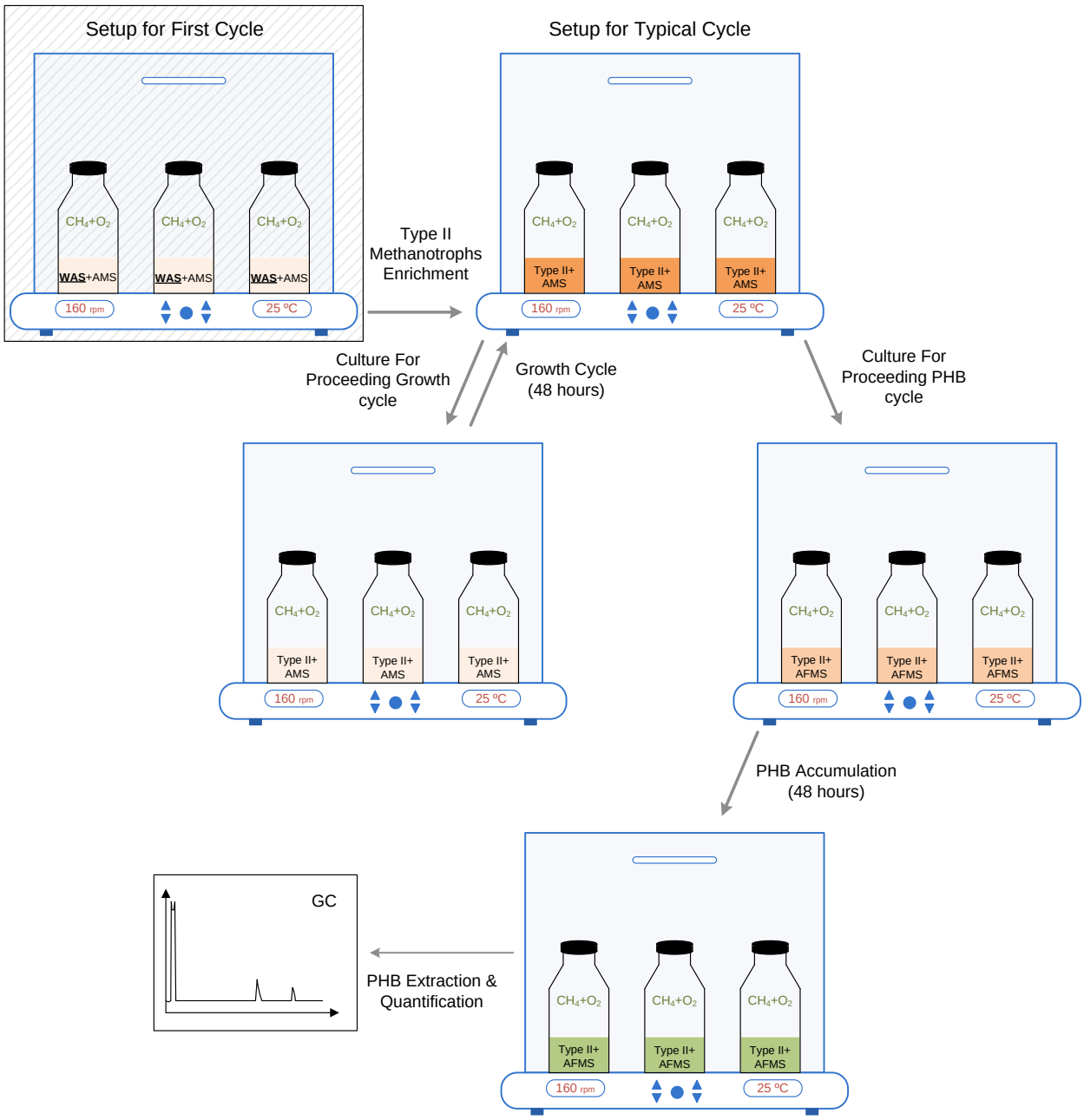

Figure 4.1: Schematic diagram for the cycles operation in different experiments

4.2.3 Effect of copper elimination and switching nitrogen source to nitrate

To discuss the effect of copper elimination on the growth conditions of the enriched culture, three 250-ml serum bottles were running using the same conditions applied in Set-3 experiments while only the copper was eliminated from the liquid medium during both the growth phase and the PHB accumulation phase.

Another triplicate was initiated using the same conditions while changing the nitrogen source from ammonium chloride with 5 mM concentration to sodium nitrate with a concentration of 20 mM. The copper concentration was kept similar to the previous experiments with a final concentration of 10 µmol. During both experiments, gases consumption was monitored as well as the optical density and the nitrogen consumption.

4.2.4 Molecular biology analysis

Five samples were taken from the enrichments during the operation of different sets to monitor the existence of either type I or type II or both in the enriched culture under different operational conditions. The First sample was taken during the end of Set-1 operation while the samples 2 and 3 were taken at the beginning and the end of Set-2. Finally, the last two samples were taken during the third and last cycle when the nitrogen source was replaced by nitrate. RNA was extracted, amplified using PCR then the products were subjected to gel electrophoresis to confirm the existence of type I and type II methanotrophs and the intensity of each band can relate to the genomic density of the target gene in the original sample. Two Specific primer sets targeting the 16s rRNA for both types [109] were used and detailed description for the primers sequence, the target genus and the procedures followed during the molecular biology analysis are described in section **A.3** of the Appendix.

4.2.5 Analytical methods

Samples withdrawn using gas tight syringe from the bottles headspace were injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD) and a molecular sieve column (Restek, Bellefonte, PA.) to monitor methane, oxygen and nitrogen concentrations. The temperature program was as following: injector, 60^{o}C; Oven, 80^{o}C; TCD, 80^{o}C and helium gas was used as carrier gas with a flowrate of 15 ml/min. External calibration curves were constructed using a known mixture of gases and used to convert the measured peak areas into gases concentrations and masses. For bacterial growth monitoring, O.D was measured using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). A correlation curve between O.D and VSS was developed where VSS was measured according to standard methods. Liquid samples were collected from the supernatant after the cultures centrifuge (4200x) for 20 min. Then, HACH methods and testing kits were used to measure inorganic nitrogen. The kinetic and stoichiometric parameters including specific growth rate, biomass yields, methane utilization rates were calculated according to the equation presented in the appendix and the electron partitioning fractions for cell synthesis (f_s) and energy (f_e) were calculated according to the model developed for methanotrophs utilizing methane and ammonium as their carbon and nitrogen sources, respectively [59].

PHB was extracted from cellular biomass and quantified with gas chromatography using the following procedure [110]. Firstly, 10-15 mg of lyophilized biomass were collected and 2 ml of acidified methanol (3% sulphuric acid) and 2 ml of chloroform were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ^{o}C for 3.5 hours then left to cool down to room temperature. Afterwards, 1 ml of deionized water was added and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for

PHB quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The temperature program was as following; 1 min 80ºC, 10ºC/min, 180ºC for 4 min. Results were compared to standard curves obtained using PHB standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy.

4.3 Results and discussions

4.3.1 Type II methanotrophs enrichment from activated sludge

Filtered activated sludge was used as a seed for methanotrophs cultivation and after approximately three transfers in fresh medium turbidity was developed and the harvested biomass was used as inoculum for upcoming experiments. Previous studies were mainly focused on determining the effect of different ammonium concentrations as a nitrogen source on the growth of methanotrophs and type II selection and reported the necessity of elevated ammonium concentrations to outcompete type I [100]. Although this higher concentration successfully selected type II but at the expense of their growth rate and PHB accumulation capacity. Accordingly, the ultimate goal of these experiments was to apply lower ammonium concentration and manipulating other operational factors rather than the ammonium concentration as the F/M ratio, N/M ratio and the SRT for type II selection without impacting their growth rates and PHB production.

The first attempt was to study the effect of the SRT on the enriched type of methanotrophs while using an ammonium concentration of 5 mM. In the cycles mode of operation employed in these experiments, the SRT is mainly controlled by the amount of biomass returned from each cycle to the proceeding one. During the first set of experiments, this control was achieved by returning 50% of the enriched culture, which corresponds to 25 ml of the liquid culture regardless, the final biomass density achieved, which resulted in a SRT of 4 days.

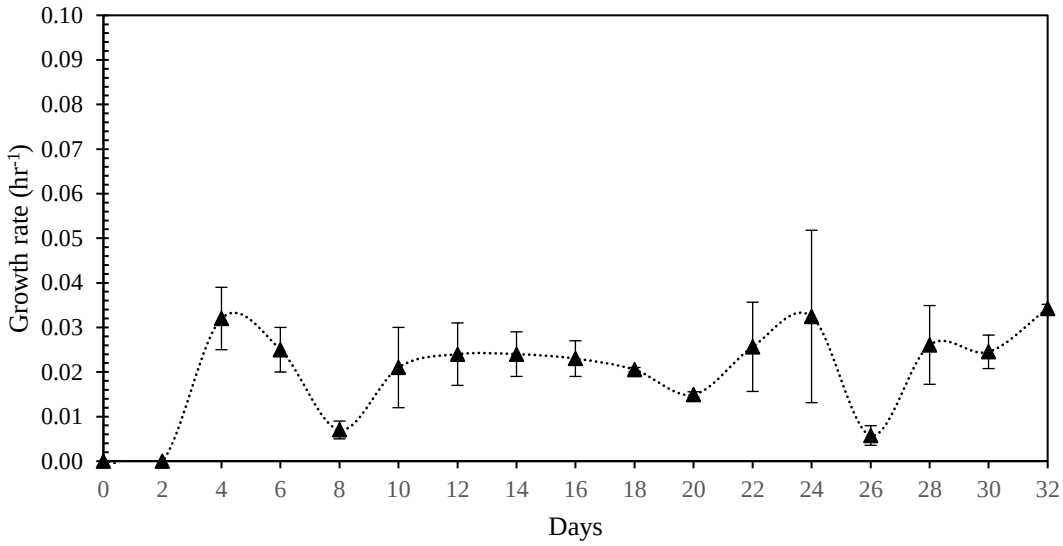

Figure 4.2: Specific growth rate for methanotrophic enrichments during each cycle of operation of Set-1

The results for Set-1 showed a slow specific growth rate as illustrated in **Figure 4.2**. After almost one month of operation, the average specific growth rate for the enrichments was 0.021±0.02 hr^{-1}, which is lower than previous studies reported for methanotrophs (0.04-0.11hr^{-1}) [16], [59]. Moreover, the enrichments pigmentation showed the dominance of the pink color known for type I [90]. Additionally, an extreme fluctuation in other growth parameters as the biomass yield and methane utilization rate was also observed (data not shown). All the tested cultures were not capable of producing PHB under nitrogen limited conditions due to the failure of Type II dominance which was also confirmed by the microbial analysis shown in **Figure 4.3** where type I methanotrophs outcompeted type II in the enriched culture.

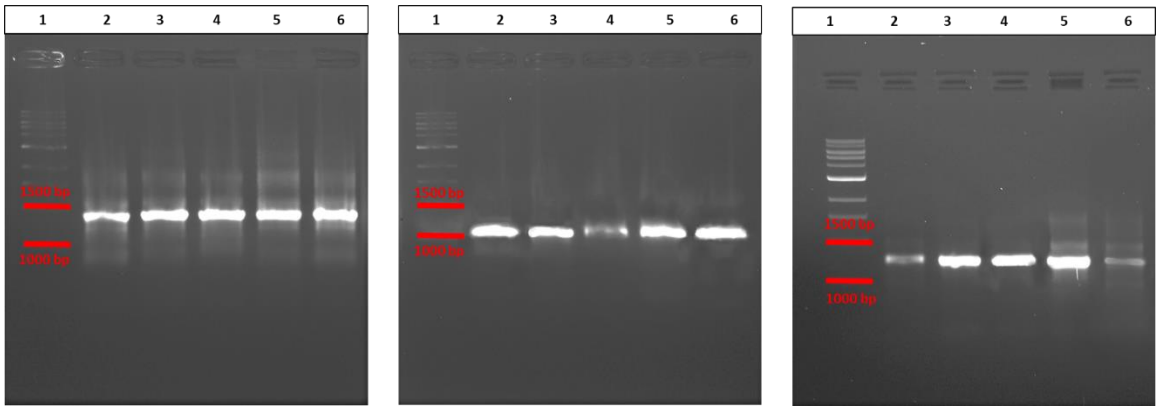

Figure 4.3: Amplified PCR products from the sample with the three sets of primers on 1 % TAE agrose gel a) Universal primer, b) Type I primer, c) Type II primer. In each figure, Lane 1 includes the DNA ladder (1 kb). PCR products for Set-1 were injected in Lane 2 while Lanes 3-4 contained the PCR products from the beginning (cycle 4) and end (cycle 20) of Set-2 operation respectively. The products from the third and last cycles of the nitrate experiments were added to Lanes 5-6 respectively

These results can be referred to two important factors. Firstly, the lower ammonium concentration which was below the inhibitory concentration for both types [99]. Secondly, to the relatively long SRT used for this set of experiment which allowed the adaptation of type I to the ammonium or in other words even if this ammonium concentration was inhibitory for type I they had enough time to utilize it after other existing methanotrophs have already consumed part of it. Afterwards, owing to their higher growth rates they would outcompete type II and dominated the enriched culture.

These findings also showed the need for another methodology that would increase the inhibition caused by ammonium on type I while promoting the growth of type II. This inhibition can be induced by considering the initial ratio between the biomass concentration and the ammonium concentration where the amount of biomass returned can be controlled by fixing it to a constant value instead of returning it as a percentage. Accordingly, Set-2 experiment was initiated by maintaining an initial O.D of 0.69±0.07 at the beginning of each cycle. This decrease in the initial O.D resulted in an increase in the initial N/M ratio, the F/M ratio and a decrease in the SRT. At the early cycles, the existence of type II in the enriched community was observed and an inhibitory

effect resulting from ammonium was noticed as shown in **Figure 4.4**. This inhibitory effect was monitored by calculating the ratio between the biomass generated and the ammonium consumed which should be theoretically around 0.12 mg-N/mg-VSS considering the empirical formula of the bacteria is $C_5H_7O_2N$ and any increase in this ratio can be referred the co-oxidation resulting from ammonium. After few cycles of operation, this inhibitory effect disappeared and a stable growth for an enrichment dominated by type II methanotrophs was achieved with the regression of Type I in the culture as shown in **Figure 4.3**.

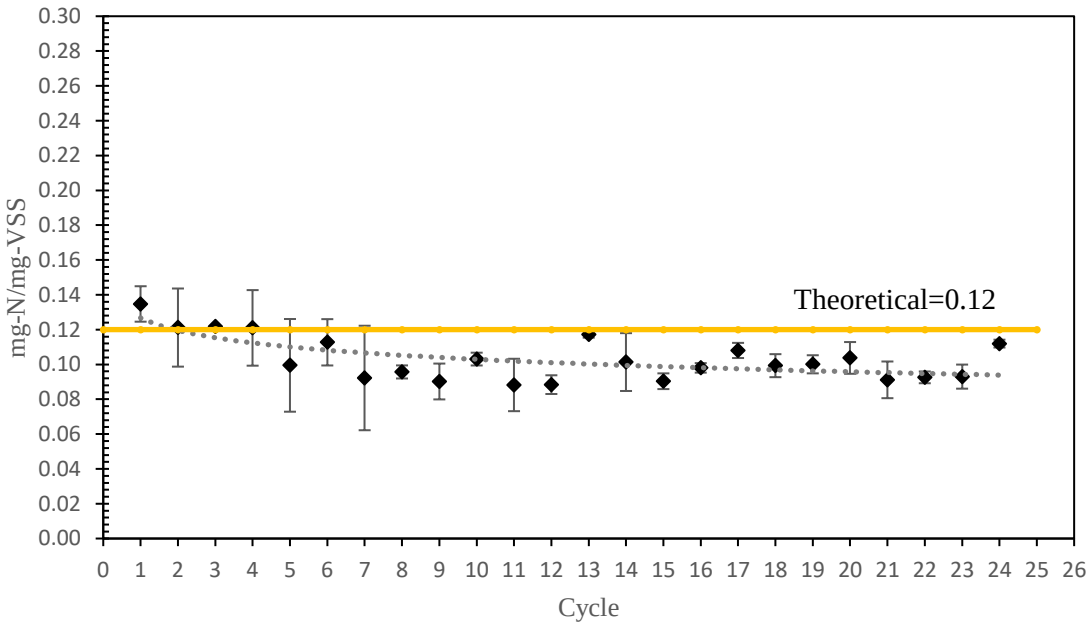

Figure 4.4: The observed ratio between the consumed ammonium and the generated biomass for Set-2 cycle

The target for conventional organics removal bioreactors is to maintain a maximum waste removal with minimum solids production and lower biomass yields. However, a methanotrophic bioreactor should be operated from a different perspective where achieving the maximum solids (biomass) production is the ultimate goal. Accordingly, this bioreactor should be operated to give higher growth rate, biomass yield, final biomass density and capable of accumulating biopolymers.

Generally, a steady-state performance was observed during 25 consecutive cycles of operation for all growth kinetics when the SRT was 2.8±0.1 days. The average specific growth rate for Set-2 experiments was 0.05±0.01 hr^{-1} while the biomass yield and the methane utilization rate were 0.67±0.09 mg-VSS/mg-CH_4 and 0.08±0.004 mg-CH_4/mg-VSS.hr, respectively with a final biomass density of 1095±88 mg-VSS/l as shown in **Figure 4.5**.

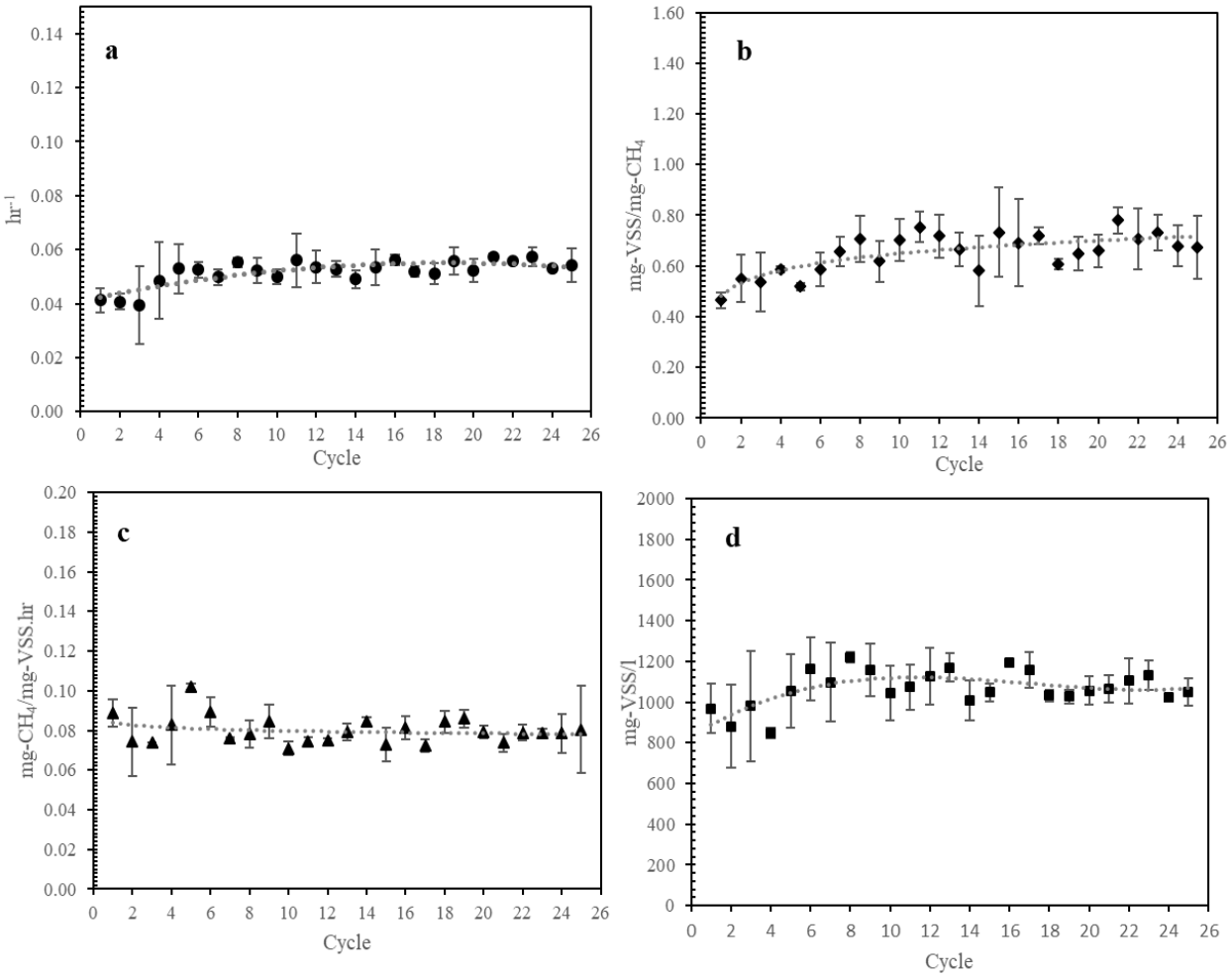

Figure 4.5: Observed a) specific growth rate, b) biomass yield, c) methane utilization rate and d) final biomass density for each cycle during the operation of Set-2

The consumption ratio between oxygen and methane can be used to calculate the electron fractions equivalents for energy (f_e) and synthesis (f_s) [59]. This ratio is equal to $1+f_e$ with a maximum theoretical value of 2 when $f_e=1$ and a minimum value of 1 when $f_s=1$. To maintain the

aforementioned state of high specific growth rate and biomass yield, it is crucial to have an enriched culture directing the consumed methane towards cell synthesis i.e. highest possible f_s. The results show that the average methane consumption rate was 1.097±0.07 mg-CH_4/hr with an average consumption ratio between oxygen and methane of 2.9±0.17 mg-O_2/ mg-CH_4 resulting in f_e and f_s of 0.55 and 0.45, respectively as illustrated in **Figure 4.6.** Although the results from Set-2 showed a successful selection for type II methanotrophs, the achieved growth conditions were still lower than previous pure culture studies. In addition, these results showed that the ammonium inhibition-based selection could be achieved by adjusting the N/M ratio instead of the elevated ammonium concentration. The impact of the SRT was also obvious where type II selection was significantly improved when the SRT was lowered from 4 days during the operation of Set-1 to 2.8±0.2 days in Set-2.

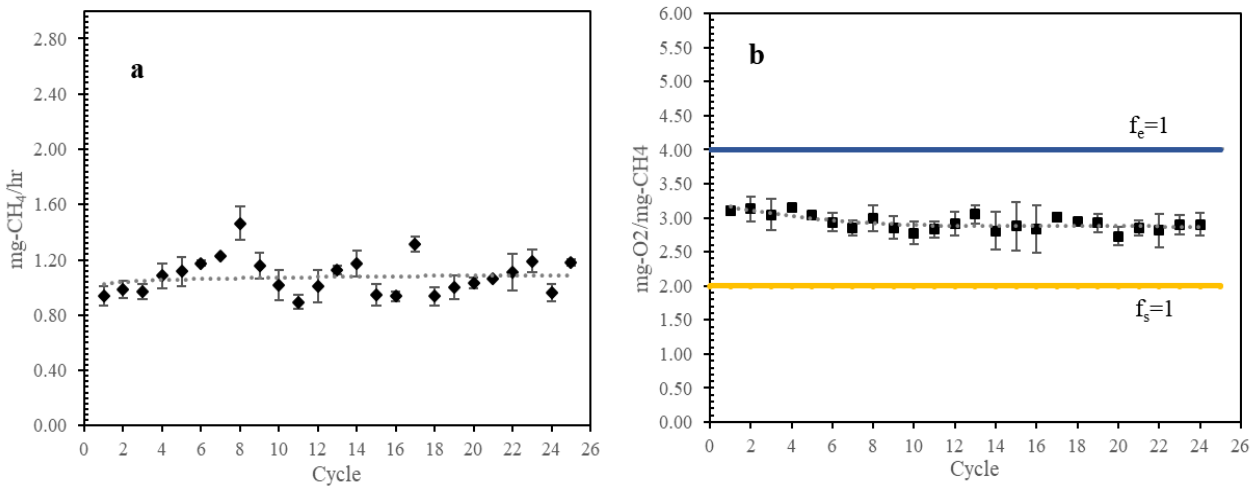

Figure 4.6: a) Methane consumption rate and b) oxygen to methane consumption ratio for Set-2 cycles

During the operation of Set-2, while all parameters were significantly changed the F/M ratio was the only parameter that had a minor difference between Set-1. To assess the effect of the F/M ratio, Set-3 was initiated by furtherly decreasing the amount of returned amount of biomass and adjusting

the initial O.D of each cycle to 0.34±0.04. Additionally, this set aimed to confirm the ability to initiate a selective ammonium inhibition for type I using the N/M ratio and the SRT. As shown in **Figure 4.7**, although the ammonium had a greater inhibitory effect in the first few cycles, type II selection was achieved afterwards, and the enriched culture adapted to the new growth conditions efficiently. Moreover, due to the higher F/M ratio Set-3 was running, a general improvement in the growth conditions was achieved compared to Set-2.

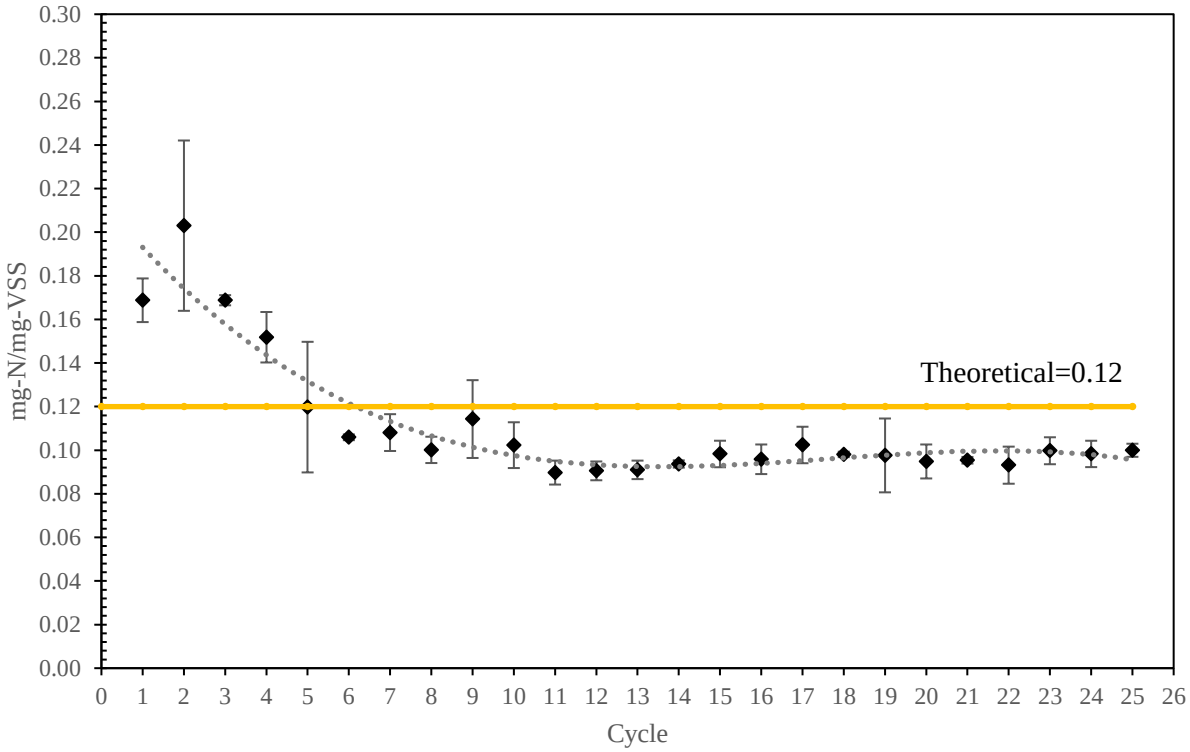

Figure 4.7: The observed ratio between the consumed ammonium and the generated biomass for Set-3 cycles

During 25 cycles of operation, the enriched type II methanotrophs had a stable behavior and the average specific growth rate was 0.077±0.005 hr^{-1}. The biomass yield and methane utilization rates were 0.81±0.08 mg-VSS/mg-CH_4 and 0.097±0.011 mg-CH_4/mg-VSS.hr, respectively, and the

final biomass density reached 943±70 mg-VSS/l as shown in **Figure 4.8**, which is comparable to previous pure culture studies.

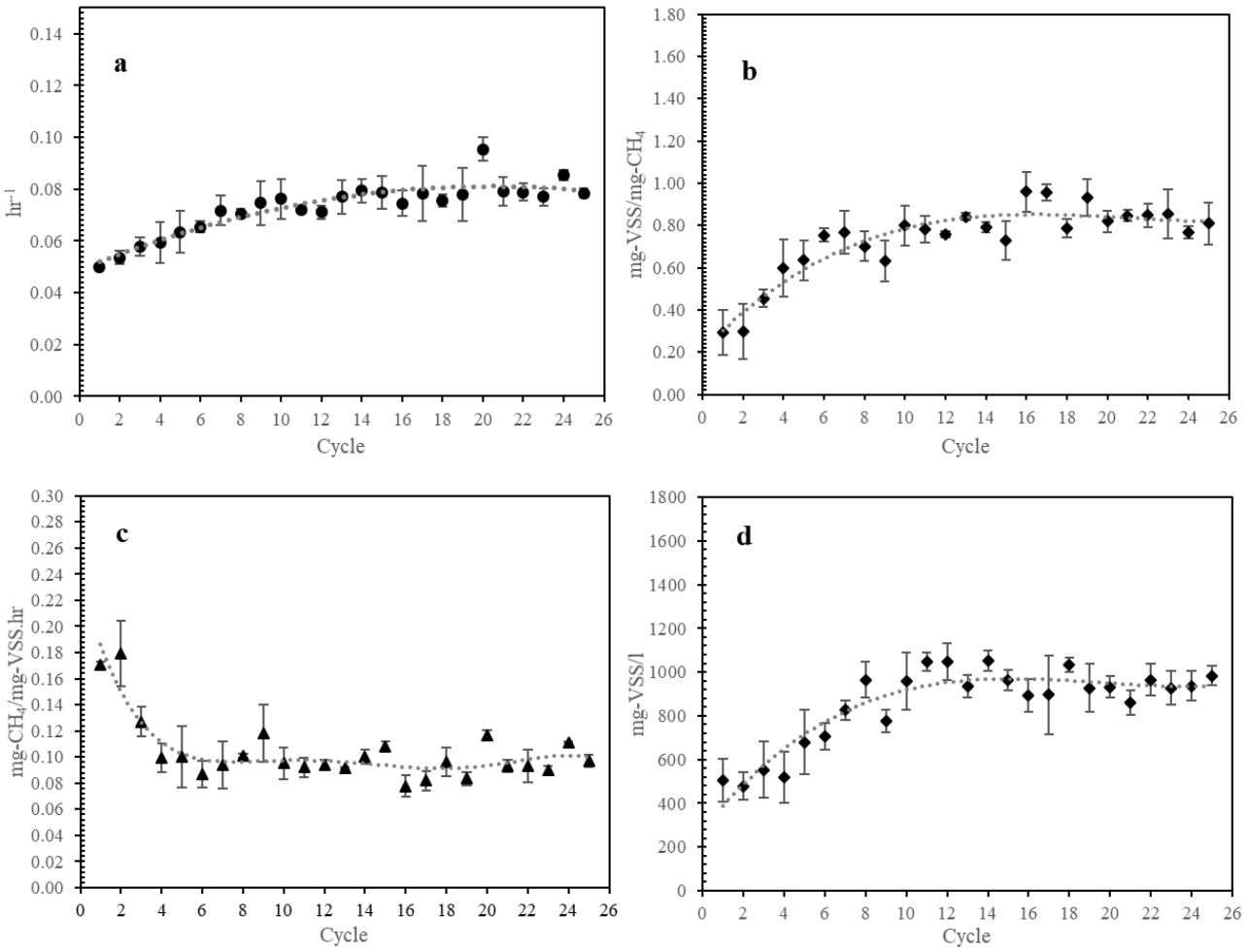

Figure 4.8: Observed a) specific growth rate, b) biomass yield, c) methane utilization rate and d) final biomass density for each cycle during the operation of Set-3

Proceeding to the gases consumption results, despite the lower biomass densities employed during Set-3 operation, the methane consumption rate was almost similar to Set-2 with an average value of 1.02 ± 0.07 mg-CH_4/hr. However, a significant reduction in the consumption ratio between oxygen and methane was noticed reaching 2.68 ± 0.13 compared to 2.9±0.17 mg-O_2/ mg-CH_4 for Set-2 which resulted in f_s of 0.66 and f_e of 0.34 as shown in **Figure 4.9**. Finally, lowering the initial

O.D during the operation of Set-3 resulted in an SRT of 2.5±0.2 days confirming the influence of this parameter on the selection of type II methanotrophs.

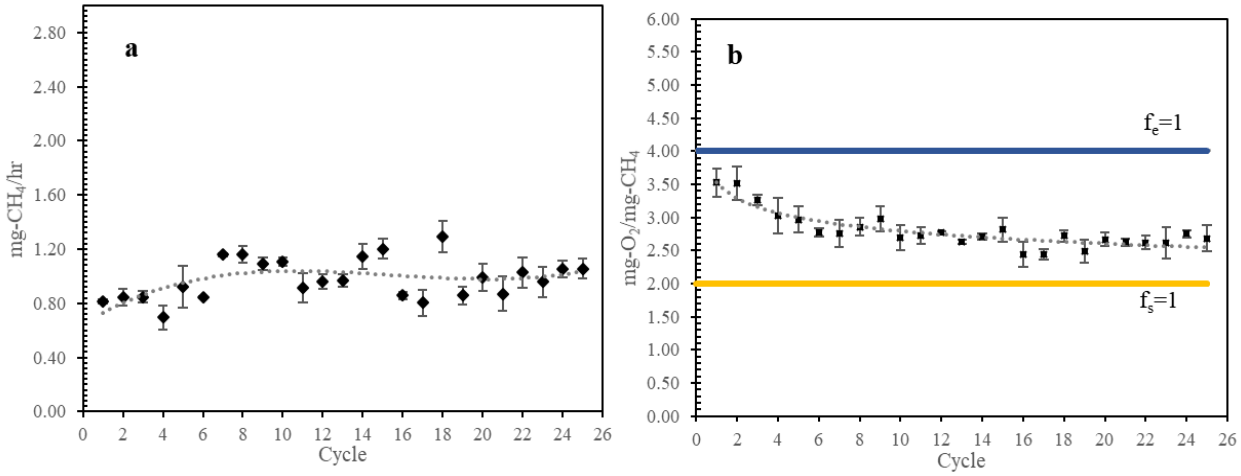

Figure 4.9: a) Methane consumption rate and b) oxygen to methane consumption ratio during the operation of Set-3 cycles

One main advantage of using the N/M ratio as a selection parameter during these experiments for type II selection is the increased F/M ratio generated. This increased F/M ratio allowed the release any competition between ammonium and methane for MMO oxidation and subsequently improved growth conditions[99]. The regression of type I observed in Set-2 and Set-3 can be referred to two main reasons; firstly, lowering the SRT during these two sets disallowed type I to adapt to their unfavorable nitrogen source which is ammonium. Secondly, the increase in the N/M ratio resulted in an inhibitory effect on type I without affecting type II.

4.3.2 PHB production by Type II methanotrophs enriched from activated sludge under different operational conditions

As previously mentioned, PHB production depends on successful dominance of type II in the enriched culture. Accordingly, no PHB accumulation was observed under nitrogen limited conditions for the biomass enriched in Set-1 due to the failure of type II selection. On the other

hand, Both Set-2 and Set-3 enriched biomass was capable of accumulating significant amounts of PHB. For both Sets, the PHB productivity was increasing while proceeding in cycles and can be classified into three phases according to the significant increase in PHB accumulation; Phase I (cycle 1-7), Phase II (cycles 8-12) and Phase III (cycles 13-25) as illustrated in **Figure 4.10**.

During Phase I in both sets a significant decrease in the biomass density was observed at the end of each cycle indicating the existence of non-producing PHB microorganisms in the enrichment culture. However, this observation was disappearing in the second phase and disappeared in Phase III for both sets as shown in **Figure 4.10**. This observation also confirms the efficiency of the methods applied for type II selection in the growth phase, which was reflected on the PHB accumulation phase.

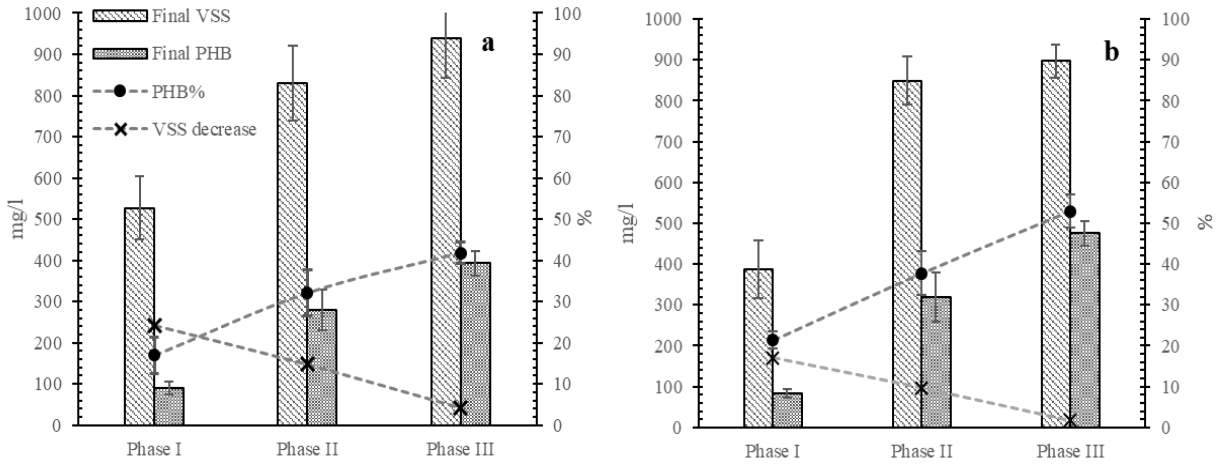

Figure 3: Change in the PHB accumulation capacity observed for a) Set-2 and b) Set-3 over cycles

One other important finding in the PHB results for both sets was the influence of the growth conditions on the PHB accumulation capacity of the enriched culture. Cultures with higher growth rates in the growth phase were capable of producing more PHB under deficiency conditions. The biomass enriched from Set-2 had an average PHB accumulation of 41.82±2.6 % while the PHB

produced by type II enriched from Set-3 reached a 52.9±4% during Phase III which is to the best of our knowledge one of the highest PHB accumulation reported for methanotrophs enriched from an activated sludge process.

Considering the methane and oxygen consumption ratio during the PHB accumulation phase, this ratio was 1.58±0.09 and 1.55±0.1 mg-O_2/mg-CH_4 for Set-2 and Set-3, respectively. The PHB yields for Set-2 was 0.49±0.1 mg-PHB/mg-CH_4 while this value was 0.54±0.12 mg-PHB/mg-CH_4 for Set-3 which confirms also the effect of the previous growth phase on the PHB accumulation phase.

4.3.3 Cultivation in copper free medium and switching the nitrogen source to nitrate

One of the most influential parameters that was widely discussed in previous studies is the effect of copper existence in the culturing medium on the growth of type II methanotrophs [104]. Results from previous studies showed that this effect is strain specific and the effect on mixed cultures was rarely discussed. Copper regulates the expression of the MMO and accordingly the step of methane to methanol oxidation [36]. The soluble form of the MMO enzyme (sMMO) is activated in copper free medium while the particulate form (pMMO) requires copper to function. Although growth on pMMO shows better results for methanotrophs in general, sMMO is mainly utilized by type II only. However, recent studies revealed the ability of some type I strains to grow under copper free conditions [111]. Accordingly, Copper was eliminated from the growing medium to study its effect on the dominance, growth rate and the PHB accumulation capacity of the previously enriched type II methanotrophs with similar conditions applied during Set-3 experiments.

Table 4.1 shows a comparison between all the conditions applied towards achieving a stable type II enrichment with high PHB accumulation production throughout this study. The results show

that the enrichment grown in copper free medium was still dominated by type II methanotrophs. However, a significant drop in the growth rate and biomass yield was observed. In comparison with Set-3, the average growth rate dropped from 0.077±0.005 hr^{-1} to 0.049±0.014 hr^{-1} while the biomass yield was 0.49±0.16 mg-VSS/mg-CH_4 compared to 0.81±0.08 mg-VSS/mg-CH_4 in Set-3 as shown in **Table 4.1**. Moreover, the PHB accumulation was affected with the new applied conditions and declined to 23.3±3 %. It is noteworthy to mention that the copper elimination experiments were employed for only five consecutive cycles using the culture enriched during Set-3 experiments. Therefore, deeper investigations are required to study this effect on type II enrichment from waste activated sludge directly for longer periods of operation.

Table 4.1: Operational parameters applied, and average results observed throughout different experiments conducted

Parameter	**Set-1**	**Set-2**	**Set-3**	**Cu-0**	**Nitrate**
NH_4 concentration (mM)	5	5	5	5	-
C/N (mg-CH_4/mg-N)	10.05±1.059	9.71±0.78	10.06±0.79	11.53±0.2	10.19±0.4
F/M (mg-CH_4/mg-VSS)	2.01±0.22	2.54±0.51	5.40±1.15	5.79±0.64	5.314±1.37
N/M (mg-N/mg-VSS)	0.13±0.01	0.26±0.02	0.54±0.05	0.52±0.03	-
Biomass control	50% return	$O.D_i$	$O.D_i$	$O.D_i$	$O.D_i$
$O.D_i$	N/A[a]	0.69±0.07	0.34±0.05	0.35±0.04	0.32±0.08
SRT (days)	4	2.8±0.1	2.5±0.2	3.05±0.2	2.53±0.3
Type II selection	no	yes	yes	yes	no
Growth rate (hr^{-1})	N/A[a]	0.053±0.01	0.077±0.005	0.049±0.014	0.166±0.06
Biomass yield (mg-VSS/mg-CH_4)	N/A[a]	0.67±0.06	0.81±0.06	0.44±0.11	0.57±0.16
O/M (molar ratio)	N/A[a]	1.45±0.08	1.34±0.06	1.6±0.15	1.54±0.14
PHB %	N/A[a]	41.8±2.6	52.9±4	23.3±3	ND[b]
PHB yield (mg-PHB/mg-CH_4)	N/A[a]	0.49±0.1	0.54±0.12	0.44±0.11	ND[b]

(a) Could not be calculated due to instable behavior of the bacterial culture.
(b) Below detection limit.

Another factor was studied during these experiments which was the effect of nitrate as a nitrogen source. Previous studies revealed that the highest growth rates that were achieved by type II methanotrophs by utilizing nitrate as their nitrogen source [59]. During these experiments nitrate was employed in an attempt to maximize the growth rate of the enriched type II methanotrophs and accordingly the final biomass density and the amount of PHB accumulated when subjected to limitation conditions. As previously mentioned, nitrate has no selection capacity on type II over type I methanotrophs [74]. Consequently, due to their higher growth rates, introducing nitrate in the growing medium released the inhibition caused by ammonium on type I during previous sets and resulted in their dominance again in the enriched culture. The growth rate increased to $0.166\pm0.06\ hr^{-1}$ and the biomass yield had an average of 0.57 ± 0.16 mg-VSS/mg-CH_4 as shown in **Table 4.1**, a noticeable increase in the methane uptake rate was observed reaching an average value of 1.51 ± 0.22 mg-CH_4/hr and an oxygen to methane consumption ratio of 3.08 ± 0.28 mg-O_2/mg-CH_4. Moreover, the pigmentation of the microbial community shifted to the pink color known for type I methanotrophs starting from the first cycle of employing nitrate with the existence of both types in the culture. However, as shown in **Figure 3.3**, using nitrate in the following cycles led to the loss of type II methanotrophs. Additionally, a severe drop in the PHB production was observed with only $9.2\pm1\%$ of PHB accumulated during the first two cycles of nitrate application followed by a complete stop in the PHB accumulation for another three cycles before the experiment was stopped. Despite type I invasion, these observations confirm the effectiveness of the strategy employed during the operation of both sets 2 and 3 in halting the growth of type I while maintaining enrichments dominated by type II and capable of accumulating elevated amount of PHB.

4.4 Conclusions

The conducted fed batch experiments showed the feasibility of enriching type II methanotrophs with high PHB production from waste activated sludge. The solids retention time seems to play an important role in type II selection as well as the fitness of the enriched culture. Achieving a stable community dominated by type II methanotrophs can be reached by increasing N/M instead of elevating the ammonium concentration. Increasing the F/M ratio by controlling the biomass return throughout the experiments mitigated any inhibition that can be caused by ammonium. Moreover, it resulted in improved growth conditions in terms of growth rates, biomass yields and substrate utilization rates. The PHB production phase is directly influenced by the growth phase and cultures with better growth conditions (Set-3) resulted in higher PHB production by almost 30%. Copper elimination did not have a significant effect on type II dominance; however, it had a negative effect on the growth of the enriched type II community as well as their PHB accumulation capability. Although the experiments were running for almost two months and a stable enrichment dominated by type II was achieved, switching to nitrate caused the invasion of type I and deteriorated the PHB production of the enrichment.

Chapter 5

Development of methane-utilizing mixed cultures for polyhydroxyalkanoates (PHAs) production from anaerobic digestion sludge[4]

5.1 Introduction

Recently, the vision for wastewater treatment plants as facilities for pollutants removal has changed. The water resources recovery facility concept (WRRF) is emerging and different applications for the biological processes involved in the wastewater treatment have been employed for energy and value-added resources recovery [112]. Anaerobic digestion (AD) of sludge is not only an essential component in any modern wastewater treatment facility for sludge stabilization, reduction of organics and pathogens but also a key component to intensify resources recovery from waste streams [113]. The AD sludge has been successfully employed for various biotechnological applications such as production of single cell proteins, biodiesel, dietary supplements and biopolymers [114]. Furthermore, AD results in the formation of methane or hydrogen which can both be considered as a valuable source for energy generation [113], [115].

Biopolymers production is one of the promising applications for the biogas generated from the anaerobic digestion process (AD) through targeting mixed cultures dominated by bacterial species with high polyhydroxyalkanoates (PHAs) accumulation capacity when subjected to feast-famine or nutrient limiting conditions [116], [117]. Containing up to 70% methane, biogas can be utilized by a special type of bacteria i.e. methanotrophs and converted into polyhydroxybutyrate (PHB) under nutrient limited conditions [38]. PHB can be then extracted from the bacterial cells and processed to be an ideal substitute for conventional resources-consuming polymers in various

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applications [118]. Moreover, these products can be bio-degraded at their end-life [119]. It was estimated that the feedstock contribution towards the cost of biopolymers production can be reduced from 50% to 20% if methane is employed for biopolymers production [120] which overcome one of the major dragging forces for the sustainability of the biopolymers production.

Methanotrophs can be classified into different types based on their terminal electron acceptor; aerobic and anaerobic methanotrophs or according to their carbon assimilation pathways; ribulose monophosphate (RuMP), Serine or Calvin Benson Bassham (CBB) pathways [20]. Previous studies revealed the capability of only aerobic methanotrophs undergoing the serine pathway for PHB accumulation which are named type II methanotrophs [47]. However, the main challenge to employ methanotrophs for PHB production in mixed cultures is preventing the invasion of other non-PHB producing methanotrophs, mainly type I, which tends to outcompete type II in mixed cultures owing to their higher growth rates and methane affinity [121]–[123].

The nitrogen driven selection for type II methanotrophs was the most successful approach on a long-term basis [16], [48]. Using nitrogen gas or ammonium as nitrogen sources provides type II methanotrophs with the required advantage to dominate in mixed cultures. Despite the existence of the nitrogen fixation genes in most of type II species ,unlike type I, growth of type II methanotrophs on nitrogen gas results in the slowest growth rate compared to other nitrogen sources [59] due to the oxygen sensitivity of enzymes involved in this process [124]. Moreover, using nitrogen gas introduces a third gaseous element to the cultivation process at the expense of the energy input requirements. On the other hand, ammonium selection depends on the higher ability of type II to handle the inhibition resulting from the ammonium co-oxidation by the methane monooxygenase enzyme (MMO) [64] which supports them to outcompete type I in mixed

cultures. However, in order to achieve an efficient ammonium based selection, ammonium concentrations should be adapted to eliminate any adverse effect on type II growth rate as well as the PHB accumulation capacity of the enriched culture [16].

The possibility of obtaining methanotrophic mixed cultures with high PHB productivity from wastewater treatment streams was mainly focused on WAS [16]. In addition, previous studies employing WAS mainly focused on deducing the operational conditions that would favor the growth of type II methanotrophs and their PHB accumulation in mixed cultures without considering the effect of the seed origin. Compared to waste activated sludge, anaerobic digestion sludge can provide a better seed for selection of type II over other non-producing PHB methanotrophs considering the higher ammonium concentrations found in the digester resulting from the ammonification process during the anaerobic digestion process [113]. Furthermore, the ammonium-rich wastewater produced from anaerobic digestion (AD centrate) can replace the traditional synthetic medium described for methanotrophs growth [123] to develop a sustainable microbial culture and feedstock within the wastewater and waste stream process.

Interestingly, biopolymers production by methanotrophs is not limited to PHB only. Introducing volatile fatty acids (VFAs) as a co-substrate to the culturing medium during the nitrogen limitation conditions results in the synthesis of other biopolymers from the PHA family [125]. For instance, addition of valeric acid supports the incorporation of hydroxyl valerate (HV) units and the formation of polyhydroxybutyrate-co-valerate (PHBV) co-polymer [79]. While the applications of PHB can be limited by its stiffness, brittleness, high crystallinity and low thermal stability resulting in a difficulty for its processing [126], PHBV has a lower melting temperature, crystallinity, water permeability and enhanced mechanical properties [81] which broaden the market for biopolymers

produced from methanotrophs in various industrial, medical and agricultural applications including food packaging, the production of water-resistant surfaces, biodegradable implants, controlled drug delivery systems and fabrication of nanocomposites [127]. Properties of the produced co-polymer PHBV mainly depends on the HV fraction incorporated in the produced biopolymer and previous studies showed that the HV fraction is mainly regulated by the co-substrate concentration in the culturing medium [16], [77], [83].

Therefore, the aim of this research is to evaluate the possibility of directing different products from AD process towards biopolymers production. First, the capability of establishing methane-utilizing mixed cultures seeded from AD sludge with steady and consistent performance is demonstrated in both the growth phase using an enhanced ammonium-driven selection approach and in the PHB accumulation phase under nitrogen limited conditions. Furthermore, the PHB accumulation capacity of the enriched culture is shown under different operational conditions. Moreover, valeric acid addition is coupled with nitrogen limited conditions to induce PHBV production and evaluate the environmental factors affecting the biopolymer accumulation and the change in the fractions of the co-polymer composition to develop an AD-dependent bioreactor for biopolymers production.

5.2 Materials and methods

5.2.1 Cultivation conditions

Sludge was collected from anaerobic digesters treating both primary and waste activated sludge at Humber wastewater treatment plant located in Toronto, Canada and used as a seed for methanotrophs cultivation. All enrichments were cultivated in the mineral salts medium (MSM) [90] and had the following concentrations of chemicals (mg/l):1000 $MgSO_4.7H_2O$, 200 $CaCl_2.H_2O$, 272 KH_2PO_4, 610 K_2HPO_4, 4 Fe-EDTA and 1 ml/l Trace metal solution. The trace

metal solution had the following concentrations of chemicals (mg/l): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.4H_2O$, 30 H_3BO_3, 3 $Na_2MoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$ and 20 $CoCl_2.6H_2O$. Ammonium chloride (NH_4Cl) was added as the sole nitrogen source to the MSM medium with a concentration of 5mM (72 mg-N/l). Copper sulphate ($CuSO_4.5H_2O$) was added from a stock solution to give a 10µmol final concentration.

Unless otherwise specified, all the experiments for type II methanotrophs enrichment from anaerobic digestion sludge were running in a fed-batch mode and in triplicates using consecutive growth cycles [16]. Enrichments were incubated in 250 ml serum bottles capped with butyl rubber stoppers. The liquid volume was 50 ml and the headspace was filled with a methane and oxygen (>99.9% purity, Praxair) mixture of volumetric ratio (1:1). All the experiments were running at a temperature of 25°C using table orbital shakers at a speed of 160 rpm. The pH was kept between 6-7 using 10% NaOH prepared solution. Samples were taken from the headspace periodically to monitor the gases consumption and helium was added to restore atmospheric pressure to eliminate any effect from the low methane solubility. The growth and ammonium concentration were measured at the beginning and the end of the experiment by measuring the optical density (O.D) and ammonium concentration as mg-N/l in the culturing medium.

5.2.2 Growth cycles

At the beginning of the experiment, anaerobic sludge was filtered through a 100 µm cell strainer to remove undesired solids. After centrifuging, the filtered sludge was added to 50 ml of the MSM. Air was removed from the headspace of the serum bottles using a vacuum pump and replaced with 100ml of O_2 and 100ml of CH_4 every 24 hours. After 48 hours of cultivation, part of the cultures were centrifuged (4000x) for 20 min then the collected biomass was used in the growth of the proceeding cycle while the rest of the biomass was tested for PHB accumulation. To stimulate the

growth of type II methanotrophs and to ensure their dominance during the growth cycles, an ammonium-driven selection approached was employed [16] with some modification [128]. The amount of biomass returned from each growth cycle was adjusted to give an initial O.D of 0.37±0.08 (175mg-VSS/l) and to initiate an inhibitory effect on type I methanotrophs without affecting type II growth by maintaining a high N/M_i ratio and keeping the C/N ratio at around 10±3 mg-CH_4/mg-N which would result in a high F/M ratio and eliminate any inhibitory effect resulting from the competition between ammonium and methane for MMO oxidation [129]. By applying those conditions, the N/M, C/N and F/M ratios were 0.52±0.08 mg-N/mg-VSS, 11.71±1.07 mg-CH_4/mg-N and 5.39±0.94 mg-CH_4/mg-N, respectively. Different growth parameters were observed during each growth cycle including; final biomass density, specific growth rate (μ), biomass yield (Y), methane consumption, oxygen consumption and methane utilization rate (q). the results from each cycle was compared with the previous one and the steady-state was confirmed when changes in different parameters between the cycles were below 10%.

5.2.3 Methane biodegradation kinetics

Biomass was collected from cycles 8, 23 and 32 to estimate the maximum specific growth rate (μ_{max}), substrate half saturation coefficient (K_s) and the maximum specific substrate utilization rate (q_{max}) of the mixed cultures. The biomass collected from each cycle was divided into ten 250-ml serum bottles. Then, each duplicate was subjected to five different methane concentrations by adding different methane volumes to the headspace of each bottle. The volumes of methane were 25, 50, 100, 150 and 200 ml resulting in aqueous methane concentration of 1.87 ± 0.1, 2.5 ± 0.14, 6.34 ± 0.27, 9.73 ± 0.6 and 13.53 ± 0.53 mg-CH_4/L, respectively by considering the dimensionless Henry's law constant (H_c) at 25^{o}C and 1 atm. of 31.4 [130]. The initial biomass density for all the bottles was the same (190 ± 20 mg-VSS/l). All other operational conditions were similar to the

previous experiment except for the duration where this experiment was running for 20 hours in a batch mode to ensure the existence of the biomass in their exponential phase based on the observations from the previous experiment. Methane, oxygen, and biomass density were measured at the beginning and after the experiment for all cultures. The kinetic parameters were estimated using the equations described by [130] where Monod equation was used to describe the microbial growth and the lineweaver-Burk correlation was used to determine maximum specific growth rate (μ_{max}) and the substrate half-saturation constant (K_s) then the maximum specific substrate utilization rate (q_{max}) was estimated using μ_{max} and the biomass yield as shown in **Figure A.1** in the Appendix section. The calculated parameters at different cycles are summarized in **Table A.3** in the Appendix section.

5.2.4 PHB and PHBV accumulation

The PHB accumulation potential of the enriched culture was monitored by subjecting the remaining biomass at the end of each growth cycle to nitrogen-limited conditions. The PHB accumulation phase was running in a batch mode for 48 hours using 250ml serum bottles and in triplicates. The headspace of each bottle was evacuated and filled with 200ml of methane and oxygen with volumetric ratio of 1:1. After a couple of cycles, the amount of biomass transferred from the growth cycle to the PHB cycle reached a stable value and the initial O.D of each cycle was 1.56±0.22 (720 mg-VSS/l) till the end of the experiments.

When the amount of PHB produced was greater than 40% and the change in PHB accumulation over cycles was below 10% (cycle 11), the effect of other operational conditions was tested using other triplicates in parallel to ensure the consistency of the PHB accumulation capacity of the enriched methanotrophs and the uniformity of the results. The effect of the biomass density on the PHB accumulation capacity of the enriched methanotrophs was tested by employing different sets

of experiments using triplicates having initial O.D ranging from 0.5-3 which is equivalent to 240-1500 mg-VSS/l. Then, the effect of methane to oxygen ratio (M/O) on the PHB accumulation was examined by changing the headspace gas composition from 1:1 (methane: oxygen) to 1:2, 2:1 and 4:1. Finally, PHB accumulation over the 48 hours experiment duration was monitored every 12 hours to estimate the PHB productivity of the enriched culture.

Similar experiments were running to test the PHBV accumulation capacity. The main difference was the valeric acid addition as a co-substrate. Concentrations ranging from 50 to 2000 mg/l were tested on the enriched methanotrophs while monitoring their effect on the amount of PHBV accumulated and the percentage of HV units incorporated in the biopolymer. Upon addition of valeric acid to the culturing medium, the pH dropped as expected. However, at high valeric acid concentrations, the pH decreased below 4 which is below the tolerance known for type II methanotrophs [38]. Accordingly, the pH of the culturing medium was adjusted between 6-7 using 10% NaOH prepared solution to eliminate any effect resulting from the pH drop accompanied with valeric acid addition [83] and to study the effect of a single factor only (co-substrate concentration). Then, different M/O ratios at different valeric acid concentrations and PHBV productivity test were operated as previously described. The effect of each tested parameter on the PHB (or PHBV) content, HV fraction and yield was statistically tested using spearman correlation test on GraphPad Prism software package where ρ represents Spearman's correlation coefficient and p represents the significance. Parameters are considered significant for $p \leq 0.05$. The p-values and correlation coefficients of those parameters are summarized in **Tables A.4-A.5** in the Appendix section.

5.2.5 Microbial community analysis

Two samples were taken from the enrichment during the cultivation to ensure the dominance of type II in the mixed culture. The first sample was taken after 10 cycles of cultivation while the

second sample was taken after 30 cycles. The DNA extraction and the amplification of the V4 region of the 16S SSU rRNA were carried out using the Earth Microbiome Project benchmarked protocols (http://www.earthmicrobiome.org/emp-standard-protocols/). The DNA extraction protocol involving mechanical and enzymatic lysis followed by a phenol-chloroform extraction and a clean-up step using MoBio PowerMag soil DNA isolation kit as per the manufacturer protocol [131].

Primers 515FB (5'-GTGYCAGCMGCCGCGGTAA-3') and 806RB (5'-GGACTACNVGGGTWTCTAAT-3') were used and the barcodes incorporated into the forward primer enabled the usage of various reverse primer constructs to obtain longer amplicons and removal of biases[132]. Briefly, each 25µl PCR mixture contained the following to amplify V4 of the 16S rRNA gene by PCR: 13µl of PCR-grade water (Sigma, USA), 10 µl of Platinum Hot Start PCR Master Mix (2x) (ThermoFisher, USA), 1 µl of Template DNA, 0.5 ml of 515FB primer (10 µM), 0.5 ml of 806RB primer (10 µM). The reaction then was run for 35 cycles (94°C for 2 min, 94°C for 30 s, 50°C for 30°C, 72°C for 30 s), with a final polymerization step at 72°C for 10 min. The products were separated by electrophoresis in a 2% agarose gel and visualized under a UV transilluminator light, and the products corresponding to the amplified V4 (300-350 bp) were excised and purified using standard gel extraction kits (Qiagen, Netherland). Products were then quantified with Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher, USA). Equal amounts of products from each sample (240 ng) were combined into a single, sterile tube then cleaned using MoBio UltraClean PCR clean-up kit. The final concentration was measured using nanodrop spectrophotometer and the products quality was checked by ensuring that the absorbance ratio at 260/280 nm is ranging from 1.8-2.

The resulting PCR products were sequenced using the Illumina MiSeq personal sequencer (Illumina Incorporated, USA) at the McMaster Genomics Facility, Ontario, Canada. Cutadapt was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality score of 30 and a minimum read length of 100bp [133]. Sequence variants were then resolved from the trimmed raw reads using DADA2, an accurate sample inference pipeline from 16s amplicon data [134]. DNA sequence reads were filtered and trimmed based on the quality of the reads, error rates were learned and sequence variants were determined by DADA2. Chimeras were removed, and taxonomy was assigned using the RDP classifier against the SILVA database version 1.2.8.

5.2.6 Analytical methods

Gas samples were withdrawn using gas tight syringe from bottles headspace then injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD) and Molecular sieve column (Restek, Bellefonte, PA.) to monitor methane, oxygen and nitrogen concentrations with a detection limit of 0.01%. The temperature program was as follows: injector, 60^{o}C; oven, 80^{o}C; TCD, 80^{o}C and helium gas was used as carrier gas with flowrate of 15 ml/min. External calibration curves were constructed where three 250 ml serum bottles were evacuated, and each was filled with 50 ml of water to mimic the conditions applied throughout the experiments. Then, about 200 ml of different gas mixes with known oxygen, methane and nitrogen compositions were injected to the headspace of each bottle. Afterwards, samples were injected to the GC and the peak areas resulting from each bottle at different gases compositions were converted into concentrations and masses. For bacterial growth monitoring, optical density (O.D) was measured using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). Correlation curves between O.D and VSS were

developed. HACH methods and testing kits were used to measure inorganic nitrogen (NH_3-N, NO_2-N, and NO_3-N) concentrations on a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA).

PHB and PHBV were measured using the method developed by Braunegg et al. [110]. Briefly, 10-15 mg of lyophilized biomass were collected and 2 ml of acidified methanol (3% sulphuric acid) and 2 ml of chloroform were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ºC for 3.5 hours then left to cool down to room temperature. Afterwards, 1 ml of deionized water was added, and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for biopolymers quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The temperature program was as follows: 1 min 80 º C, 10 º C/min, 180 º C for 4 min. Results were compared to standard curves obtained using PHB and PHBV standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy and maintain a control for all errors.

5.3 Results and discussions

5.3.1 Methanotrophs cultivation from AD sludge

Filtered sludge obtained from AD was used for methanotrophs cultivation directly without performing any additional adaptation procedures before starting the growth cycles. Then, an increase in the (O.D) was observed during early cycles of the growth phase as shown in **Figure 5.1a**. The pigmentation of the culture shifted towards the tan color known for type II methanotrophs [90]. This visual observation was also confirmed by the conducted microbial analysis where the only methanotrophic genus detected was *Methylocystis* which belongs to type II without any existence for type I genera.

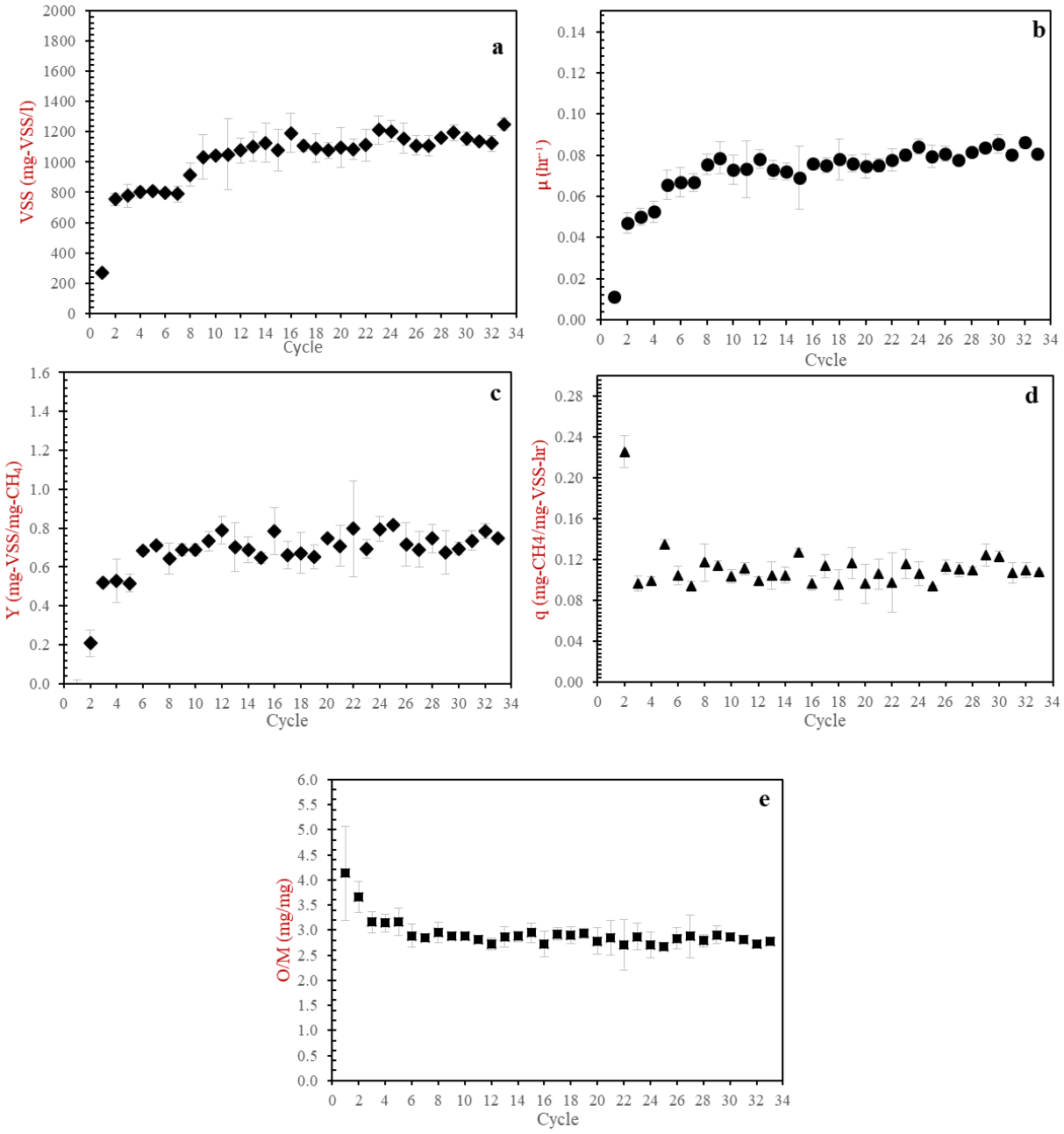


Figure 5.1: The average a) final biomass density b) specific growth rate c) biomass yield d) methane utilization rate and e) O/M consumption ratio observed for AD enrichments during cycle operation. The error bars represent the standard deviation for triplicate enrichments.

As shown in **Figure 5.1a,** a steady-state specific growth rate started after almost 5 cycles of operation and the fluctuations were below 10% till the end of the experiments. Similar behavior was also noticed for other growth parameters including biomass yield, methane and oxygen uptake, substrate utilization rate and final biomass density as illustrated in **Figure 5.1b-1e**. While the

behavior of these parameters was stable for enriched culture in total, the abundance of *Methylocystis* genus increased from 28% at cycle 10 to reach 56% after 30 cycles of operation as shown in **Figure 5.2**.

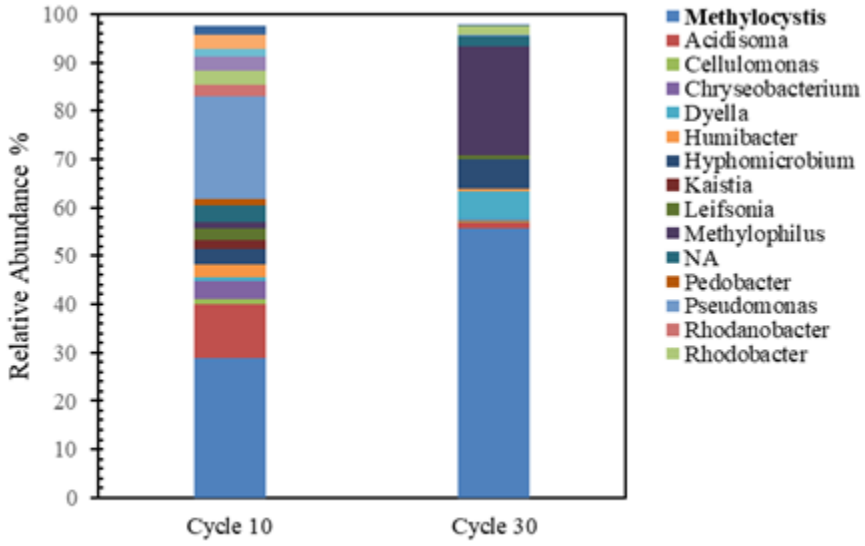


Figure 5.2: Relative abundance of the bacterial community resulting from 16S rRNA gene sequences at genus level with relative abundance above 1% at cycles 10 and 30.

This increase in the *Methylocystis* genus in the mixed culture had a positive effect on the methane affinity and biodegradation kinetics of the enriched culture. As shown in **Figure A.3**, the substrate half saturation constant (K_s) decreased from 2.63±0.3 mg-CH_4/l at cycle 8 to 1.82±0.2 mg-CH_4/l at cycle 23. The maximum specific methane utilization rate (q_{max}) at cycles 8 and 23 were 0.1±0.01 and 0.13±0.01 mg-CH_4/mg-VSS-hr, respectively. On the other hand, the K_s and q_{max} were 1.78±0.2 mg-CH_4/l and 0.14±0.03 mg-CH_4/mg-VSS-hr, respectively at cycle 32. As this study mainly focused on applying conditions to ensure the dominance of type II methanotrophs, further studies should consider applying different molecular techniques to investigate the relationships and co-operation between different members in the mixed culture. For instance, the other two key genera detected at cycle 30 were *Methylophilus* and *Hyphomicrobium* with relative abundances of 22 and

6.5%, respectively. Both facultative methylotrophs have the ability to grow on methanotrophs metabolites [48] and *Hyphomicrobium* can accumulate up to 59% PHB [135]. In addition to *Hyphomicrobium*, other genera including *Sphingomonas, Rhodobacter, Pseudomonas* that were detected, have even higher PHB accumulation capacity than methanotrophs [48]. Thus, optimizing conditions where methanotrophs act as a biocatalyst to support metabolites for other PHB-accumulating bacteria in addition to methanotrophs ability can result in an improvement in the methane biodegradation and PHB productivity for the whole mixed culture.

Different values for the kinetic and stoichiometric parameters for type II methanotrophs were reported in previous studies according to different operational modes Generally, the main advantage noticed for AD enrichments, compared to WAS, was the shorter period achieved to reach a stable performance. AD enrichments reached a steady-state after 10 days of cultivation while WAS enrichments needed 24-30 days when ammonium was employed as a nitrogen source [128] and up to 220 days under nitrogen fixation conditions [48]. Considering full scale application, this shorter adaptation (start-up) period could be one of the factors affecting the choice of type of sludge employed for methanotrophs cultivation. In particular, when similar operational conditions were applied on AD and WAS enrichments, the methane affinity, methane uptake rate and methane utilization rates were 1.5 times higher in the AD enrichments [128]. In addition, considering the same cultivation period under the same conditions, the abundance of type II in AD enrichments was double the WAS enrichments after 30 cycles of operation. Moreover, despite the relatively lower ammonium concentration adopted during these experiments, there was no existence for type I methanotrophs throughout the cultivation of AD enrichments unlike WAS enrichments where a transition period took place and both types of methanotrophs existed in the mixed culture and consequently affected the PHB productivity [136]. Collectively, AD

enrichments can be more efficient for combining the need for methane mitigation and recovery of value-added products.

In comparison with previous studies on either pure or mixed cultures, the reported maximum specific growth rates (μ_{max})and maximum utilization rates (q_{max})ranged from 0.012 to 0.19 hr^{-1} and 0.021-0.26 mg-CH_4/mg-VSS-hr, [16], [59] respectively while the biomass yields ranged from 0.3 to 0.9 mg-VSS/mg-CH_4 [59], [83], [137], [138]. The maximum values reported in those ranges where associated with pure cultures studies. The attained results for the AD enrichments during this study were in the upper range of these values and among the highest reported for different methane-utilizing mixed cultures where the maximum specific growth rate was 0.098±0.007 hr^{-1} and the maximum methane utilization rate was 0.14±0.05 mg-CH_4/mg-VSS-hr while the biomass yield was 0.7±0.08 mg-VSS/mg-CH_4 which also confirms the potential of AD enrichments and the convenience of the applied conditions to type II methanotrophs growth.

5.3.2 PHB production by type II methanotrophs enriched from AD sludge

The ability of PHB accumulation by the AD enrichments was induced by applying a nitrogen-limited phase for 48 hours after the growth phase starting from the first cycle of operation. The PHB accumulation phase had a similar behavior to the growth phase. During the early cycles (till cycle 6) small amounts of PHB were accumulated with an average of 15±4 %. Afterwards, a significant improvement in the PHB accumulation capacity was achieved with a stable behavior while reaching 48±6% till the end of the experiments as shown in **Figure 5.3**.

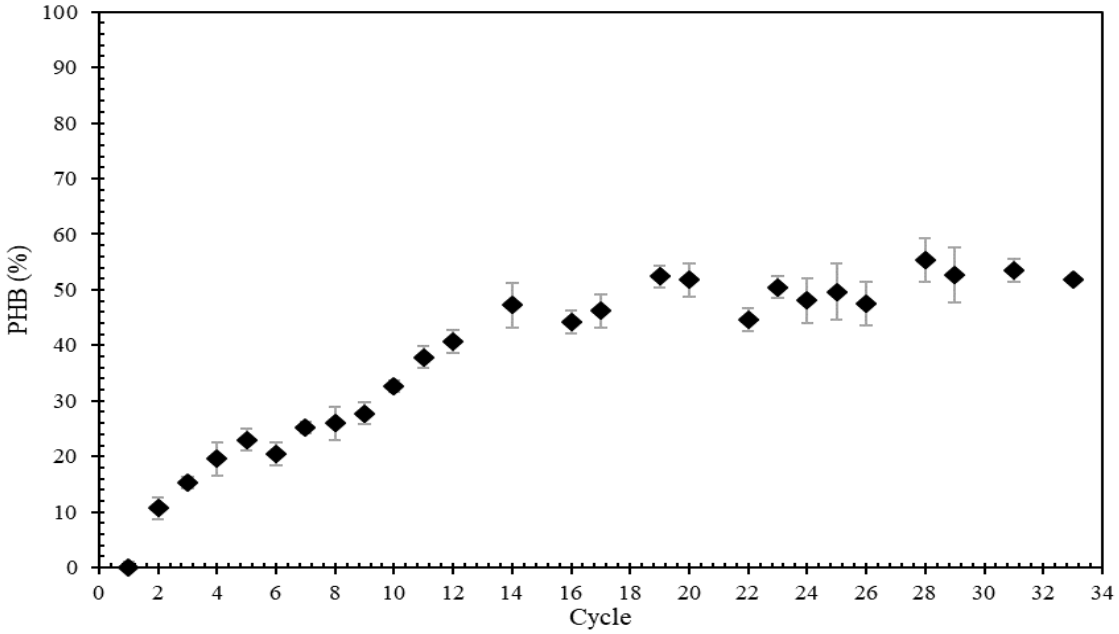


Figure 5.3: Average PHB accumulation for AD enrichments during the cycles operation

The effect of manipulating different M/O on the PHB production was tested by changing the gases composition in the headspace. As shown in **Figure 5.4**, the highest PHB accumulation was observed at M/O ranging from 1:1 to 2:1 and the enriched methanotrophs could accumulate up to 46±4% PHB in their cellular biomass. However, increasing the M/O mainly affected the PHB yield ($p= 0.0026$, $\rho= -0.782$), where a decrease from 0.49±0.04 to 0.33±0.03 mg-PHB/mg-CH_4 was observed when the M/O increased from 1:1 to 2:1. Further increase in the M/O to 4:1 caused a severe drop in the PHB accumulation capacity of the culture with only 10±2% PHB produced ($p= 0.0033$, $\rho= -0.772$). High M/O is considered as one of the favorable conditions for type II methanotrophs growth [102], [139]. However, coupling lower oxygen concentrations put another stress condition on type II methanotrophs and the energy intensive PHB accumulation step in addition to nitrogen limitation and consequently affected their PHB accumulation which can be also confirmed by the lowest yield observed 0.13±0.02 mg-PHB/mg-CH_4 at M/O of 4:1. On the

other hand, decreasing the M/O to 1:2 declined the amount of PHB accumulated to 34±4%. The decrease in the PHB accumulation can be mainly contributed to the decrease in the methane fraction in the headspace, however, increased oxygen availability allowed the PHB yield to be less vulnerable to this change and was maintained at 0.43±0.06 mg-PHB/mg-CH_4.

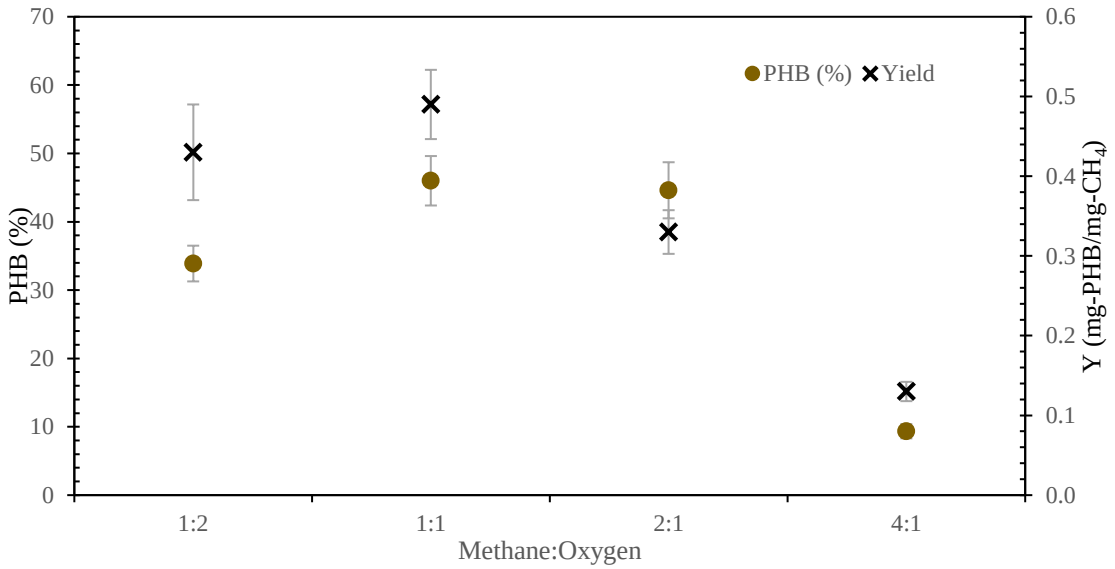
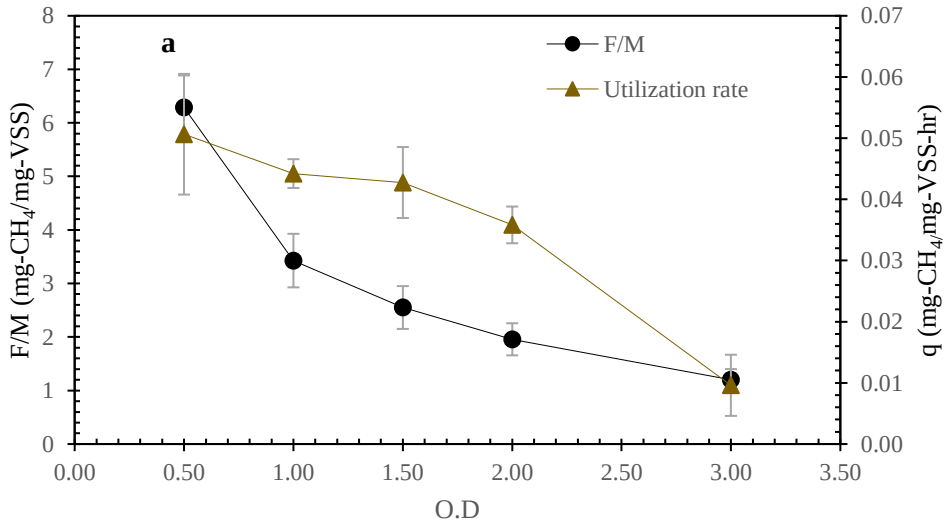


Figure 5.4: Effect of the methane-to-oxygen ratio on the PHB accumulation and yield

Changing the biomass density can affect methanotrophs in two ways; firstly, previous studies revealed that methanotrophic bioreactors are usually mass transfer limited (liquid-gas transfer) at higher biomass densities, which may cause a decline in the bacterial activity [140]. Secondly, changing the biomass density also alters the F/M ratio and consequently the growth of the enriched culture [141]. As shown in **Figure 5.5a**, increasing the biomass density (O.D) from 0.5 (238 mg-VSS/l) to 3 (1430 mg-VSS/l) decreased the F/M ratio from 6.29±0.6 mg-CH4/mg-VSS to 1.29±0.2 mg-CH4/mg-VSS. However, as shown in **Figure 5.5b**, the enriched culture could maintain a stable amount PHB accumulation and the average value was 51±2% till an O.D of 2. In addition, the PHB yield increased from 0.49±0.02 to 0.61±0.1 mg-PHB/mg-CH_4 when the biomass density increased

from 0.5 to 2. Further increase in the O.D to 3 resulted in a decrease in the amount of biopolymer accumulated (p= 0.006, ρ= -0.714) where only 10% PHB was produced by the enrichment while the yield was more tolerant to this change (p= 0.274, ρ= -0.328) and had a value of 0.46±0.05 mg-PHB/mg-VSS. Interestingly, both experiments O.D = 3 and M/O ratio (1:2) had almost the same F/M ratios and similar yields while the PHB accumulation in the M/O experiment was almost 3-fold the attained one for the O.D = 3 experiments which shows that the F/M ratio mainly affects the yield not the amount of the PHB accumulated in the biomass. On the other hand, the negative effect of methane solubility at increased biomass densities was mainly reflected on the rate of methane utilized by the enriched culture which was slightly decreasing from 0.51±0.01 to 0.43±0.006 mgCH4/mg-VSS-hr before O.D=1.5 followed by a sharp decrease to reach 0.01±0.005 mgCH4/mg-VSS-hr when the optical density was 3 as illustrated in **Figure 5.5a**.



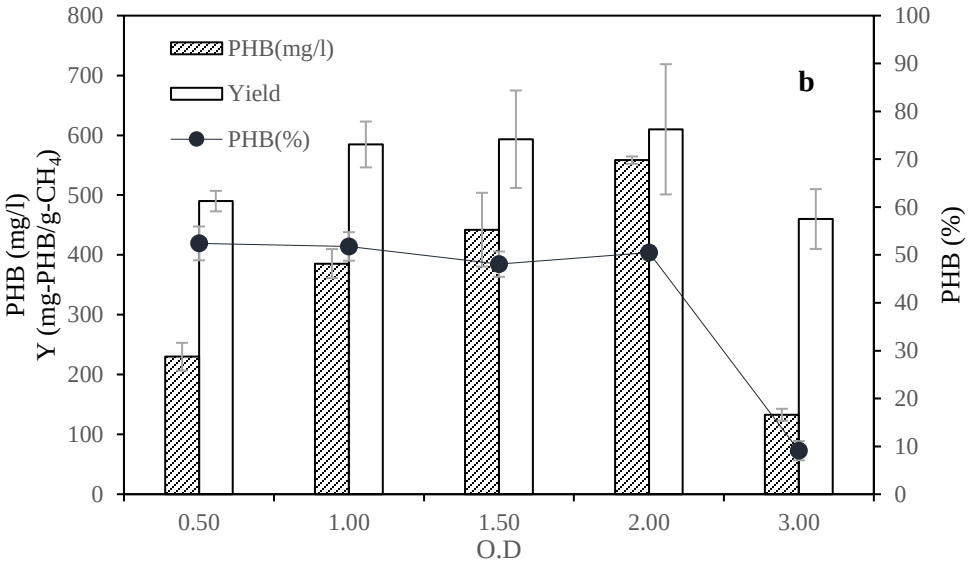


Figure 5.5: Observed a) F/M and methane utilization b) PHB accumulation, concentration and yield at different biomass densities. The error bars represent the standard deviation for triplicate enrichments.

To assess the PHB productivity of the enriched type II methanotrophs from AD sludge, PHB accumulation was measured every 12 hours during the 48 hours of nitrogen-limited cultivation. The results show that the PHB accumulation was increasing with the time course of the experiment and the PHB reached its maximum after 48 hours as illustrated in **Figure 5.6**. In addition, the maximum productivity was achieved after 36-48 hours of cultivation in nitrogen limited conditions reaching 9.5±0.8 mg-PHB/l-hr. Furthermore, increasing the cultivation period for more than 48 hours did not show any noticeable increase in the PHB accumulation and therefore resulted in lower PHB productivity. Similar observations were also noticed in previous studies where the PHB accumulation reached the maximum value after 48 hours of cultivation in nutrient limited conditions followed by either a stability or a slight increase in the amount of PHB produced by type II methanotrophs [142].

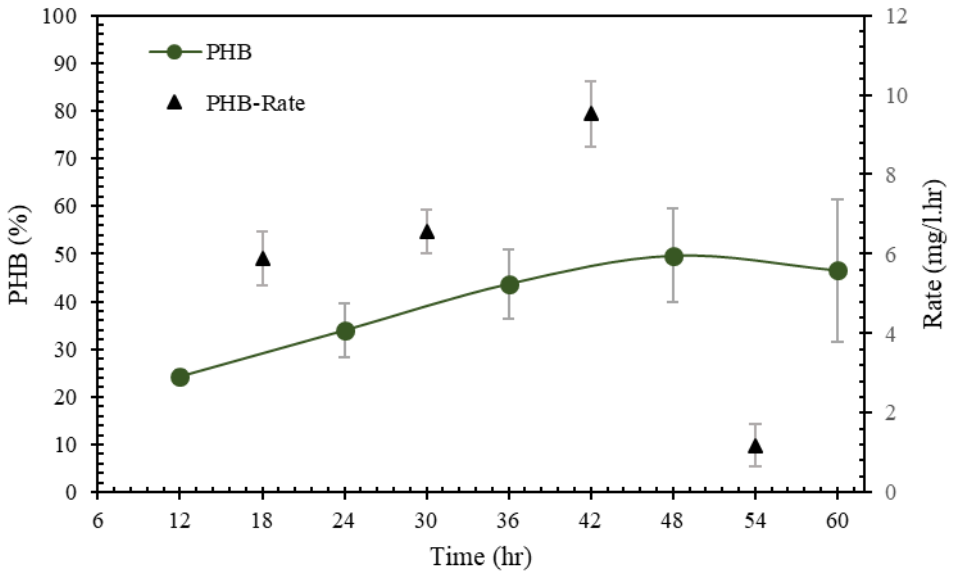


Figure 5.6: Change in the PHB accumulation over time with the corresponding PHB productivity during each period.

5.3.3 PHBV production by type II methanotrophs enriched from AD sludge

Valeric acid was introduced to the nitrogen-limited cultivating phase to induce the accumulation of PHBV. Valeric acid was chosen as a model for volatile fatty acids (VFAs) as the maximum amount PHBV accumulated by pure type II strains was achieved by using valeric compared propionic acid or their salts [77], [83]. PHBV is mainly formed by the polymerization of two units; 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV). While methane assimilation secure the formation of the first one only, the main role of the added co-substrate is providing precursors for HB units (3-hydroxybuturyl-CoA) or HV units (3-hydroxyvaleryl-CoA) for incorporation in the produced biopolymer and apparently valeric acid regulates the second one [77], [79], [83]. As shown in **Figure 5.7**, increasing the valeric acid concentration from 50 to 100 mg/l increased the amount of PHBV accumulated from 34±3% to 47±4% while the percentage of the HV units increased from 25±2% to 35±3%. Further increase in valeric acid concentration deteriorated the amount of PHBV accumulated and a severe decline in the accumulation was noticed at 2000 mg/l with only 15% PHBV produced ($p= 0.004$, $\rho= -0.645$). Generally, valeric acid addition introduced

a second energy demanding step for its uptake in addition to the methane to methanol oxidation. Although these requirements are fulfilled through the methane oxidation pathway (at the expense of increased oxygen requirements), increasing the valeric acid concentration resulted in higher energy demands and the amount of CH_4 oxidised for energy generation increased the substrate partitioning coefficient for energy (f_e) to reach 0.86 at 2000 mg/l compared to 0.4 at 50 mg/l. This increase did not only affect the amount of the biopolymer accumulated but also the noticeable decrease in the observed PHBV yield. The observed yield declined from 0.68±0.03 mg-PHBV/mg-CH_4 to 0.17±0.01 mg-PHBV/mg-CH_4 at concentrations of 50, 2000 mg/l, respectively ($p= 0.0001$, $\rho= -0.789$). Despite the drop noticed for the overall PHBV production, the percentage of the incorporated HV units was not affected and remained 49±3% at concentrations above 100 mg/l as shown in **Figure 5.7**. Similar behavior was also observed for the strain *Methylocystis parvus* OBBP with the maximum observed PHBV accumulation at a sodium valerate concentration of 100 mg/l (54%) followed by a decline in the biopolymer production and a stability in the HV units incorporation [79]. In addition, the maximum PHBV accumulation for *Methylocystis hirsuta* was 53.8% when the concentration of valeric acid was 130 mg/l [83].

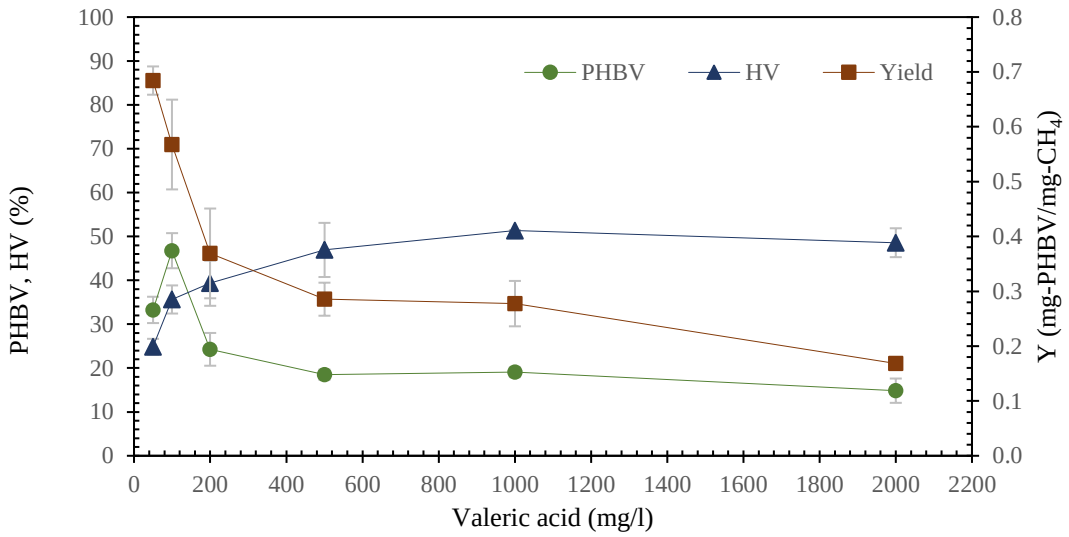


Figure 5.7: PHBV accumulation, HV units incorporation and yield at different valerate concentrations

The aim of manipulating different M/O ratios during PHBV production was not only to study the effect on the amount of the accumulated biopolymer. It was also studied to discuss the capability of manipulating another factor rather than the valeric acid concentration that would change the fractions of the PHBV without decreasing the overall production for a tuned product. As shown in **Figure 5.8a**, when the methane to oxygen ratio was 1:2 and the valeric acid concentration was 100 mg/l, the amount of PHBV accumulated by the enriched methanotrophs was 44±3% with almost the same to the amount accumulated when this ratio was 1:1 at a valeric acid concentration of 100 mg/l, however, the HV units increased from 34±1% to 39±2% by changing the M/O to 1:2 which can be referred to the increased availability in oxygen and more favorable conditions for the energy intensive valeric acid uptake by type II methanotrophs during PHBV accumulation [79]. Further increase in oxygen ratio to 1:3 resulted in a decline in the amount of PHBV to reach 25±3% while the HV incorporation was maintained at 38±2%. These observations show that the presence of conditions favoring valeric acid uptake as high acid concentration or oxygen availability will direct methane oxidation to serve the synthesis of HV units till it reaches its maximum even if it affects

the overall PHBV accumulation. On the other hand, increasing M/O to 2:1 was expected to result in a decrease in the HV monomers in the accumulated biopolymer due to increased availability of methane. However, the percentage of the incorporated HV units was almost constant at 35±1% while the overall PHBV accumulation slightly increased from 43±2% to 47±3% as shown in **Figure 5.8b**.

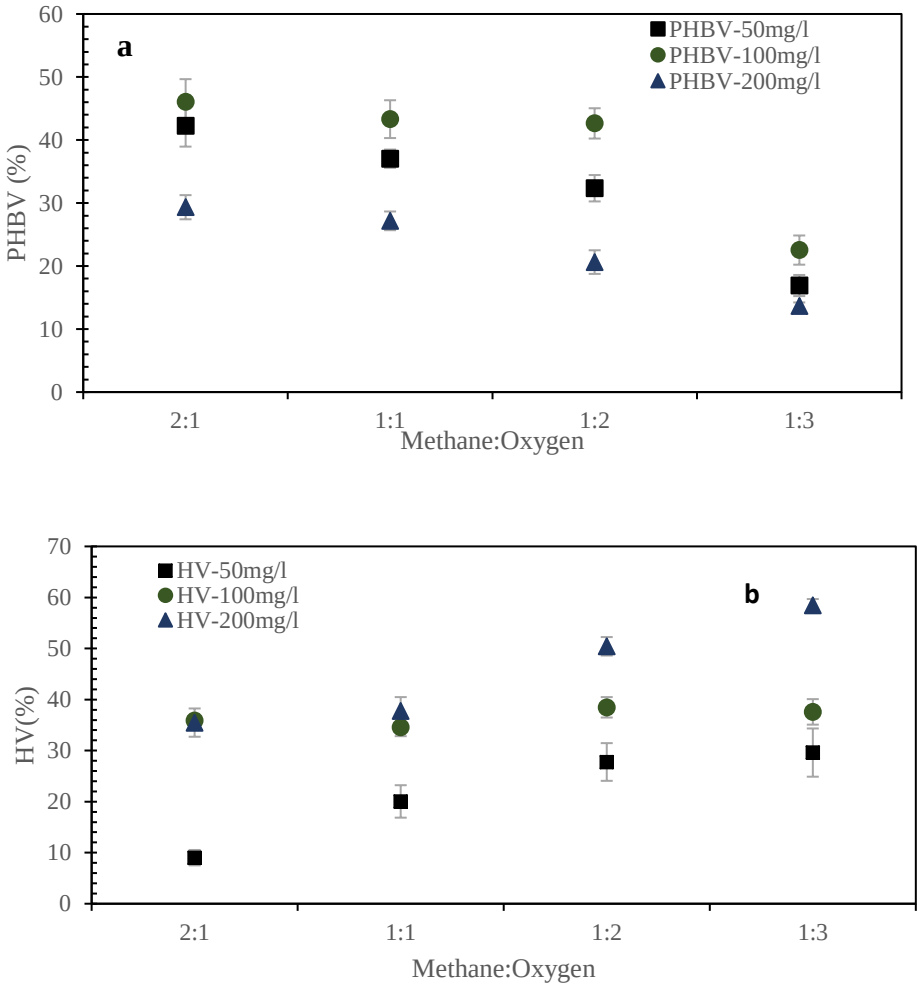


Figure 5.8: Effect of methane to oxygen ratio on a) PHBV accumulation b) HV fraction at different valeric acid concentrations

To elaborate the previous observations, similar M/O were tested at valeric acid concentrations of 50 and 200 mg/l. While at both concentrations the overall amount of PHBV followed a similar

trend to what has been observed in the previous experiment at 100mg/l **Figure 5.8a**, the incorporation of the HV units had a different behavior. At 50 mg/l, increasing the M/O to 2:1 decreased the HV fraction from 20±3% to 9±2% while almost no change in this fraction was observed at 200mg/l and the HV units represented 36±2% of accumulated biopolymer as shown in **Figure 5.8b**. On the other hand, M/O of 1:2 increased the HV fraction at both concentrations. However, at 50 mg/l changing the M/O from 1:2 to 1:3 did not affect the PHBV composition and the maximum HV fraction was 29±2%. Contrarily, at 200 mg/l, further increase in the oxygen ratio to 1:3 elevated the HV content to 58±4% which is higher than the fraction observed in the previous experiment running at a valeric concentration of 2000 mg/l supporting the significance of oxygen on the PHBV composition taking into consideration that the PHBV accumulation at both conditions was almost the same (14%).

The PHBV productivity profile throughout the 48 hours duration of the experiment had a different behavior compared to the observed one for PHB. While the amount of the produced PHBV by the enriched methanotrophs and the percentage of the incorporated HV units was increasing and reached the maximum at the end of the 48 hours experiment with values of 52±6% and 33±5% of PHBV and HV respectively. The rate of HV incorporation reached its maximum after 24-36 hours of operation followed by a decrease in the subsequent period which can introduce another method for controlling the characteristics of the accumulated biopolymer by controlling the exposure time to the nitrogen limited conditions. On the other hand, the overall productivity of the PHBV had the highest value between 12-24 hours (10±2 mg-PHBV/l-hr) followed by a slight decrease during the following periods of the experiment (7±1.9 mg-PHBV/l-hr) as shown in **Figure 5.9**.

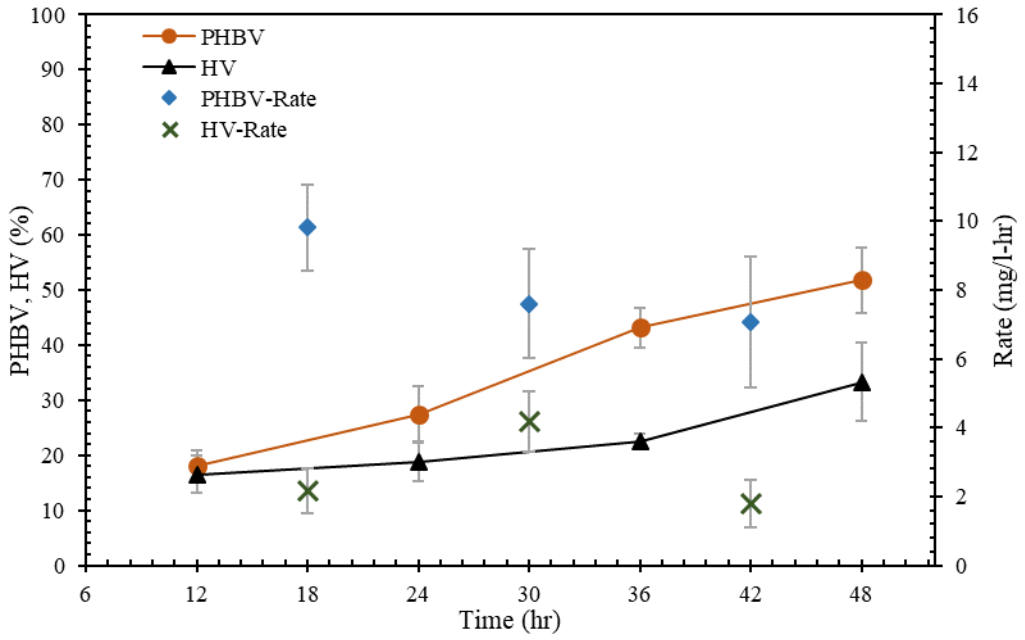


Figure 5.9: Change in PHBV accumulation, HV incorporation and productivity overtime.

5.4 Conclusions

The conducted fed-batch experiments showed the applicability of enriching a PHB-accumulating and methane-utilizing mixed culture with high and stable growth rates, biomass yields and gases uptake rates from the sludge obtained from AD process. Interestingly, the AD cultures had a shorter adaptation period compared to WAS enrichments Moreover, the enriched cultures were able to accumulate elevated quantities of PHB and PHBV up to 52% under different operational conditions. Not only did the methane to oxygen ratio affect the overall biopolymer accumulation but also a noticeable change in the monomer composition was observed especially at high oxygen ratios. This study provides a closer step towards the choice of the optimum scenarios for attaining sustainable biopolymers production from different AD process products to be implemented in WRRFs.

Chapter 6

Growth and PHB accumulation of methanotrophic mixed cultures using wastewater treatment streams resources

6.1 Introduction

Bioplastics have attained the focus of many researchers in the last years as a green alternative for petroleum based plastics in various applications [143]. Wide range of microorganisms have been explored for their ability to accumulate biopolymers under unbalanced growth conditions and most of these bacteria showed great potential to produce different types of polyhydroxyalkanoates (PHAs) as a carbon and energy reserve [81]. Employing methane as a cheap feedstock showed a great potential for biopolymers production. Under unbalanced growth conditions, a special type of microorganisms named methanotrophs can convert their carbon source i.e. CH_4 into polyhydroxybutyrate (PHB) as an energy reserve which can be extracted from the bacterial cells and processed to develop a target product [81]. Moreover, these products can be bio-degraded when subjected to anaerobic environments at their end-life. However, not all types of methanotrophs could accumulate PHB and this property is limited to aerobic methanotrophs utilizing the Serine cycle for methane assimilation i.e. type II [128].

Generally, the PHB production process requires two phases; a growth phase where all the essential nutrients are provided for methanotrophs to sustain their growth increase their biomass density [128]. Then, the cultivated biomass is subjected to deficiency conditions to prevent their cellular replication and induce PHB accumulation. To start-up a methane-driven bioreactor for PHB accumulation, four essential elements are required; the inoculum, methane, nutrients-sufficient medium (during the first phase) and nutrients-deficient medium (during the second phase). Most

of the previous studies employed either pure strains of type II methanotrophs, synthetic mediums or pure methane for cultivation which does not mimic real operational conditions. Hence, the aim of this research is to overcome the aforementioned challenges and to develop a sustainable microbial culture and feedstock within the wastewater and waste stream process using the following strategies: (i) find an alternative source of type II methanotrophs inoculum (i.e. anaerobic digestion (AD) sludge), (ii) identify a self-resource of the nutrients-sufficient medium using AD sludge centrate to replace ammonium mineral salts medium (AMS) in the growth stage and an effluent from final sedimentation tank (FST) was utilized to induce the PHB accumulation, and (iii) utilize and convert the real biogas into polyhydroxybutyrate.

6.2 Materials and methods

6.2.1 Cultivation conditions

A mixed culture dominated by type II methanotrophs and enriched from AD sludge was used in a series of batch tests to examine the effect of different conditions on their growth and PHB accumulation capacity. Sludge was collected from (AD) working in Humber wastewater treatment plant located in Toronto, Canada and subjected to anaerobic conditions and the generated biogas (61±4% CH_4, 38±3% CO_2) was collected and used in upcoming experiments.

Different combinations between different cultivation conditions were employed throughout the batch experiments as shown in **Table 6.1**. Enrichments were incubated in 250 ml serum bottles capped with butyl rubber stoppers. The liquid volume was 50 ml and the headspace was filled with a methane or biogas and oxygen mixture of volumetric ratio (1:1). All the experiments were running at a temperature of 25°C using table orbital shakers at a speed of 160 rpm. pH was kept between 6-7 using 10% NaOH prepared solution. Samples were taken from the headspace periodically to monitor the gases consumption. The growth and ammonium concentration were

measured at the beginning and the end of the experiment by measuring the optical density (O.D) and ammonium concentration as mg-N/l in the culturing medium.

The 50 ml liquid volume contained the biomass with initial optical density (O.D) of 0.43±0.06 and either AMS or the AD centrate during the growth phase. The main characteristics of the centrate are summarized in **Table A.6** in the Appendix section. Unless specified, the methane, nitrogen and biomass concentrations were adjusted to give a carbon to nitrogen ratio (C/N), Food to microorganisms ratio (F/M) and Nitrogen to microorganisms ratio (N/M) of 12±2 mg-CH4/mg-N, 5±1 mg-CH4/mg-VSS and 0.4±0.1 mg-N/mg-VSS, respectively [136]. During the PHB accumulation phase, the enriched biomass was collected, centrifuged and subjected to either nitrogen free mineral salts medium (NFMS) or the FST effluent. The effect of each tested condition on different growth parameters was statistically tested using spearman correlation test on GraphPad Prism software package where ρ represents Spearman's correlation coefficient and p represents the significance. Parameters are considered significant for $p \leq 0.05$. The p-values and correlation coefficients of those parameters are summarized in **Tables A.7-A.8** in the appendix section.

Table 6.1: Different conditions applied throughout the experiments

Experiment	**Inoculum**	**Growth phase**		**PHB phase**	
		Carbon source	Nutrients	Carbon source	Nutrients
1	Type II methanotrophs enriched from AD	CH_4	AMS	CH_4	NFMS
			AD centrate (10% v/v)		
		Biogas	AMS	Biogas	
			AD centrate (10% v/v)		

2		Biogas	AD centrate	-	-
			AD centrate (50% v/v)	-	-
			AD centrate (25% v/v)	-	-
			AD centrate (5% v/v)	-	-
3		Biogas	AD centrate (10% v/v)	Biogas	Effluent
					Effluent (50% v/v)
					Effluent (25% v/v)
					Effluent (10% v/v)

6.2.2 Analytical methods

Samples were withdrawn using gas tight syringe from bottles headspace then injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD) and Molecular sieve column (Restek, Bellefonte, PA.) to monitor methane, oxygen and nitrogen concentrations. The temperature program was as follows: injector, 60^{o}C; oven, 80^{o}C; TCD, 80^{o}C and helium gas was used as carrier gas with flowrate of 15 mL/min. External calibration curves were constructed using a known mixture of gases and used to convert the measured peak areas into gases concentrations and masses. For bacterial growth monitoring, optical density OD_{600} was measured using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). Correlation curves between O.D and VSS was developed. HACH methods and testing kits were used to measure inorganic nitrogen (NH_3-N, NO_2-N, and NO_3-N).

PHB was measured using the method developed by[110]. 10-15 mg of lyophilized biomass were collected and 2 ml of acidified methanol (3% sulphuric acid) and 2 ml of chloroform were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ºC for 3.5 hours then left to cool down to room temperature. Afterwards, 1 ml of deionized water was added and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for biopolymers quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The temperature program was as follows: 1 min 80 º C, 10 º C/min, 180 º C for 4 min. Results were compared to standard curves obtained using PHB standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy and maintain a control for all errors.

6.3 Results and discussions

6.3.1 Growth and PHB accumulation of biogas supplemented cultures

The main aim of the first set of experiments was to study the effect of switching the carbon source for methanotrophs from pure methane to real biogas. In addition, replacing the synthetic AMS media with AD centrate which was chosen owing to the high ammonium concentrations. Ammonium concentration in the AD was 526±50 mg-N/l and was diluted to maintain the C/N, N/M ratios that favors the growth of type II methanotrophs [136].

As shown in **Figure 6.1a**, neither the biogas nor the AD centrate had an obvious inhibition on type II methanotrophs growth. Despite the lower methane content in the biogas (61±4%) compared to pure methane, the biogas supplemented enrichments were able to maintain almost the same specific growth rate of 0.063±0.01 hr^{-1} ($p= 0.125$, $\rho= -0.588$) and biomass yields of 0.64±0.09 mg-VSS/mg-CH_4 ($p= 0.120$, $\rho= -0.594$) which confirms the role of carbon dioxide in enhancing the

growth of type II methanotrophs as input to the serine cycle for methane assimilation pathway and the elimination of further biogas upgrading or cleaning-up processes [10]. On the other hand, adjusting the ammonium concentration of the AD centrate by dilution supported the growth of type II methanotrophs using methane or biogas.

All the cultures were capable of accumulating the same amount of PHB regardless the methane source type as shown in **Figure 6.1b**. The PHB accumulation reached 45±3% with a yield of 0.46±0.09 mg-PHB/mg-CH_4. Generally, the results for both the growth and PHB accumulation phases were comparable to previous studies conducted on either pure strains or mixed cultures enrichments of type II methanotrophs [144].

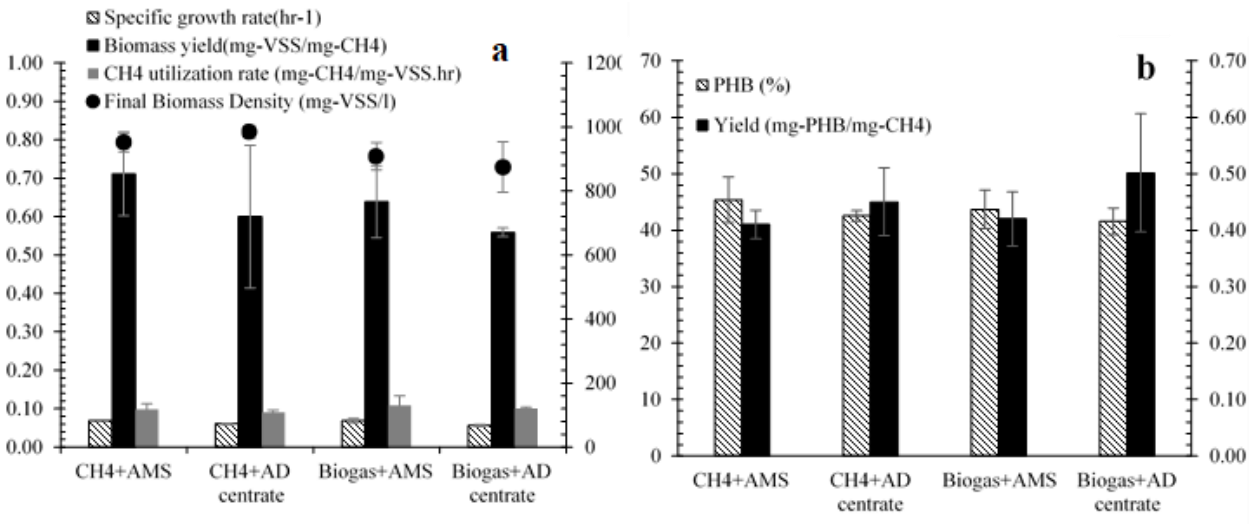


Figure 6.1: Effect of biogas and centrate on a) the growth and b) PHB accumulation of type II methanotrophic mixed cultures

6.3.2 Effect AD centrate on Type II methanotrophs

The second experiment aimed to explore the effect of using biogas and AD centrate at different dilutions on the growth of type II methanotrophs and to confirm that the growth observed during the first set of experiments was mainly derived by the existing type II methanotrophs in the mixed cultures. As shown in **Figure 6.2**, AD centrate concentration had a significant effect on the growth

of the mixed culture (p= 3.64E-06, ρ= -0.970), applying AD centrate without dilution resulted in a complete inhibition in the methane uptake of the enriched culture and consequently the growth. This inhibition was mainly caused by the elevated ammonium concentration found in the centrate (534±50 mg-N/l) while the concentration of phosphorus (120±15 mg-PO_4/l) was in the range required for methanotrophs [10].

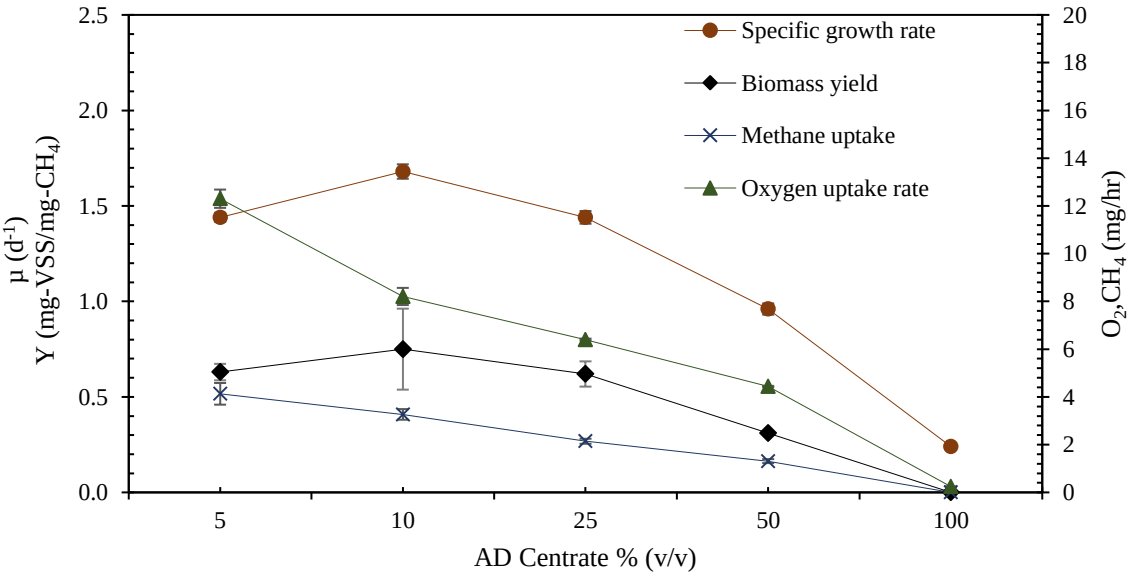


Figure 6.2: Effect of centrate dilution on the specific growth rate, biomass yield and gases uptake rates.

On the other hand, diluting the centrate with deionized water initiated the methane consumption and biomass growth and the highest growth rates were observed between 5-25% v/v centrate concentrations. It is noteworthy to mention that centrate concentrations of 5% and 25% had almost the same specific growth rate (0.06±0.001 hr^{-1}) and biomass yields (0.62±0.066 mg-VSS/mg-CH_4) and the maximum value for both growth parameters was observed at 10%v/v with specific growth rate of 0.07±0.002 hr^{-1} and 0.75±0.212 mg-VSS/mg-CH_4, respectively. However, the methane uptake rate at 5% was almost double than the value obtained at 25%. This observation can be referred to the release of competition between ammonium and methane for methane

monooxygenase enzyme but at the expense of the growth rate due to the decrease in the available nitrogen source to be utilized for cell synthesis. Despite the lower methane uptake associated with the increase in the centrate concentration, it is important to maintain this ammonium inhibition to ensure the dominance of type II methanotrophs in the enriched culture [128]. Nonetheless, when the AD centrate was diluted at 10 % (v/v) to give similar N/M, C/N and F/M ratios to the synthetic medium experiment almost 90% of the microbial activity was restored. While this study mainly focused on the impact of the nitrogen source (ammonium) in the AD centrate, further studies are required to elucidate the effect of other micronutrients and trace metals on the growth especially if dilution is applied which may be the main reason for the lower growth rates for AD centrate at adjusted conditions. To validate the previous findings, AD centrate at concentration of 10 % (v/v) was supplemented for the enriched culture during 10 consecutive growth cycles where consistent performance for the enriched culture was observed without any sign of inhibition as summarized **Table 6.2**.

Table 6.2: Average values for different kinetic and stoichiometric parameters observed for the enriched culture during the cycle operation after reaching steady state

Parameter	**MSM**	**AD centrate 10% (v/v)**
Specific growth rate (hr^{-1})	0.078±0.005	0.06±0.002
Biomass yield (mg-VSS/mg-CH_4)	0.7±0.08	0.6±0.11
Final biomass density (mg-VSS/l)	1099±80	984±19
Specific substrate utilization rate (mg-CH_4/mg-VSS-hr)	0.11±0.02	0.09±0.01
O/M (mg-O_2/mg-CH_4)	2.86±0.12	2.97±0.22
f_e	0.44±0.06	0.48±0.09
f_s	0.56±0.06	0.52±0.09

6.3.3 Employing FST effluent for PHB accumulation

As shown in **Figure 6.3**, applying the deficiency conditions required for PHB accumulation using FST effluent at different dilutions was explored. The maximum amount of PHB was achieved without dilution (32±3%). Further dilution decreased the PHB accumulation to 22±1%. Since nitrate and ammonium concentrations in the FST effluent were 15±3 and 1.7±0.3 mg-N/l, respectively, PHB accumulation would have been expected after their consumption. Despite the lower amount of nitrogen associated at higher dilutions, lowering other nutrients affected the PHB accumulation of the culture which was also reflected on the final biomass density at each FST concentration. The final biomass density was 785±20 mg-VSS/l when the FST effluent was not diluted compared to 635±8 mg-VSS/l at 10%(v/v) taking into consideration that all the experiments had almost the same initial biomass density (575±20 mg-VSS/l) and the same duration (48 hours). In addition, the maximum PHB value noticed throughout this set of experiments was lower than the observed values observed during the previous experiments even though the cultures were the same.

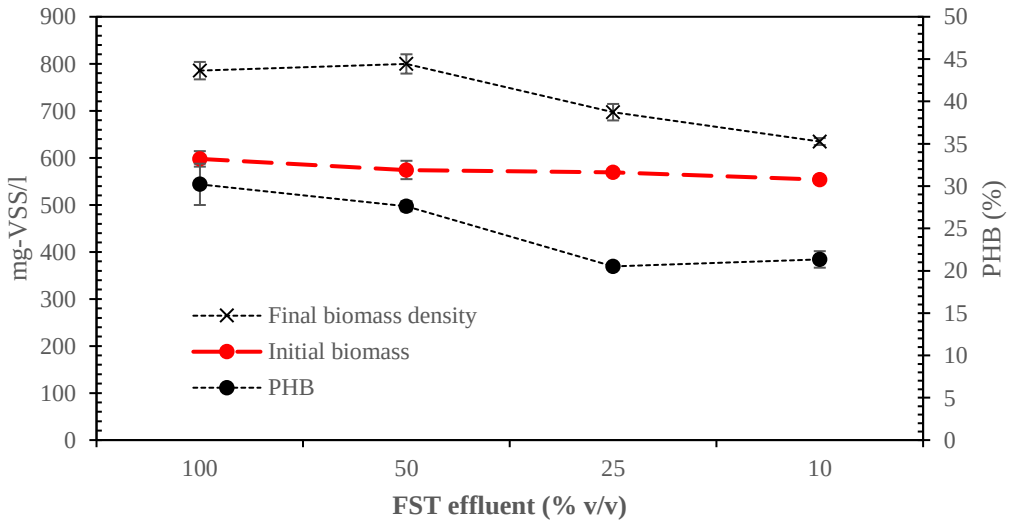


Figure 6.3: PHB accumulation and final biomass density of type II enrichments at different FST effluent dilutions.

The PHB accumulation, nitrogen concentration and biomass density were monitored overtime to estimate the maximum PHB accumulation achieved by the FST effluent without dilution. As shown in **Figure 6.4**, the time course of the experiment can be divided into a growth phase where the enriched culture utilized the nitrogen existing for cellular synthesis in the effluent for cellular synthesis for the first 24 hours of cultivation and delayed the PHB accumulation where the final biomass density increased from 575±20 to 940±60 mg-VSS/l while the PHB accumulation did not exceed 10%. Then, after nitrogen depletion from the medium PHB accumulation was initiated and reached its maximum between 48 to 60 hours at a value of 51±3%. However, it is important to mention that as most of the nitrogen found in the FST effluent is in the form of nitrate and the long-term application of the FST effluent for supporting both the growth and PHB accumulation might result in the invasion of type I methanotrophs. Accordingly, a combination between applying AD centrate during the first phase to ensure both the growth and dominance of type II methanotrophs and FST effluent during the second phase to increase biomass density and PHB accumulation can be tested as the optimum scenario for long-term operation.

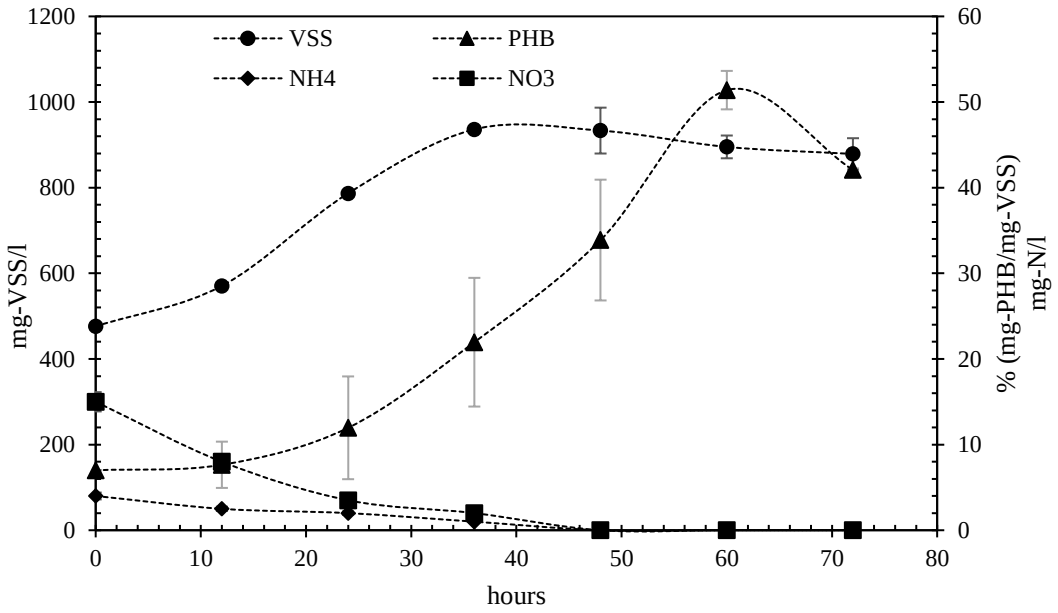


Figure 6.4: Time course of Biomass density, nitrogen concentration and PHB production using FST effluent

6.4 Conclusions

The conducted batch tests showed the potential of providing all the requirements to support methane-driven biopolymers production. Biogas successfully supported the growth of type II methanotrophs during both the growth and PHB phases. AD centrate can be used as ammonium-rich medium to ensure the selection and growth of type II methanotrophs in the mixed culture. The FST effluent increase the final biomass density and establish the conditions required to induce PHB accumulation. However, the long-term effect on the microbial community structure and the PHB accumulation should be investigated.

Chapter 7

Utilization of PHB as intracellular reducing power for methanol production to alleviate the reliance of external energy sources by *Methylocystis hirsuta*

7.1 Introduction

Over the past few years, the adverse effect of human activities on polluting the environment have been unmissable. Recently, substantial efforts are undertaken to minimize such effects by employing green sources of energy, minimizing the discharged waste and finding alternative means for waste conversion into reusable products. From this perspective, methane can be considered as an obvious practice for waste valorization. Capturing methane emissions from landfills and anaerobic digestion processes, which is viewed as waste or being flared, would minimize its atrocious effect as a major greenhouse gas. In addition to its mitigation, methane can be employed in various sustainable applications such as electricity generation, chemicals or biofuels production.

Furthermore, recent discoveries and studies on methanotrophic bacteria increased the interests for biological methane utilization [145]. Methanotrophs are gram-negative bacteria descending from the methylotrophic family where they oxidize methane to generate their energetic and replication needs through a series of interconnected reactions. Detailed information for the metabolic pathways can be found elsewhere in the literature [20], [35], [146]. Briefly, methane is initially oxidized into methanol with the aid of the methane monooxygenase (MMO) enzyme which is then converted into formaldehyde with the aid of the methanol dehydrogenase (MDH). At this level, formaldehyde is either directed towards cell synthesis (through the ribulose monophosphate (RuMP) cycle or the Serine or the CBB cycle) or furtherly oxidized into formate then carbon

dioxide for energy generation [144]. It is noteworthy that all the steps in the methane to carbon dioxide reactions are energy producing steps except for the initial step of methane to methanol conversion which requires two electrons. The energy required from this step is secured from the latter three reaction which produce six electrons and the rest of the energy i.e. electrons enters the electron transport chain to reduce the electron acceptor (oxygen for aerobic methanotrophs) to generate the cellular energy requirements [147].

Methanotrophs are considered as the main biological sink for methane emissions mineralization [20] . They can also couple methane uptake with multiple biotechnological applications. A non-exhaustive list of those applications include chemicals, biofuels, single cell proteins, ectoine, hydroxyectoine and bioplastics [38]. One of the key applications that has been fairly studied over the course of the previous decade is the methane conversion into methanol. While methane applications are limited by its low handling capability and its adaptability to existing infrastructure due to its gaseous nature, methanol is advantageous due to its better storability and security [148]. Besides, the energy content of the methanol molecule (15.8 MJ/l) is 400 times higher than methane (38.1×10^{-3} MJ/l). As a result, methanol can be then employed either for biofuel production or converted into other chemicals such as ethanol, acetic acid, formaldehyde and olefins [149]. Compared to other thermochemical techniques, biological methane to methanol conversion has several advantages as it has lower energy consumption, higher conversions, lower equipment cost. More importantly, it eliminates the need for gas pre-treatment if biogas from anaerobic digestion as a source of methane is employed [144].

For extracellular methanol accumulation, the methane oxidation shall be halted beyond the first step (methanol production). Different inhibitors for the MDH enzyme have been tested on either

pure methanotrophic strains or mixed cultures with varied concentrations including phosphate, magnesium chloride, EDTA, sodium chloride and cyclopropane or a combination between any of them. The inhibitors concentrations were optimized to ensure the stoppage of the reaction beyond methanol while minimizing any negative effects on the MMO enzyme for higher methanol production [150]. In addition, an energy source must be supplied to the reaction medium to secure the required energy for methane to methanol conversion (the energy demanding step as previously described) and maintain cell vitality [148]. Exhaustively in the previous studies, formate was supplied as an external energy source where the energy derived from its oxidation to carbon dioxide for simultaneous methanol production. Concentrations ranging from 40 to 100mM of sodium formate resulted in the highest methanol concentrations for different methanotrophic strains or mixed cultures while the methanol concentrations were up to 500 mg/l [144]. Nonetheless, the addition of external energy source remains one of the bottlenecks for commercializing such a technology at a larger scale.

Another appealing capability of methanotrophs is the intracellular carbon storage in the form of polyhydroxybutyrate (PHB) in response to the unbalanced conditions as an energy reserve. In fact, PHB production has been always viewed as a promising application for methanotrophs to be extracted and employed as a raw biodegradable material for bioplastics production using a cheap feedstock [151]. Apart from extraction, PHB can play an important role as an energy reserve and a source for reducing equivalents in other methanotrophic applications. It was reported that the degradation ability of *Methylocystis trichosporium* OB3B for Trichloroethylene (TCE) increased 16 times when PHB was present in their cells[152]. In addition, the methane oxidation capacity and growth rates of *Methylocystis parvus* OBBP also increased in the presence of PHB inside the cells[57]. However, the effect of PHB existence and its role to support methanol production was

barely discussed. It was only reported that cells having PHB were able to produce trace amounts of methanol, however, CO_2 was used instead of methane [153]. To ascertain high methanol concentrations, high MMO activity should be maintained. Thus, most of the previous studies considered the influence of the added inhibitors on the MMO activity [148]. Nonetheless, the biomass state, or its activity represented by its kinetics such as methane oxidation rates, prior to inhibitors application has not been considered. Biomass growth passes through different phases lag, exponential, stationary, and decay [154]. The maximum microbial activity would be expected to take place when the biomass is growing exponentially before stabilizing at the stationary phase which would indeed affect the methanol production of the harvested cells.

Accordingly, in this study, *Methylocystis hirsuta* is employed for methane to methanol conversion. This α-proteobacteria methanotrophic strain is known for its ability to accumulate significant amount of PHB (up to 55%) under nitrogen limited conditions [40]. Hence, it can be used to test the potential of employing PHB as an alternative source of energy to mediate the methane to methanol conversion and to study the conditions that would trigger the PHB consumption for this purpose. In addition, insights on the influence of the biomass state, influenced by the cultivation duration, on the subsequent methanol production phase is investigated.

7.2 Materials and methods

7.2.1 Strain used and Cultivation conditions

The strain *Methylocystis hirsuta* was purchased from Cedarlane labs (Burlington, Canada) and cultivated as per instructions. Briefly, the biomass pellet was suspended in 50 ml of the mineral salts medium (MSM) described by [146] and had the following concentrations of chemicals (mg/l):1000 $MgSO_4.7H_2O$, 200 $CaCl_2$.H20, 272 KH_2PO_4, 610 K_2HPO_4, 4 Fe-EDTA and 1 ml of trace metal solution. The trace metal solution had the following concentrations of chemicals

(mg/l): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.4H_2O$, 30 H_3BO_3, 3 $Na_2MoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$ and 20 $CoCl_2.6H_2O$. Ammonium chloride (NH_4Cl) was added as the sole nitrogen source to the MSM medium with a concentration of 270 mg/l. 250 µl of Copper were added from a stock solution having a concentration of 500 mg $CuSO_4.5H_2O$/l. The headspace of the 250 ml serum bottle was evacuated and filled with 100 ml of methane and 100 ml of oxygen (>99.9% purity, Praxair). Every day, the headspace was changed with a new gas mixture and the consumption was monitored. After 4-5 days, growth was observed, and the cultivated bacteria was allowed to grow until the biomass density stabilized. Afterwards, the biomass was divided into six other bottles and resuspended in fresh MSM medium for 48 hours cycles of feeding and wasting [128] till a stable growth rate and gases uptake rates were observed then the wasted biomass was used as an inoculum for the upcoming experiments. To induce the PHB accumulation, biomass collected from each growth cycle was centrifuged and resuspended in nitrogen free MSM medium under the same operational conditions.

Unless otherwise specified, all the experiments for studying the capability of the strain *M. hirsuta* for methanol production were running in a batch mode for 6-8 hours. The harvested biomass from the previous growth phase was centrifuged then incubated in 250 ml serum bottles capped with butyl rubber stoppers. The liquid volume was 50 ml and the headspace was filled with a methane and oxygen (>99.9% purity, Praxair) mixture of volumetric ratio 1:1. Magnesium chloride was used as an inhibitor for MDH enzyme at a concentration of 10 mM and sodium formate (if used) was added with a concentration of 100 mM.

All the experiments were running under septic conditions to avoid any contamination and to maintain the culture purity. The temperature was maintained between 25-28°C using table orbital

shakers and a speed of 160 rpm. pH was kept between 6-7 using 10% NaOH prepared solution. Samples were taken from the headspace at the beginning and the end of each experiment to monitor the gases consumption and carbon dioxide production. In addition, the growth was observed at the beginning and the end of the experiment by measuring the optical density (O.D). Upon the completion of each experiment, the biomass was collected then centrifuged and the formed pellet was tested for PHB quantification while the supernatant was used to estimate the methanol concentration.

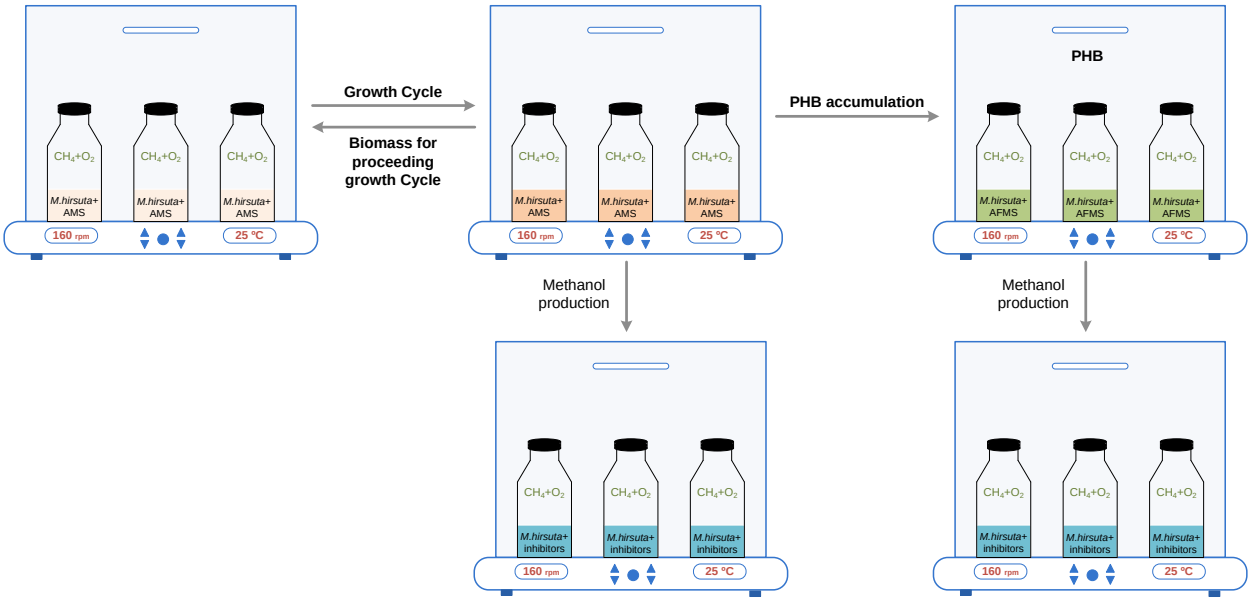


Figure 7.1: General schematic diagram for the employed experimental setup for methanol production

7.2.2 Effect of cultivation period and PHB content on methanol production

To evaluate the effect of biomass state and cultivation duration on methanol production, the wasted biomass from the seed bottles after the growth phase was collected then centrifuged. The bacteria were re-suspended into fresh MSM medium with methane and oxygen and allowed to grow for 12, 24, 48 and 72 hours where each timing represent an experiment running in triplicate. Gas samples were withdrawn periodically, and the headspace was evacuated and refilled in case of

methane or oxygen depletion. At each time point, biomass was centrifuged and subjected to methanol accumulation conditions as previously described. On the other hand, other experiments were running in parallel where the seed biomass was centrifuged and subjected to nitrogen free MSM to induce PHB accumulation for similar time periods (12, 24, 48 and 72 hours) then tested for methanol production phases under similar operational conditions.

7.2.3 Methanol production in PHB accumulating cells in the absence of formate

During these set of experiments, the harvested biomass from the seed bottles was resuspended in nitrogen free MSM for the same time durations as the previous experiment. After the different PHB accumulation periods (12, 24, 48 and 72 hours), the PHB-rich cells were harvested. However, during the methanol production test, sodium formate was omitted from the culturing medium to examine the possibility of employing PHB as an alternative electron source to ensure the continuity of the methane to methanol oxidation step.

7.2.4 Effect of formate concentration on methanol production

This experiment aimed to discuss the influence of different formate concentrations on the PHB consumption and the methanol production of *M. hirsuta*. Five different formate concentrations (0, 25, 50, 100, 150 and 200 mM) were applied during the methanol production phase. During these experiments, based on the results from the previous experiments, the biomass harvested from the seed bottles were subjected to 24 hours of growth in normal MSM containing ammonium as nitrogen source then the biomass was re-suspended in nitrogen free medium to induce PHB accumulation for 24 hours. In addition, the methanol production phase was extended to 24 hours compared to 8 hours in the previous experiments to ensure sufficient time for PHB consumption especially at higher formate concentrations.

7.2.5 Methanol production over time under different formate concentrations

Within these batch of experiments, three different formate concentrations (0, 25 and 100 mM) were applied during the methanol production phase to see the combined effect of PHB content and formate on the methanol productivity of *Methylocystis hirsuta*. In addition, these concentrations were applied on biomass collected from the seed bottles then subjected to either 24 hours of growth in MSM or 24 hours of PHB accumulation under nitrogen limited conditions. Furthermore, the methanol concentrations, methane and oxygen consumption, carbon dioxide production and PHB utilization were tested after 6, 12, 24 and 48 hours from the start of the methanol production phase. Each time point was tested for both types of biomass and each experiment was running in triplicates as summarized in **Table 7.1**.

Table 7.1: Conditions applied to test the effect of formate concentration on methanol production by *Methylocystis hirsuta*

Formate (mM)	Growth (MSM) duration (hrs)	PHB (N-Free) duration (hrs)	Methanol production duration (hrs)
0	24	0	(6- 48)
	0	24	
25	24	0	
	0	24	
100	24	0	
	0	24	

7.2.6 Analytical methods

Samples were withdrawn using gas tight syringe from bottles headspace then injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD) and Molecular sieve column (Restek, Bellefonte, PA.) to monitor methane, oxygen, carbon dioxide and nitrogen gases concentrations. The temperature program was as following: injector, 60^{o}C; Oven, 80^{o}C; TCD, 80^{o}C and helium gas was used as carrier gas with

flowrate of 15 ml/min. External calibration curves were constructed using a known mixture of gases and used to convert the measured peak areas into gases concentrations and masses. For bacterial growth monitoring, optical density (O.D) was measured using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). Correlation curves between O.D and volatile suspended solids (VSS) were developed. Nitrogen and phosphorus ions (NH_4, NO_2, NO_3, and PO_4) were measured using a Dionix Integrion HPIC Ion chromatography system (Thermo Fischer Scientific Massachusetts, United States).

PHB was measured using the method developed by [110]. 10-15 mg of lyophilized biomass were collected and 2 ml of acidified methanol (3% sulphuric acid) and 2 ml of chloroform were added in a glass vial. After gentle mixing, the cocktail was heated at 100 ºC for 3.5 hours then left to cool down to room temperature. Afterwards, 1 ml of deionized water was added, and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for biopolymers quantification using Agilent 7890a gas chromatography equipped with a flame ionization detector (Agilent Technologies, California, United States) and DB-wax column (Restek, Bellefonte, PA.). The temperature program was as following; 1 min 80 º C, 10 º C/min, 180 º C for 4 min. Results were compared to standard curves obtained using PHB standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy and maintain a control for all errors. Afterwards, the amount of the obtained PHB was divided by the VSS to estimate the PHB accumulation of the bacterial culture.

After the completion of each methanol production test, the biomass was collected and centrifuged. Then the supernatant was directly injected into Agilent 7890a gas chromatography equipped with a flame ionization detector (Agilent Technologies, California, United States) and DB-wax column (Restek, Bellefonte, PA.). The temperature program was as following; 1 min 50º C, 10 º C/min,

100 º C for 4 min. Results were compared to standard curves obtained using methanol for quantification (Sigma Aldrich). Methanol conversion efficiency was estimated by dividing the number of methane moles consumed by the number of methanol moles produced while the PHB mineralization (conversion) was calculated by dividing the PHB consumed (mg-C) using the chemical formula $C_4H_6O_2$ by the CO_2 (mg-C) generated.

7.3 Results and discussions

7.3.1 Effect of biomass growth duration on the methanol production

To produce methanol, three important factors are of great importance. Firstly, the inhibition of methanol dehydrogenase (MDH) enzyme and blocking methanol to be furtherly oxidized into formaldehyde. Secondly, ensuring the presence of the energy source (i.e., electrons) required to mediate the energy-demanding methane to methanol conversion step. Typically, formate has been employed to fulfil this role [155]. Finally, maintain the methane monooxygenase enzyme at its highest activity levels for higher methane conversion [156]. These factors have been widely studied through testing different inhibitors and formate concentrations while monitoring their effect on the enzymes' activities (MMO and MDH) and methanol production activity. However, in these experiments, another factor was not taken into consideration which is the biomass state. The collected biomass was subjected to different cultivation periods and then, the methanol production was tested at different time points. Such cultivation periods in fed-batch tests denote the anticipated operating biomass retention time in the system or the sludge age. Sludge age has been intensively studied as a key parameter in any biotechnology [154]. Nonetheless, this is the first study to explore the biomass state or the cultivation duration as an indication to the sludge age on methanol production.

To investigate such effect, the strain *M. hirsuta* is a type II methanotroph (α-proteobacteria) which has been successfully tested for various methane-based biotechnological applications [40], [83]. To activate the culture and start the experiments, the purchased strain was allowed to reach steady-state conditions. In addition, to ensure the consistency of the results, the biomass was exposed to typical-repeated growth cycles and the excess biomass from the growth cycle would be used for methanol production experiments. Before the methanol experiments, the collected biomass was subjected to nitrogen limited conditions for PHB accumulation. After almost 30 days of this procedure, the growth rate of *M. hirsuta* was $0.12\pm0.02 hr^{-1}$ with a biomass yield of 0.55 g-VSS/g-CH_4 during the growth phase and the PHB reached 52±5% during the nitrogen limited conditions. These values are comparable to the literature [144] and indicate that the culture had reached the steady-state for both the growth phase and the PHB accumulation phase.

As shown in **Figure 7.2a**, the biomass density was increasing exponentially during the first 24 hours of cultivation before entering a stationary phase for almost another 48 hours of cultivation. The highest methanol production was observed when the collected biomass was subjected to methanol production conditions after 12-24 hours of growth. The attained methanol concentration reached 350±39 mg/l and 303±6 mg/l for the biomass grown for 12 and 24 hours, respectively, as shown in **Figure 7.2b**. Given that process conditions have not been optimized prior to this experiment, the obtained concentration are comparable to the reported values in the literature for methanol production [148]. On the other hand, when the biomass was harvested in the stationary phase after 48 and 72 hours the methanol concentration was severely decreased by almost 50%. At which, the concentrations were 186±7 mg/l for the 48 hours grown biomass then it was declined to 141±23 mg/l for the 72 hours biomass. The methane to methanol conversion also followed the same pattern where the highest conversion efficiency decreased from 70±10% to 40±12% when

the biomass was subjected to methanol production after 12 and 72 hours of cultivation, respectively.

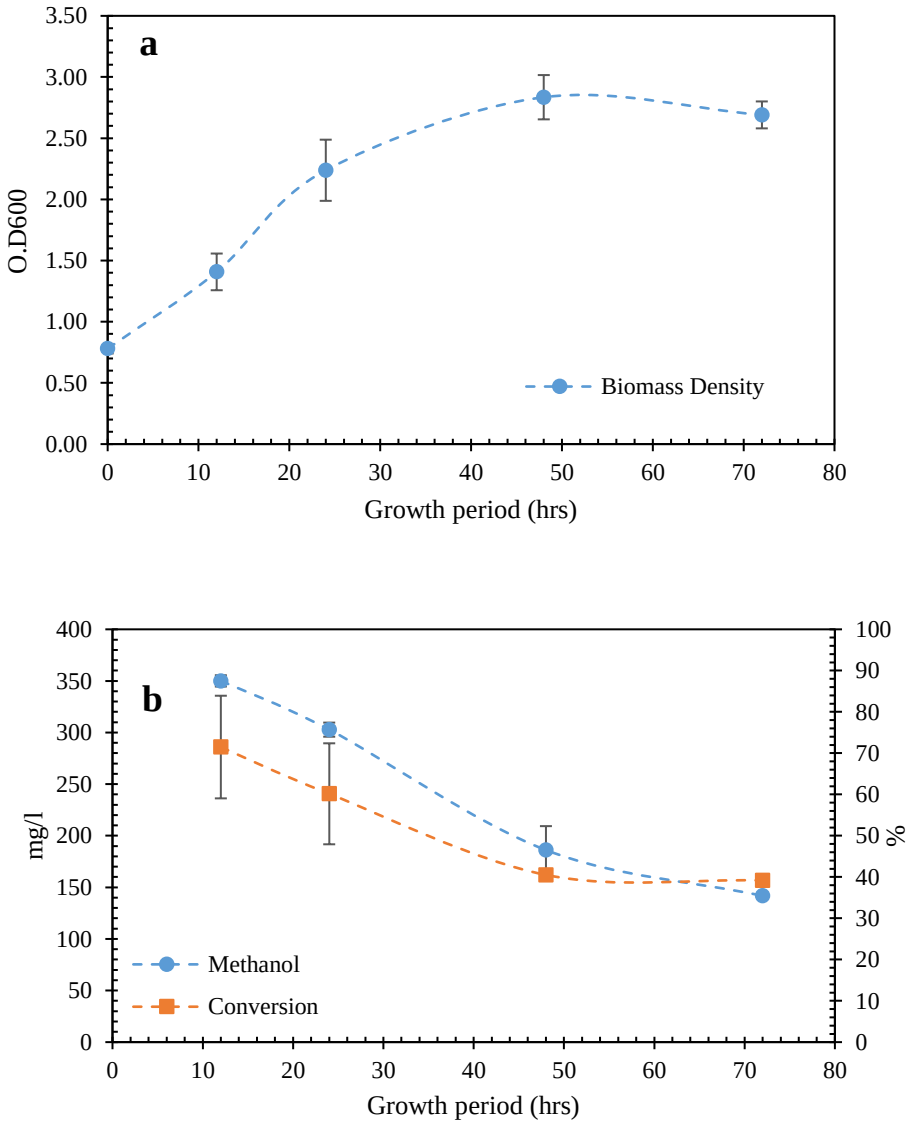


Figure 7.2: a) Biomass density profile during the growth stage under nutrient sufficient conditions. b) Methanol concentration and methane conversion efficiency of the biomass harvested at different growth periods prior to methanol production (8 hours).

The previous experiment was performed directly with the collected biomass after the growth phase which have not been subjected to PHB accumulating condition. Hence, to examine the effect of PHB content in the cells of *M. hirsuta* on its methanol production capability, the cells were subjected to nitrogen limited conditions to initiate the PHB accumulation. Then, cells were tested for methanol production while formate (100mM) was present in the reaction mixture. As shown in **Figure 7.3a**, the PHB increased from 19±2% to 34±4% during the first 12 hours of incubation then stabilized at almost 50±3% from 24 to 72 hours. Regarding methanol production, As shown in **Figure 7.3b**, when the PHB-containing cells tested for methanol production, the highest methanol concentration was observed for the cells subjected to 12 hours of nitrogen limited conditions. It possessed a PHB content of 34±4% and the resulting methanol concentration was 183±16 mg/l. After the methanol production experiment, a slight decrease in the PHB content was observed as the PHB content decreased to only 27±4%. Similar observations were also observed for the strain *Methylocystis parvus* OBBP in which the existence of formate delayed the PHB consumption during growth when they were both present in the culturing medium [57]. In addition, as both formate and PHB consumption yields CO_2, the results show that the PHB mineralization ratio was only 0.31±0.03 g /g (**Figure 7.3c**). On the other hand, subjecting the biomass for longer periods of nitrogen limited conditions i.e. increasing the PHB content resulted in lower methanol concentrations. The obtained methanol concentrations were 145±7 and 89±3 mg/l for the cells subjected to 24 and 72 hours of nitrogen limited conditions, respectively. However, the PHB consumption had an opposite behavior, for the 24-hours cells, almost 30% of the PHB content was consumed during the methanol production experiment while the 72-hours cells consumed 42% of their stored PHB. In addition, the PHB mineralization increased from 0.58±0.02 to 0.85±0.1.

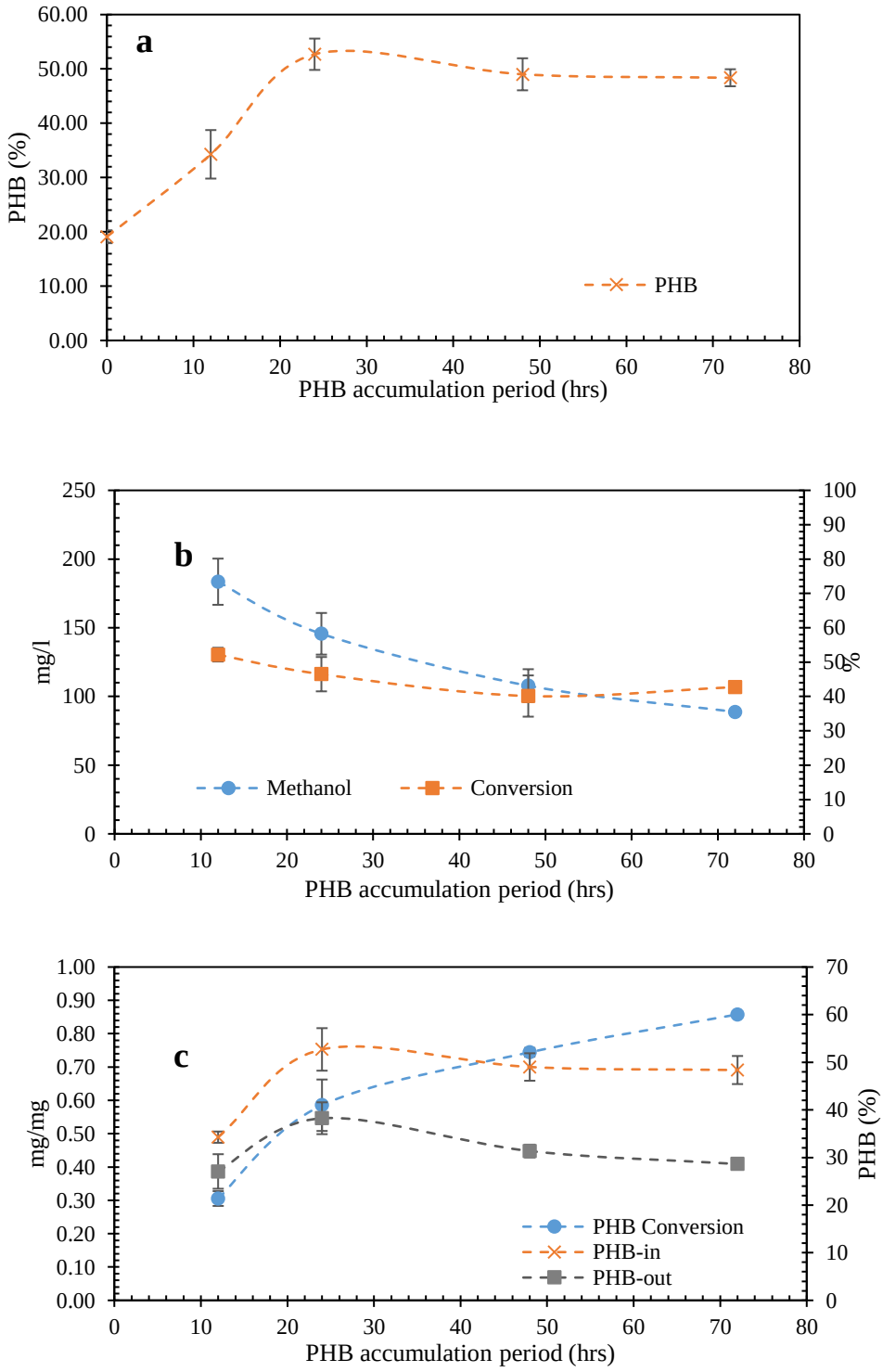


Figure 7.3: a) PHB accumulation profile under nitrogen limited conditions. b) Methanol concentration and methane conversion efficiency for the biomass harvested at different periods prior to methanol production c) PHB content at the beginning and the end of the methanol

production phase and the PHB mineralization for the biomass harvested at different periods prior to methanol production.

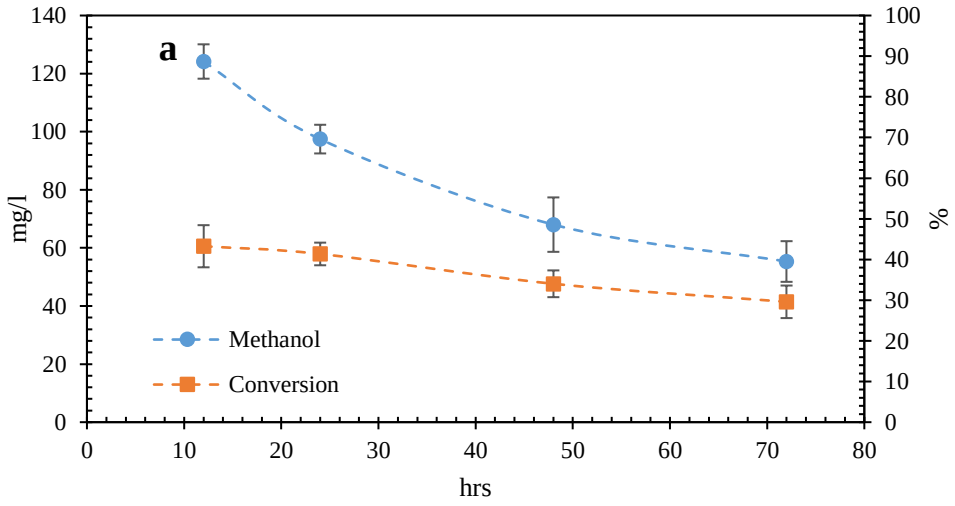
Generally, during these set of experiments, the methanol concentrations were almost 50 % less than the concentrations achieved for cells that were not subjected to nitrogen limited conditions (without high PHB content). However, the methane to methanol conversion efficiency was less vulnerable to this decrease for cells cultivated for 12 and 24 hours in both conditions prior to the methanol production (25% decrease). Supported with the first experiment results, one possible explanation for the lower methanol concentration for the PHB-cells is the long cultivation period (48 hrs) in the seed bottles before the PHB accumulation was initiated which affected the *M. hirsuta* activity. Moreover, if the whole cultivation duration is taken into consideration, the biomass subjected to 72 hours of cultivation under normal growth condition had almost the same methanol concentration (145±5 mg/l) of the biomass grown under nitrogen-limited conditions for 24 hours (72 hours in total). Interestingly, cells cultivated for longer periods under nitrogen and nitrogen-limited conditions (48-72 hours) had almost the same methane to methanol conversion ratio (40%) where the increased PHB consumption and PHB mineralization ratio confirms the possibility of obtaining the energy required for methanol production from the stored PHB inside the cells.

7.3.2 Effect of biomass growth duration on methanol production in the absence of formate

During the previous experiments, the methanol production phase was performed while the reaction medium was supplied with 100mM of sodium formate as the main energy source to mediate the methane to methanol conversion. In the contrary, during this set of experiments, the cells cultivated in either nitrogen or nitrogen-free medium were subjected to methanol production in a formate

free medium to confirm the role of PHB consumption in supplying the required energy for methanol generation.

Regardless of the cultivation period, cells grown under nitrogen sufficient conditions were not able to produce notable amounts of methanol when they were transferred to the formate free medium. The maximum concentration observed was as low as 25±5 mg/l. On the other hand, when *M. hirsuta* was subjected to nitrogen free medium inducing PHB accumulation, the harvested cells after 12 hours produced 124±6mg/l of methanol when transferred to the production phase without formate as shown in **Figure 7.4a**. However, like the previous experiment, the methanol decreased with the increase in the duration of the PHB accumulation. The methanol concentration was only 55±9mg/l for the cells cultivated for 72 hours then subjected to the methanol production phase.



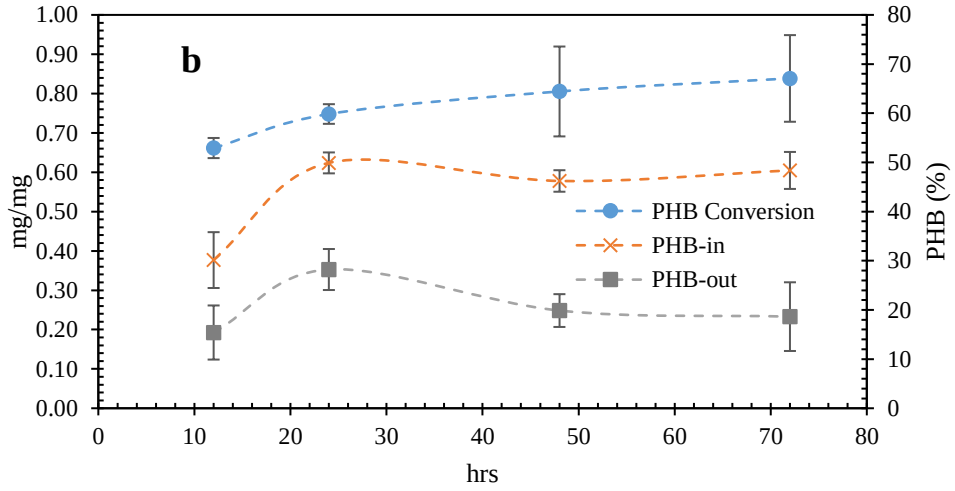


Figure 7.4: a) Methanol concentration and methane conversion efficiency for the biomass harvested at different periods prior to methanol production. b) PHB content at the beginning and the end of the methanol production phase and the PHB mineralization for the biomass harvested at different periods prior to methanol production.

While the low methanol concentrations can be related to the need for adjusting the total cultivation period as previously discussed, the results from these experiments confirms the feasibility of producing methanol in the absence of an external energy source depending on the intracellular PHB. During these experiments, the 12-hours cultivated cells consumed almost 50% of their PHB, as shown in **Figure 7.4b**, compared to only 20% when formate was present in the methanol production step. Moreover, the PHB mineralization was 0.66±0.03 when formate was not present in the methanol step compared to 0.31±0.03 in the formate supplemented medium for the 12-hours cultivated cells. However, the negative effect of cultivation duration was still present where the methane to methanol conversion efficiency decreased from 44±2% to 29±4% when the cells were subjected to nitrogen limited conditions for 12 and 72 hours, respectively as shown in **Figure 7.4b**.

7.3.3 Effect of formate concentration on methanol production by PHB-accumulating cells

The previous experiments explored the effect of the cultivation period on the methanol production of cells having both high and low PHB concentrations and showed the potential of coupling PHB

consumption with the oxidation of methane into methanol. According to those results, the growth period was shortened to only 24 hours and the PHB-accumulation period under nitrogen-limited conditions also for 24-hours. By applying those conditions, the PHB accumulation for *M. hirsuta* was 42±4% prior to the methanol production phase. Its noteworthy to mention that during this experiment the methanol production was extended to 24 hours (8 hours in the previous experiments) to ensure enough time for PHB consumption under different formate concentrations.

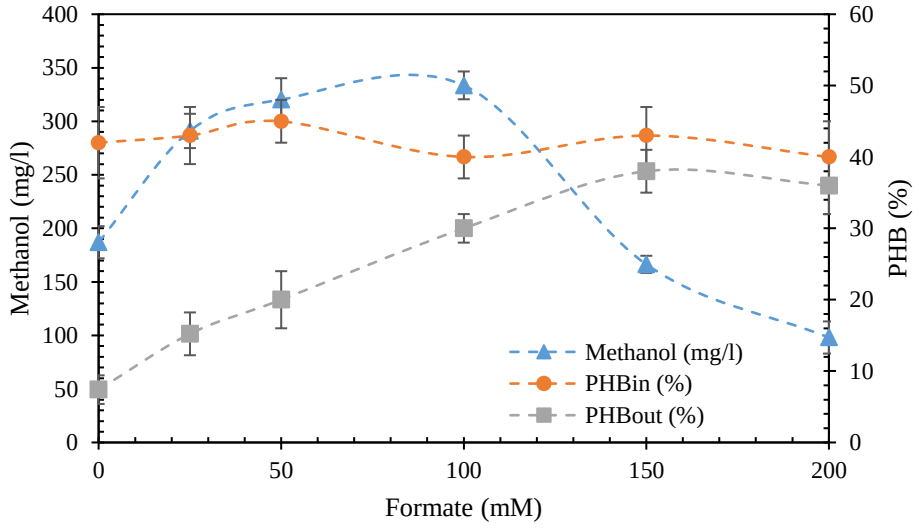


Figure 7.5: Effect of different Formate concentrations on the PHB consumption and the methanol production of *Methylocystis hirsuta*

Generally, As shown in **Figure 7.5**, Applying the new cultivation conditions had a positive effect on the methanol production of *M. hirsuta*. In addition, extending the methanol production period improved the methanol concentration when formate was absent from the reaction medium (relying only PHB for energy supplement). The methanol concentration was 187.04±15 mg/l while the highest concentration achieved during the previous experiment was 124.17±6 mg/l. In addition, almost 80% of the stored PHB was consumed to support methane oxidation. However, addition of formate gradually decreased the amount of PHB consumed where only 25% of the stored PHB

was consumed at 100 mM formate. On the other hand, the increase in methanol concentrations were minor when formate was supplemented at concentrations between 25-100 mM. Considering the amount of PHB consumed at 25mM (65%), this result indicates the positive effect of PHB existence during the methanol production phase where the amount of external carbon can be reduced by 75% while the same methanol production can be achieved. At higher formate concentrations, not only the methanol concentration was negatively affected but also the PHB consumption was stopped.

7.3.4 Effect of methanol production period on PHB consumption under different formate concentrations

For further elaboration on the effect of PHB on the methanol production, three different formate concentrations (0, 25 and 100mM) were tested on cells with and without PHB at different methanol production durations.

For PHB-rich cells having 0 mM formate, PHB consumption was rapidly initiated as shown in **Figure 7.6a**. the highest methanol concentration was achieved after 48 hours and reached 241±15 mg/l while the methane conversion efficiency reached 47.5±4%. In addition, PHB consumption was increasing overtime during the experiment where almost 80% of PHB was utilized by the end of the experiment and the PHB mineralization reached 0.77±0.06 as shown in **Figure 7.6b**.

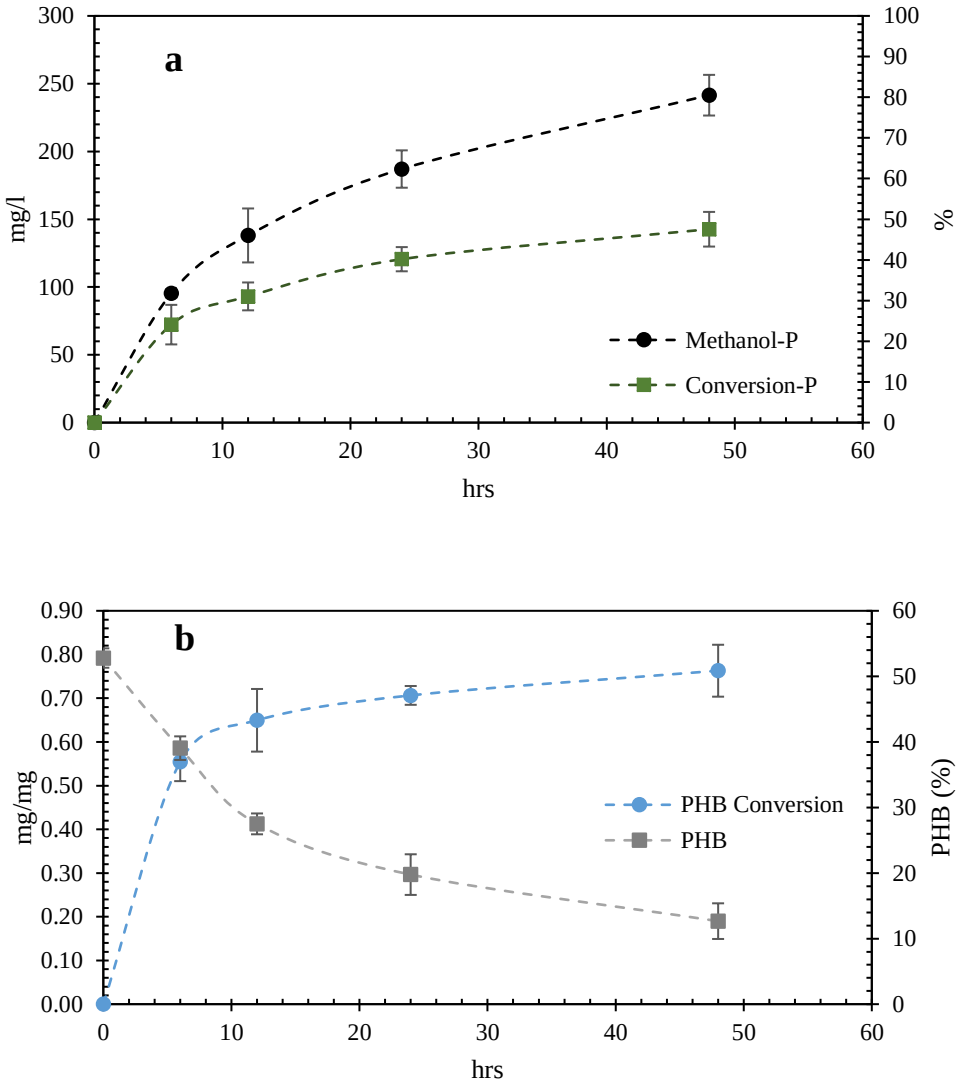
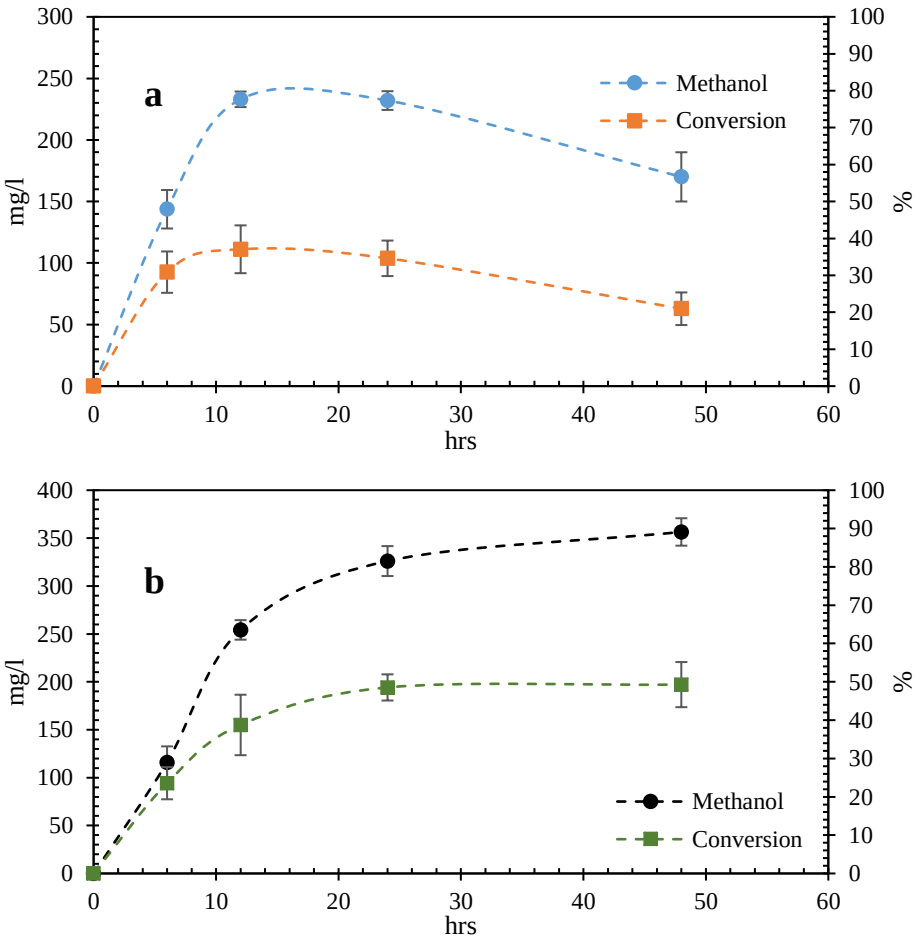


Figure 7.6: a) the methanol concentration and conversion efficiency 0 mM formate. b) changes in PHB content and PHB mineralization over time at 0 mM formate

On the other hand, the positive effect of PHB at formate concentration of 25 mM was apparent. For cells lacking PHB, the maximum methanol concentration was achieved after 12 hours of cultivation. It reached 233±6 mg/l and the conversion efficiency 37±6%. Further increase in the methanol production period did not increase the concentration. Contrarily, the methanol concentration declined to 170±20 mg/l when the duration was extended to 48 hours as shown in **Figure 7.7a**. Interestingly, despite having the same methanol concentration after 12 hours, PHB-

containing cells continued to produce methanol afterwards and the methanol was 326±16 mg/l with a conversion efficiency of 48±4% after 24 hours and further increase in the incubation period allowed the methanol to reach a concentration of 356±14 mg/l after 48 hours. However, the conversion efficiency did not change (**Figure 7.7b**). Similar to the formate free experiment PHB consumption was initiated at the beginning of the cultivation and increased with the increase in the incubation period. By the end of the experiment, 75% of the stored PHB was consumed to support methanol production and the PHB mineralization reached 0.82±16 as shown in **Figure 7.7c**.



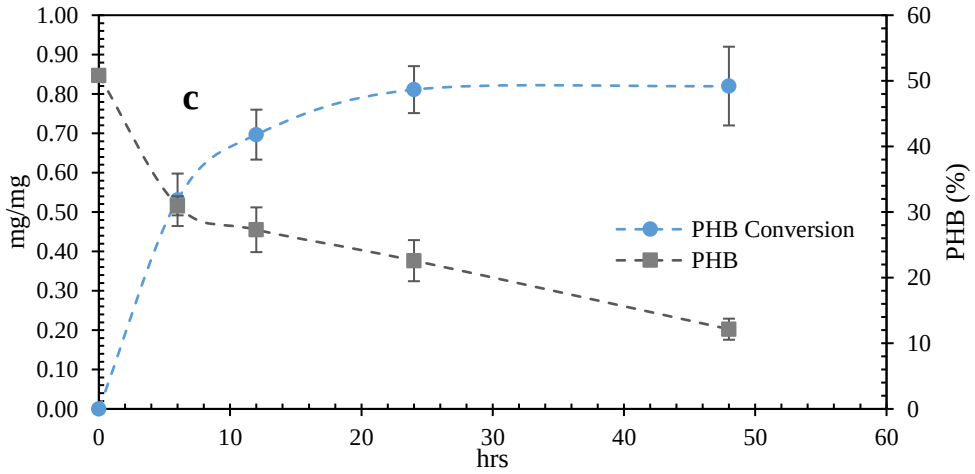
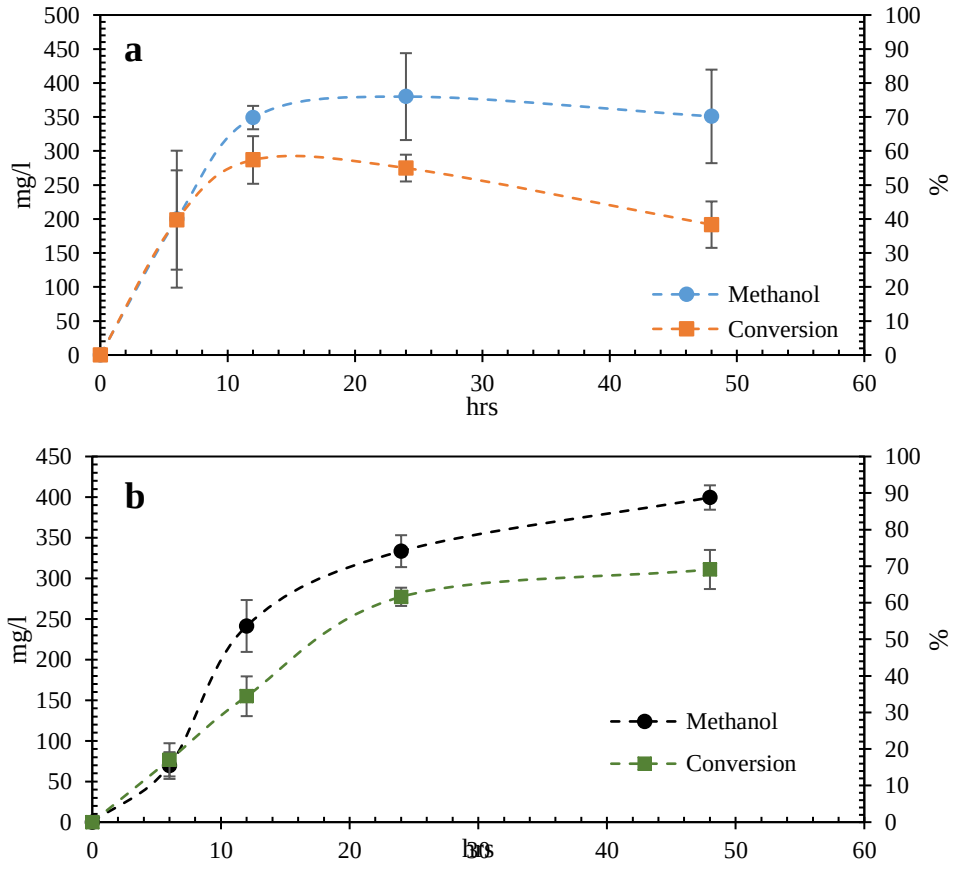


Figure 7.7: a) methanol concentration and conversion efficiency for PHB-lacking cells at 25 mM formate b) methanol concentration and conversion efficiency for PHB-rich cells at 25 mM formate. c) changes in PHB content and PHB mineralization changes over time at 25 mM formate

One of the issues reported in the literature that is associated with methanol production is the decline in the biomass density at the end of the production [155]. Interestingly, this was only observed for PHB lacking cells where the biomass density decreased from 662±30 mg-VSS/l to 476±26 mg-VSS/l by the end of the experiment. Whilst, the biomass density was almost stable for PHB-rich cells at 671±38 mg-VSS/l (VSS from PHB is not taken into consideration).

For the PHB-lacking cells, similar pattern was observed at 100 mM formate to the one observed at 25 mM, however, the methanol concentration was higher. The methanol concentration peaked after 12 hours at a value of 350±18 mg/l with a conversion efficiency of 57±7%. In addition, due to excess availability of external energy source at 100 mM formate, the decline in the methanol concentration was negligible compared to 25mM at extended durations of the experiment as shown in **Figure 7.8a**.When PHB-rich cells were monitored for methanol production overtime at a formate concentration of 100 mM, there was a delay in the methanol production compared to the 25 mM formate conditions at early stages of the experiment. However, As shown in **Figure 7.8b**, the methanol concentration reached 333±19 mg/l after 24 hours of cultivation then increased to

399±15 mg/l after 48 hours with a conversion efficiency of 69±5% which are the highest results among all the experiments performed during this study. In addition, PHB consumption was affected by formate existence at higher concentration where it was delayed compared to the 25 mM as shown in **Figure 7.8c**. However, by the end of the experiment 65% of the stored PHB was consumed and the PHB mineralization reached 0.75±0.02 mg-PHB/mg-CO_2. The same observation for the biomass density stability for the PHB-rich cells was also noticed at this formate concentration, the density of PHB lacking cells decreased from 650±30mg-VSS/l to 450±20mg-VSS/l while the biomass density for PHB-rich cells was constant at 850±38mg-VSS/l.



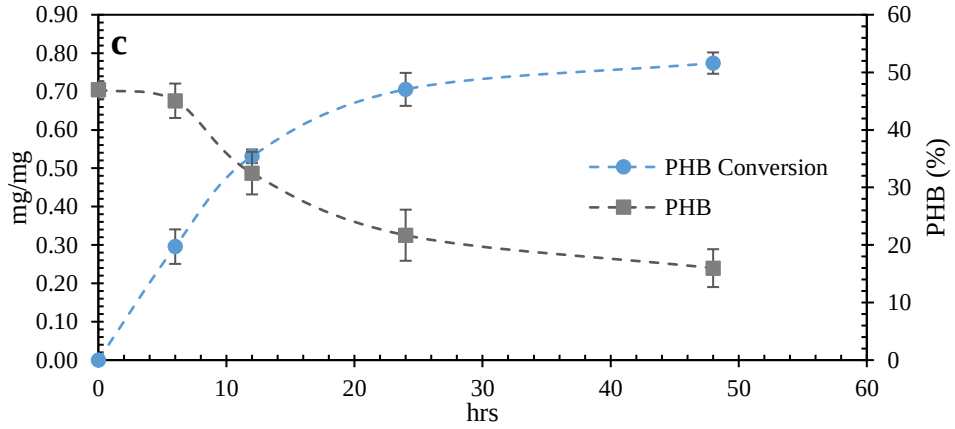


Figure 7.8: a) methanol concentration and conversion efficiency for PHB-lacking cells changes over time at 100 mM formate b) the methanol concentration and conversion efficiency for PHB-rich cells changes over time at 100 mM formate. c) changes in PHB content and PHB mineralization changes over time at 100 mM formate

Within all the formate concentrations employed throughout this set of experiments, it was obvious that PHB consumption is time dependent and increased with increasing the methanol production duration. However, increasing the formate concentration decreased the amount PHB consumed especially at earlier stages of the production. In addition, the results showed that the same methanol concentrations can be achieved at lower formate concentrations or even without adding formate. For instance, PHB rich cells had a methanol concentration of 242±15 mg/l when no formate was supplemented in the reaction mixture while almost the same concentration was achieved (232±5 mg/l) when 25 mM formate was added to PHB lacking cells. Furthermore, PHB rich cells subjected to 25 mM formate could produce up to 356±16 mg/l which is almost 65% more than what have been produced by PHB-lacking cells. To the best of our knowledge, this is the highest methanol concentration attained in the literature with formate addition less than or equal to 25 mM [157].

At 100 mM formate, both types of cells could produce up to 399±15 mg/l throughout the incubation period. It is noteworthy to mention that despite the high methanol concentration that can be achieved by relying on PHB rich cells under lower formate concentration conditions, the

main drawback is associated with the time required to achieve these concentrations. Relying on PHB as an alternative to eliminate or reduce the amount of external energy source i.e. formate would indeed sacrifice with the productivity of the process. However, the aim of these experiments was to demonstrate if its possible for the methanol production to occur while taking advantage of the stored energy inside the cellular biomass but indeed other experiments are required to manipulate other factors that would enhance the productivity of such an approach. For instance, the effect of different types of inhibitors and their concentrations can be tested to enhance the productivity while other PHB accumulating methanotrophic strains rather than *Methylocystis hirsuta* can be employed.

7.4 Conclusions

In this study, methanol production capacity of *Methylocystis hirsuta* was demonstrated over the course of diversified batch experiments. Regardless the PHB content, the methanol production was strongly affected by the previous cultivation duration reflecting sludge age significance in methanol production process design. Increasing the growth period (nitrogen sufficient or limited) decreased the methanol production capacity of the bacteria. Balancing the growth and PHB accumulation durations while allowing sufficient duration for the methanol production phase improved the methanol concentration. While depending on PHB alone as an energy source resulted in lower methanol concentration, PHB-rich bacteria supplemented with 75% less formate had almost the same methanol production. Increasing the methanol production duration for PHB-rich cells increased methanol concentration and PHB consumption regardless the formate concentration unlike PHB-lacking cells. Furthermore, utilizing PHB-rich cells resulted in a stable biomass density during the methanol production phase. Collectively, it can be deduced that the concept of PHB utilization in methanol production has been demonstrated showing its feasibility

in reducing the process cost and enhancing its stability. Nonetheless, further investigations are needed to enhance the productivity of such a process.

Chapter 8

Coupled effects of phosphorus and oxygen limitation on the PHB accumulation capacity of *Methylocystis hirsuta*

8.1 Introduction

Recently, the global attention to the severe impact of plastics on our environment have risen. Images and recordings for suffering animals and marine inhabitants are being released to increase the awareness of such problem. In agreement, many governments took the initiative to ban the single use plastics or add an additional cost for their use as means to decrease the impact of their pollution [158]. It was estimated that 12 million tons of plastics are released annually into oceans where it can take hundreds of years for a plastic bottle to degrade naturally [159].Accordingly, several researchers are now focusing on developing environmentally friendly alternatives that have the required physical and chemical properties [126].

Polyhydroxyalkanoates (PHAs) are biodegradable polymers which can be naturally synthesized while providing competitive physical and chemical properties to the conventional plastics in the packaging, medical and agricultural sectors [10]. PHAs are microbial inclusions which is formed by a wide range of bacteria in response to unbalanced growth conditions as a carbon and energy reserve [126]. Then, these intracellular granules can be harvested and processed to the desired product. Different types of biopolymers from the PHA family can be produced depending on the substrate composition [77], however, polyhydroxybutyrate (PHB) remains the most common one. Untill now the search is still on going to identify not only a cheap substrate that would make the process feasible but also optimum running scenarios that would maximize the yields of the employed biomass.

One of the cheap alternatives that were proposed as a suitable substrate for PHB production is Methane [119]. Methane emissions are often considered as waste and their release to the atmosphere have a 20-30 times worse effect compared to carbon dioxide and considered as the second major greenhouse gas [144]. Thus, employing methane for PHB production has a dual beneficial effect on the environment. For this conversion to occur, a biocatalyst is required and methanotrophic bacteria could play this role. Methanotrophs are unique microorganisms in their capability to utilize methane as their sole carbon and energy source.

Under balanced growth conditions, they assimilate methane through the ribulose monophosphate (RuMP) cycle or the serine cycle or the CBB cycle and based on these cycles methanotrophs are classified. It was assumed that only methanotrophs undergoing the serine cycle (type II) are the main types of methanotrophs involved in the PHB production, however, contradicting studies showed the capability of some methanotrophic strains employing the RuMP cycle (type I) with the ability to accumulate PHB [47]. Nonetheless, most of the studies in the literature agreed on the higher efficiency of type II as PHB producers [10]. Acetyl-CoA plays a central role in PHB-accumulating methanotrophs metabolism. Under balanced growth conditions, it is directed towards the TCA cycle to generate cellular energy requirements. On the other hand, when one or more essential nutrient is absent, Acetyl-CoA is directed towards the PHB cycle where its synthesis is initiated as an alternative reducing equivalent reserve [144].

Developing appropriate strategies for selecting PHB-accumulating methanotrophs is a key milestone for the process scale-up. However, applying those conditions on pure cultures first can anticipate the success or failure of the proposed approach. In most of the studies, the methanotrophic cells are cultivated in the presence of all essential nutrients allowing growth, then

they are transferred to another medium lacking one or more nutrients to induce PHB accumulation [128]. Different nutrients were tested to establish the limitation conditions including (nitrogen, phosphorus, potassium, iron and magnesium) and higher PHB accumulation was always associated with nitrogen limitation [128]. In addition, for long-term operation, higher PHB values were achieved when the growth phase was decoupled from the PHB phase which limits the selective pressure choice if the mixed culture mode of operation is applied [151]. In the coupled mode, when the cells were exposed to cycles of nitrogen limitation, the PHB accumulation decreased overtime [76]. On the other hand, other strategies which are known to induce the selection of other types of PHB accumulating bacteria as feast-famine regime or electron acceptor cycling did not show high PHB production for methanotrophs [76].

While most of the previous studies were focused on nitrogen limitation conditions for PHB accumulation, phosphorus can provide a better alternative. Polyphosphate degradation is associated with the generation of ATP to supply the cells with its energy needs and its consumption is associated with the production of PHB in different microbial genera such as the *Actinobacter* or *Candidatus Accumulibacter* under anaerobic conditions [160]. Interestingly, Poly-P inclusions have been visualized in many of PHB-accumulating methanotrophic strains [161], [162], moreover, they possess the enzymes required for polyphosphates (Poly-P) storage (Poly-P kinases) and even the enzymes required for their degradation (polyphosphatases) [163]–[166]. Collectively, taking advantage methanotrophs ability to accumulate polyphosphates and linking it with the PHB accumulation would lead to new strategies not only for PHB production but also a new selective pressure that could be applied on mixed cultures. However, no data is found in this context and the conditions that would impact the intracellular phosphorus content of methanotrophs are yet unknown. Furthermore, no data is available if there is a link between Poly-P consumption and

PHB synthesis within the methanotrophic cells and if it requires oxygen availability/absence to occur like other polyphosphate accumulating microorganisms (PAOs).

Accordingly, the strain *Methylocystis hirsuta* was employed in this study to discuss its PHB accumulation capacity under phosphorus limited conditions in the presence/absence of oxygen. *Methylocystis hirsuta* is a type II methanotroph and known for its ability to accumulate high levels of PHB that can reach up to 50% [83] and also the presence of polyphosphate inclusions in their cells was evident [162]. In addition, this study aims to elaborate if there is a correlation between the cellular phosphorus content and the PHB accumulation capacity under aerobic/anaerobic conditions. Moreover, to demonstrate the impact of nitrogen source on the phosphorus and PHB storage in the cells. Finally, to develop strategies based on phosphorus limitation where the biomass from growth and PHB accumulation phases are coupled with stable performance.

8.2 Materials and methods

8.2.1 Strain used and Cultivation conditions

The strain *Methylocystis hirsuta* was purchased from Cedarlane labs (Burlington, Canada) and activated as per instructions. Briefly, the bacterial pellet was suspended in 50 ml of the mineral salts medium (MSM) described by (Bowman, 2006) and had the following concentrations of chemicals (mg/l):1000 $MgSO_4.7H_2O$, 200 $CaCl_2.H_2O$, 272 KH_2PO_4, 610 K_2HPO_4, 4 Fe-EDTA and 1 ml Trace metal solution. The initial phosphorus concentration in the MSM was 170 mg-P/l. The trace metal solution had the following concentrations of chemicals (mg/l): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.4H_2O$, 30 H_3BO_3, 3 $Na_2MoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$ and 20 $CoCl_2.6H_2O$. sodium nitrate ($NaNO_3$) or ammonium chloride (NH_4Cl) were added as the sole nitrogen source to the MSM medium with a concentration of 1000 mg/l (NMSM) and 270 mg/l (AMSM),

respectively. 250 µl of Copper were added from a stock solution having a concentration of 500 mg $CuSO_4.5H_2O$/l.

The headspace of the 250 ml serum bottle was evacuated and filled with 100 ml of methane and 100 ml of oxygen (>99.9% purity, Praxair). Every day, the headspace was changed with a new gas mixture and the consumption was monitored. After 4-5 days, growth was observed, then the biomass was divided into six other bottles and resuspended in fresh MSM medium for 48 hours cycles of feeding and wasting till a stable growth rate and gases uptake rates were observed. Afterwards, part of the wasted biomass was used as an inoculum for the upcoming experiments. To induce the PHB accumulation, the other wasted part of the biomass collected after each growth cycle was centrifuged then resuspended in phosphorus free MSM medium under the same operational conditions for 24 hours.

Unless otherwise specified, all the experiments for studying the effect of oxygen, nitrogen source and phosphorus concentration on the total phosphorus (TP) content and the PHB accumulation capacity of *Methylocystis hirsuta* were in a batch mode. The harvested biomass at the end of either the growth phase or the PHB phase of the repeated cycles was centrifuged then incubated in 250 ml serum bottles capped with butyl rubber stoppers. The liquid volume was 50 ml and the headspace was filled with a methane and oxygen (>99.9% purity, Praxair) mixture of volumetric ratio 1:1. If oxygen-limited conditions were established, the same operational conditions were applied except for the headspace gas composition which was filled with a methane and helium (>99.9% purity, Praxair) mixture of volumetric ratio 1:1. A schematic diagram for the general experimental setup employed for this study is presented in **Figure 8.1**.

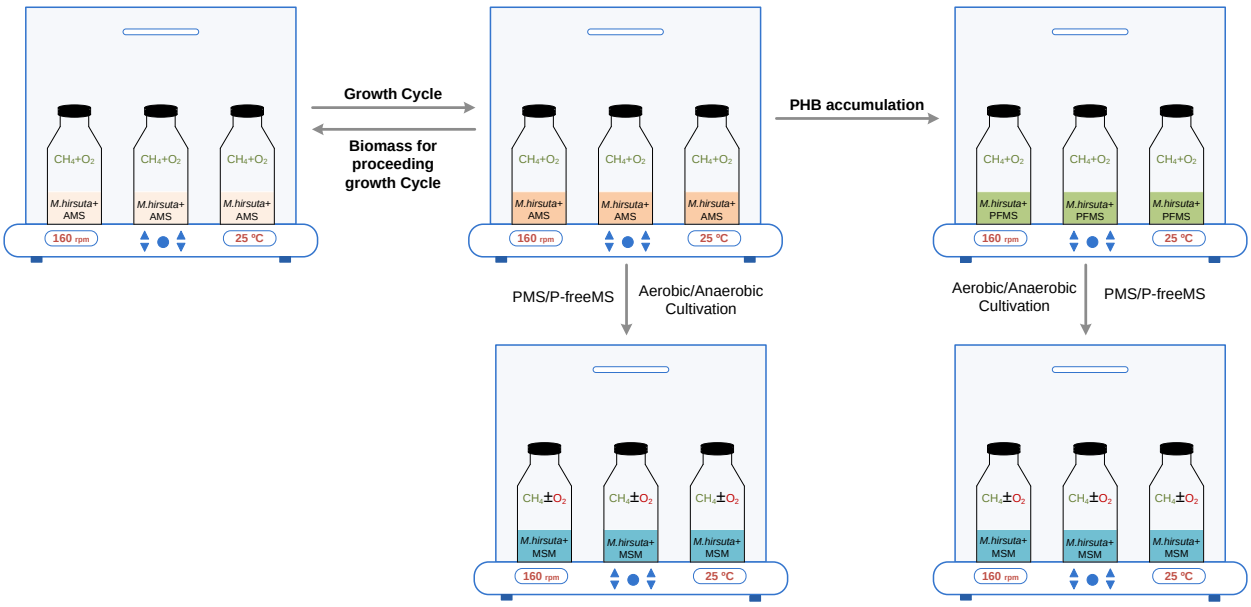


Figure 8.1: Schematic diagram for the experimental setup used to study the PHB accumulation of *Methylocystis hirsuta*

All the experiments were under septic conditions to avoid any contamination and to maintain the culture purity. The temperature was maintained between 25-28°C using table orbital shakers and a speed of 160 rpm. pH was kept between 6-7 using 10% NaOH prepared solution. Samples were taken from the headspace at the beginning and the end of each experiment to monitor the gases consumption and carbon dioxide and, nitrous oxide (in case of oxygen limitation) production. In addition, the growth was observed at the beginning and the end of the experiment by measuring the optical density (O.D). Upon the completion of each experiment, the biomass was collected then centrifuged and the formed pellet was tested for PHB quantification and total phosphorus content while the supernatant was used to estimate nitrate, ammonium and phosphate ions concentrations.

8.2.2 Effect of oxygen limitation on the total phosphorus and PHB contents of *Methylocystis hirsuta*

The aim of this experiment was to explore the conditions that would affect the total phosphorus and PHB levels inside the cells of *Methylocystis hirsuta* in response to oxygen availability. To

monitor the effect of oxygen on the growth and PHB accumulation potential of *Methylocystis hirsuta*, biomass was collected at the end of each growth phase in the repeated cycles. Cells were subjected to four different conditions while the total phosphorus content in the cells was monitored. In the first set, cells were subjected to aerobic conditions in the NMS while in the second set phosphorus was deprived from the growth medium to induce PHB accumulation in the presence of oxygen. In the third set, helium replaced oxygen in the headspace of the culturing bottles to establish anoxic conditions (since NO_3 is present in the NMS). Finally, in the fourth set, phosphorus was eliminated from the NMS in absence of oxygen. The four experiments were running in parallel in batch mode and lasted for 24 hours then the biomass was harvested for further analysis.

8.2.3 Impact of initial PHB content on the growth of *Methylocystis hirsuta*

The aim of this set of experiments is to study the combined effects of initial PHB content and oxygen presence on the TP levels of *Methylocystis hirsuta*. Similar to the previous experiments, four different conditions were applied on the cells harvested after the PHB accumulation phase (from the repeated cycles). The conditions were aerobic with NMS, aerobic with P-free NMS, anoxic with NMS and anoxic with P-free NMS.

8.2.4 Impact of ammonium as nitrogen source on the TP and PHB contents of *Methylocystis hirsuta*

Since nitrate can be utilized by methanotrophs as an alternative electron acceptor in absence of oxygen, ammonium was employed during these set of experiments to establish anaerobic conditions. In addition, to compare the effect of the nitrogen source with the observations from previous experiments. Similar to the previous experiments, four different conditions were applied on the cells harvested after the growth phase (from the repeated cycles). The conditions were

aerobic with AMSM, aerobic with P-free AMS, anerobic with AMS and anaerobic with P-free AMS.

8.2.5 Effect of phosphorus concentration on the TP content and the PHB accumulation under aerobic and anoxic conditions

During this experiment, four different phosphorus concentrations (43, 170, 340 and 680 mg-P/l) were employed. Those concentrations were used to study the PO_4 concentration effect on TP and PHB content for the cells harvested after both the growth and PHB accumulation phases. For both types of biomass aerobic conditions were applied to the cells harvested after the PHB phase while oxygen-limited conditions were applied on cells taken after the growth phase and the TP and PHB levels were monitored under different phosphorus concentrations. Moreover, the cells harvested at the end of each experiment was resuspended in P-free medium for 24 hours for PHB accumulation to examine the effect of the previous cultivation conditions on the PHB accumulation capacity.

8.2.6 Developing operational cycles for PHB accumulation under phosphorus limited conditions

Within these experiments, two operational regimes were designed according to the results obtained from the previous experiments and tested for 14 consecutive days (6 cycles each). In the first operational regime, cells of *Methylocystis hirsuta* having high PHB levels and lower TP content were subjected to aerobic conditions while all other nutrients were present for 24 hours for growth. Afterwards, the cells were resuspended in P-free medium for another 24 hours to re-accumulate PHB again under aerobic conditions then the cycle was repeated. The phosphorus concentration during the aerobic growth phase was 43 mg-P/l while ammonium was employed as the sole nitrogen source with a concentration of 270 mg-N/l. The other regime was running in parallel and was initiated with cells having low PHB accumulation and high TP contents. During the first 24

hours, both phosphorus and oxygen-limited conditions were applied to induce anerobic PHB accumulation then oxygen was supplied for another 12 hours to maximize the PHB content. Afterwards, cells were cultivated in aerobic conditions in the presence of all other nutrients to stimulate their growth.

8.2.7 Analytical methods

Samples were withdrawn using gas tight syringe from bottles headspace then injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD) and Molecular sieve column (Restek, Bellefonte, PA.) to monitor methane, oxygen, carbon dioxide and nitrogen gases concentrations while electron capture detector (ECD) was used for nitrous oxide monitoring. The temperature program was as following: injector, 60^{o}C; Oven, 80^{o}C; TCD, 80^{o}C and helium gas was used as carrier gas with flowrate of 15 mL/min. External calibration curves were constructed using a known mixture of gases and used to convert the measured peak areas into gases concentrations and masses. For bacterial growth monitoring, optical density (O.D) was measured using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). Correlation curves between O.D and volatile suspended solids (VSS) were developed. Nitrogen and phosphorus ions (NH_4, NO_2, NO_3, and PO_4) were measured using a Dionix Integrion HPIC Ion chromatography system (Thermo Fischer Scientific Massachusetts, United States). The total phosphorus content (TP) was estimated by collecting the biomass at the end of each experiment. The biomass was then centrifuged and washed three times before using the Hach measurement kit for total phosphorus (HACH Company, Loveland, Colorado, USA) as per manufacturer protocol.

PHB was measured using the method developed by[110]. 10-15 mg of lyophilized biomass were collected and 2 ml of acidified methanol (3% sulphuric acid) and 2 ml of chloroform were added

in a glass vial. After gentle mixing, the cocktail was heated at 100 ºC for 3.5 hours then left to cool down to room temperature. Afterwards, 1 ml of deionized water was added and the mixture was vortexed for 1 minute and then left until phase separation was achieved. The lower organic phase was tested for biopolymers quantification using Agilent 7890a gas chromatography equipped with a flame ionization detector (Agilent Technologies, California, United States) and DB-wax column (Restek, Bellefonte, PA.). The temperature program was as following; 1 min 80 º C, 10 º C/min, 180 º C for 4 min. Results were compared to standard curves obtained using PHB standards (Sigma Aldrich). Benzoic acid was used as an internal standard to increase accuracy and maintain a control for all errors. Afterwards, the amount of the obtained PHB was divided by the VSS concentration to estimate the PHB accumulation of the bacterial culture.

8.3 Results and discussions

8.3.1 Effect of oxygen availability on the TP and PHB levels of *Methylocystis hirsuta*

After being activated, cells of *Methylocystis hirsuta* have been adapted to continuous growth cycles. At the end of the 24 hours growth phase, part of the cells (10% vol/vol) were resuspended in fresh medium having all the essential nutrients. The other remaining cells were then cultivated in phosphorus free medium to initiate PHB accumulation. The experiments performed on the cells collected at the end of either the growth phase or the PHB accumulation phase did not start until a stable performance was observed where the fluctuations in the growth rates, biomass yields, methane and oxygen uptake rates , PHB production and yields were below 10%. After almost 30 days of applying the mentioned conditions, the growth rate of *Methylocystis hirsuta* was $0.12\pm0.02 hr^{-1}$ and the PHB reached 40±5% under the P-free conditions before starting the upcoming experiments.

The TP in the harvested cells from the repeated growth cycles was measured prior to the experiments and was found to be 2.45±0.15% (mg-P/mg-VSS). It's worthy to mention that the PHB content in the cells was low (8.5±0.5%) as expected since they were collected after the growth phase of the repeated cycles. As shown in **Table 8.1**, when the cells were exposed again to normal growth conditions where methane, oxygen and all the other nutrients were present (experiment 1), the biomass density increased from 698±15 mg-VSS/l to 1163±12 mg-VSS/l and the biomass yield was 0.61±0.02 mg-VSS/mg-CH_4. On the other hand, the TP content in the biomass increased from 2.45±0.05% to reach 3.07±0.1% by the end of the experiment. In addition, the phosphorus mass balance performed for PO_4 consumed and TP content increase was in agreement and the closure error was 6.2±0.9%. The PHB content was barely changed and it was 11±2% at the end of the experiment.

Table 8.1: Carbon and Phosphorus mass balances for biomass harvested after growth phase in the presence of nitrate

Experiment	**1**	**2**	**3**	**4**
Harvest cycle	Growth			
Oxygen	aerobic		anoxic	
Carbon-balance (mg-C)				
Biomass (mg-C)	12.3±0.08	4.51±0.34	5.58±0.011	2.29±0.93
PHB (mg-C)	2.29±0.17	12.35±0.5	-0.37±0.001	2.41±0.5
CH_4 (mg-C)	-28.6±0.88	-28.61±0.43	-16.76±0.9	-8.92±1.2
CO_2 (mg-C)	14.9±0.04	14.7±1.02	13.24±1.5	4.74±0.25
Closure	0.93±0.6	2.94±0.25	1.69±0.63	0.52±0.48
PHB_i (%)	8.3±2	8.2±2	7.8±1.5	8.9±1
PHB_f (%)	11±2	37±3	4.9±2	17±2

PHB yield (mg-PHB/mg-CH_4)	0.11±0.02	0.58±0.03	-	0.36±0.09
Phosphorus balance (mg-P)				
PO_4 (mg-P)	-0.86±0.045	-0.35±0.02	-0.45±0.045	0.44±0.025
TP (mg-P)	0.92±0.089	0.42±0.03	0.49±0.05	0.39±0.022
Closure	0.06±0.13	-0.076±0.003	0.046±0.051	0.05±0.045
TP_i (%)	2.45±0.05	2.42±0.13	2.3±0.05	2.65±0.12
TP_f (%)	3.07±0.1%	0.97±0.07	2.82±0.09	1.26±0.09

When the cells were deprived from phosphorus under similar operational conditions (experiment 2), PHB accumulation was initiated as expected (**Table 8.1**). The PHB content increased from 8.2±2% to reach 37±3% by the end of the experiment with a yield of 0.58±0.03 mg-PHB/mg-CH_4. Contrarily, the TP levels in the biomass decreased from 2.42±0.13% to reach 0.97±0.07% by the end of the cultivation where phosphorus was detected in the liquid phase of the culturing medium at with a concentration of 22±5 mg-PO_4/l.

During the oxygen-limited (anoxic) experiments, similar behavior was observed to the previous experiments but at lower rates as illustrated in **Table 8.1**. When *Methylocystis hirsuta* was subjected to anoxic conditions and all other nutrients (including phosphorus) were present (experiment 3), the TP content increased from 2.3±0.05% to reach 2.82±0.09% and the PHB content was slightly decreased to 4.9±2% by the end of the experiment. However, unlike the aerobic conditions, the increase in the biomass density was almost 50% of the increase observed under aerobic conditions. It was previously reported that different types of methanotrophic strains have the ability to switch their terminal electron acceptor from oxygen to either nitrate or nitrite under anoxic conditions as a survival strategy which can be supported by the lower biomass yield observed under these conditions (0.44±0.03 mg-VSS/mg-CH_4) [144]. In addition, it was recently

reported that *Methylocystis hirsuta* possess all the required genes to perform partial denitrification to convert nitrate into nitrous oxide which supports the detection of N_2O (data not shown) found in the headspace by the end of the experiment [167]. Despite the lower methane uptake rate observed during this experiment 0.93±0.14 mg-CH_4/hr compared to 1.59±0.2 mg-CH_4/hr under aerobic conditions there was still slight increase of almost 25% in the TP content under both conditions.

On the other hand, when the cells of *Methylocystis hirsuta* were subjected to the limitation of both phosphorus and oxygen (experiment 4), PHB accumulation was still observed but also at lower rates compared to aerobic conditions. As shown in **Table 8.1**, the PHB levels increased from 8.9±1% to 17±2% while the PHB yield was 0.36±0.09 mg-PHB/mg-CH_4. Interestingly, there was still a decrease in the TP content under these conditions and the TP content decreased 2.65±0.12% to 1.26±0.09%. As the phosphorus stored in the biomass cells (mainly polyphosphates) is considered as source of energy (ATP) which is also required for the PHB synthesis, further investigations should consider their role in PHB-accumulating methanotrophs as almost the same amount of TP was consumed under both conditions (aerobic and anoxic) while different PHB amounts were accumulated.

8.3.2 Effect of initial PHB level on the growth of *Methylocistis hirsuta* under aerobic and anoxic conditions

These set of experiments aimed to investigate the effect of applying the exact four condition of oxygen and phosphorus presence and absence to cells having high PHB levels. During these experiments, cells were harvested after the end of the PHB accumulation phase of the repeated cycles and their PHB was as high as 36±1%. Interestingly, when the TP content of those cells was

measured it was only 1.06±0.06% compared to 2.45±0.15% when the cells harvested after the growth phase in the previous experiments (1-4).

Table 8.2: Carbon and Phosphorus mass balances for biomass harvested after PHB accumulation phase in the presence of nitrate

Experiment	**5**	**6**	**7**	**8**
Harvest cycle	PHB			
Oxygen	aerobic		anoxic	
Carbon-balance (mg-C)				
Biomass (mg-C)	17.64±1.41	1.91±1.53	7.76±0.85	1.85±0.07
PHB (mg-C)	-3.39±0.5	10.87±0.42	-1.38±0.56	2.17±1.28
CH_4 (mg-C)	-32.9±2.17	-29.46±1.68	-20.78±1.17	-10.56±0.47
CO_2 (mg-C)	18.20±1.71	15.69±0.48	11.52±1.29	5.01±0.21
Closure	-0.53±0.45	-0.98±2.31	-2.96±0.4	-1.53±0.53
PHB_i (%)	37±2	36.6±3	37±3	35.7±3
PHB_f (%)	15.4±2	47±4	25±4	36±2
PHB yield (mg-PHB/mg-CH_4)	-	0.5±0.03	0.66±0.2	0.27±0.15
Phosphorus balance (mg-P)				
PO4 (mg-P)	-1.66±0.06	0.072±0.01	-1.06±0.011	0.04±0.01
TP (mg-P)	1.45±0.04	-0.091±0.001	0.91±0.05	-0.02±0.003
Closure	-0.2±0.016	-0.018±0.01	-0.15±0.04	0.02±0.01
TP_i (%)	1.06± 0.013	1.04±0.01	1.08±0.09	1.04±0.06
TP_f (%)	2.78±0.12	0.7±0.05	2.84±0.12	0.87±0.05

When the PHB-containing cells were cultivated in aerobic conditions and all the nutrients were present, there was a general improvement in the growth conditions compared to PHB-lacking cells running under the same conditions (experiment 5). As shown in **Table 8.2**, PHB was consumed

and the PHB levels decreased from 37±2 to 15±3. The biomass density increased from 680±30 mg-VSS/l to 1300±220 mg-VSS/l while the biomass yield reached 0.76±0.01 mg-VSS/mg-CH4 compared to 0.58±0.03 mg-VSS/mg-CH_4 when the cells had lower PHB content. It was previously reported that PHB mainly functions as a source for reducing equivalents for methanotrophs and PHB existence supported higher methane uptake rate for *Methylocystis parvus* OBBP [57]. Similarly, the methane uptake rate of *Methylocystis hirsuta* increased from 1.59±0.1 mg-CH_4/hr (experiment 1) to 1.84±0.12 mg-CH_4/hr in the presence of PHB. On the other hand, the TP content increased from 1.06± 0.013% to reach 2.78±0.12% which is almost double the increase observed for PHB-lacking cells. The PHB accumulation of *Methylocystis hirsuta* cells subjected to aerobic conditions in P-free medium (experiment 6) continued where the PHB increased from 36.6±3% to reach 47±4% by the end of the experiment. On the other hand, the TP content was not affected during this experiment where the TP content was initially 1.04±0.01% and slightly decreased to 0.7±0.05% at the end of the experiment.

Under oxygen-limited conditions, when the PHB-rich cells were cultivated in nutrient-sufficient conditions (experiment 7), PHB consumption was also initiated. The PHB content decreased from 37±3% to reach 25±4% after 24 hours of cultivation as shown in **Table 8.2**. While the activity was still lower compared to aerobic conditions, PHB existence increased the methane uptake rate by almost 33% compared to PHB-lacking cells under anoxic conditions. The methane uptake rate increased from 0.9±0.05 (experiment 3) mg-CH_4/hr to 1.2±0.04 mg-CH_4/hr when PHB when present in the cells of *Methylocystis hirsuta*. In addition, the TP content in the cellular biomass increased from 1.08±0.09% to 2.84±0.12% which is similar to the increase observed for PHB-rich cells under aerobic conditions (experiment 5) and double the amount accumulated by PHB-lacking cells under the same anoxic conditions (experiment 3).

Finally, cultivating the PHB-rich cells under both phosphorus and oxygen limited conditions (experiment 8) resulted in the least activity observed during all the experiments performed. Not only was the methane uptake only 0.5±0.05 mg-CH4/hr but also the PHB was not consumed or produced and stabilized at 36±2%. In addition, the TP content did not significantly change and was 0.87±0.05% at the end the experiment unlike the PHB lacking cells (experiment 4) where they had higher TP contents and a relatively higher microbial activity.

8.3.3 Effect of oxygen availability on the TP and PHB of cells cultivated with ammonium as nitrogen source

To explore if nitrogen source has an implication on the observations found during the previous experiments, nitrate was replaced with ammonium for the cells harvested after the end of the growth phase of the repeated cycles. The aforementioned four conditions were experimented on *Methylocystis hirsuta* cells having TP and PHB contents of 2.57±0.08% and 13.7±2%, respectively. As illustrated in **Table 8.3**, when the cells were suspended in a medium having all the essential nutrients under aerobic conditions (experiment 9), growth was initiated where the biomass density increased from 730±30 mg-VSS/l to 1265±0.4 mg-VSS/l while the biomass yield was 0.69±0.03 mg-VSS/mg-CH_4. Similar to the experiments running under the same conditions using nitrate (experiment 1), the TP content changed from 2.6±0.1% to 2.9±0.08% and the PHB slightly declined to 10±1%. Under P-free conditions (experiment 10), PHB accumulation was initiated as expected and it reached 40±3% at the end of the experiment and the yield was 0.54±0.05 mg-PHB/mg-CH_4. PO_4 was again detected in the medium with a concentration of 26 mg-P/l accompanied with a decrease in the TP content from 2.58±0.07 to 1.31±0.03%. While the behavior under both nitrogen sources was the same, a slight improvement in the biomass yield and PHB production was observed when ammonium was employed. In addition, the conducted experiments under aerobic conditions confirmed the changes in the TP levels (accumulation and

consumption) during both the growth and PHB accumulation phases independent of the nitrogen source applied.

Table 8.3: Carbon and Phosphorus mass balances for biomass harvested after growth phase in the presence of ammonium

Experiment	**1**	**2**	**3**	**4**
Harvest cycle	Growth			
Oxygen	aerobic		anoxic	
Carbon-balance (mg-C)				
Biomass (mg-C)	14.2±0.88	-0.05±0.64	0.27±1.03	-0.29±1.14
PHB (mg-C)	0.74±0.64	10.3±0.45	3.67±0.99	4.96±0.85
CH_4 (mg-C)	-28.9±0.11	-25.63±1.1	-9.03±0.06	-9.48±1.14
CO_2 (mg-C)	16.04±0.12	16.56±0.82	4.63±0.62	4.27±0.38
Closure	2.05±0.25	1.18±1.8	-0.45±0.72	-0.54±1.23
PHB_i (%)	13.83±2	13.7±1	13.59±1	13.64±1
PHB_f (%)	10±1	40±3	24.78±3	28.75±2
PHB yield (mg-PHB/mg-CH_4)	-	0.54±0.03	0.44±0.2	0.57±0.15
Phosphorus balance (mg-P)				
PO4 (mg-P)	-0.85±0.25	0.42±0.01	0.68±0.08	0.6±0.06
TP (mg-P)	0.88±0.035	-0.47±0.075	-0.61±0.05	-0.54±0.006
Closure	0.031±0.21	-0.054±0.075	0.074±0.13	0.06±0.06
TP_i (%)	2.6±0.1	2.58±0.07	2.56±0.06	2.56±0.07
TP_f (%)	2.9±0.08	1.31±0.03	0.92±0.05	1.13±0.06

Interestingly, an opposite behavior was observed under oxygen-limited (anaerobic) conditions where ammonium presence had a significant effect on the cells cultivated with or without phosphorus. In the previous experiments, nitrate had a dual role by providing the required nitrogen

source and an alternative electron acceptor, however, ammonium supported only the first role and anaerobic conditions were present during these experiments. As shown in **Table 8.3**, in the presence/absence of phosphorus (experiment 11 &12), cellular TP declined and PHB was accumulated in the presence of ammonium unlike the nitrate-fed cells were TP decrease was only observed in P-free conditions. The TP content decreased from 2.56±0.04% to 1.03±0.03% while the PHB increased from 13±3% to 27±4% and the PHB yield was still high at 0.55±0.1 mg-PHB/mg-CH_4 under both conditions. Despite the lower PHB accumulation observed under oxygen-limited conditions, the observed TP consumption (P-release) activity during this experiment in combination with the TP accumulation (P-uptake) under aerobic conditions would be of great interest not only to employ methanotrophs for nutrients removal but also to develop strategies to select for PHB-accumulating methanotrophs in mixed cultures.

8.3.4 Effect of phosphorus concentration on TP content and PHB accumulation of *Methylocystis hirsuta*

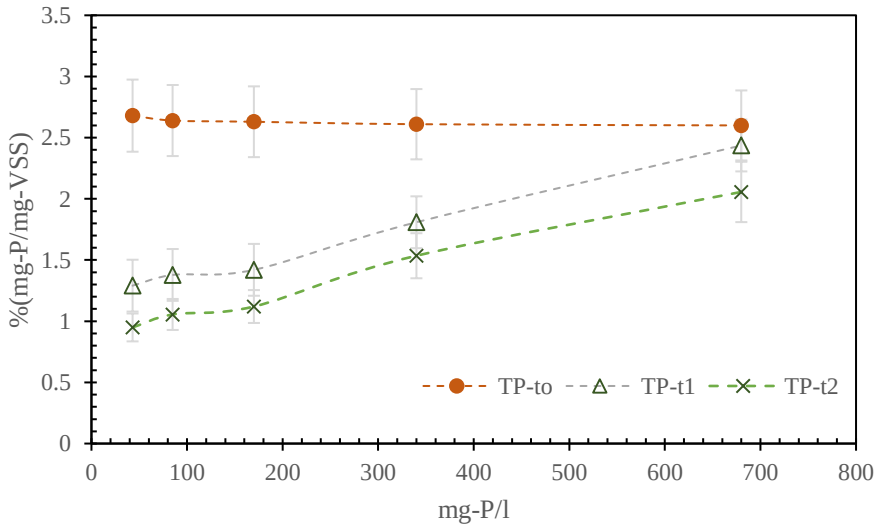
Based on the results obtained from the previous experiments, ammonium was employed as the nitrogen source for the rest of the experiments. In addition, the impact of different phosphorus concentrations under anaerobic conditions were tested on the cells harvested at the end of the growth phase of the repeated cycles. At the start of the experiment, cells had a TP content of 2.63±0.07% while the PHB was 17±2%. As shown in **Table 8.4**, no growth was observed under the oxygen-limited as expected. The methane uptake rate was 0.63±0.02 mg-CH_4/h at PO_4 concentration of 43mg-P/l, afterwards, a decline in the methane uptake was observed with the increase in phosphorus concentration at it was only 0.14±0.01 mg-CH_4/hr at 680 mg-P/l. Previous studies also revealed the negative effect of elevated phosphorus concentrations on the methane oxidation rates more specifically on the methane monooxygenase enzyme [155]. In addition, the negative effect was apparent on the TP content, cells cultivated at lower phosphorus concentrations

consumed higher amounts of their cellular TP. At 43 mg-P/l, the TP content decreased from 2.63±0.08% to 1.29±0.12 while the TP content was almost stable, and it was 2.44±0.05% at 680 mg-P/l. On the other hand, the highest PHB content was also in agreement with the decrease in the TP levels and the PHB was 38±3% at 43 mg-P/l which shows the negative effect of PO_4 at higher concentrations.

Table 8.4: Carbon and Phosphorus mass balances under different PO_4 concentrations in absence of oxygen

PO4 concentration (mg-p/l)	**43**	**170**	**340**	**680**
Harvest cycle	Growth			
Oxygen	anaerobic			
Carbon-balance (mg-C)				
Biomass (mg-C)	-0.83±0.3	-1.28±0.19	-1.87±0.8	-2.31±0.26
PHB (mg-C)	5.35±0.36	3.37±0.95	0.5±0.05	-0.21±0.5
CH_4 (mg-C)	-11.37±0.32	-6.97±0.37	-3.64±0.24	-2.53±0.01
CO_2 (mg-C)	7.32±0.09	5.35±0.0.042	5.2±0.23	4.67±0.1
Closure	0.46±0.25	0.48±0.42	0.19±0.82	-0.39±0.23
PHB_i (%)	16±2			
PHB_f (%)	36±2	28±3	20±2	17±3
PHB yield (mg-PHB/mg-CH_4)	0.59±0.05	0.54±0.03	0.18±0.2	-
Phosphorus balance (mg-P)				
PO4 (mg-P)	0.368±0.02	0.43±0.4	0.27±0.04	0.056±0.06
TP (mg-P)	-0.4±0.07	-0.39±0.01	-0.29±0.01	-0.024±0.001
Closure	-0.039±0.029	0.04±0.4	0.024±0.038	0.096±0.2
TP_i (%)	2.63±0.1			
TP_f (%)	1.29±0.08	1.38±0.03	1.81±0.15	2.41±0.06

To study the effect of the applied conditions on aerobic PHB accumulation, cells at the end of the anaerobic cultivation under different PO_4 concentrations were harvested and resuspended in P-free medium under aerobic conditions. As shown in **Figure 8.2a**, the cells were influenced by their previous cultivated conditions. the cells harvested from lower phosphorus concentrations had higher PHB accumulation levels when subjected to P-free conditions unlike the cells harvested from higher phosphorus concentrations. The cells cultivated at 43 mg-P/l were able to elevate their PHB from 38±3% to 52±4% under P-free conditions. In addition, *Methylocystis hirsuta* cells subjected to 680 mg-P/l had a final PHB accumulation of 33±4% under P-free conditions. The increase in the PHB content was almost the same for all the cells (20%), the decrease in the TP content was also the same as shown in **Figure 8.2b**. Contrarily, the PHB yield for the cells harvested from lower phosphorus concentration cultivations were higher. The PHB yield was at its maximum for the 43 mg-P/l cells at 0.48±0.14 mg-PHB/mg-CH_4 compared to only 0.22±0.06 mg-PHB/mgCH_4 for the 680 mg-P/l cells when resuspended in P-free medium.



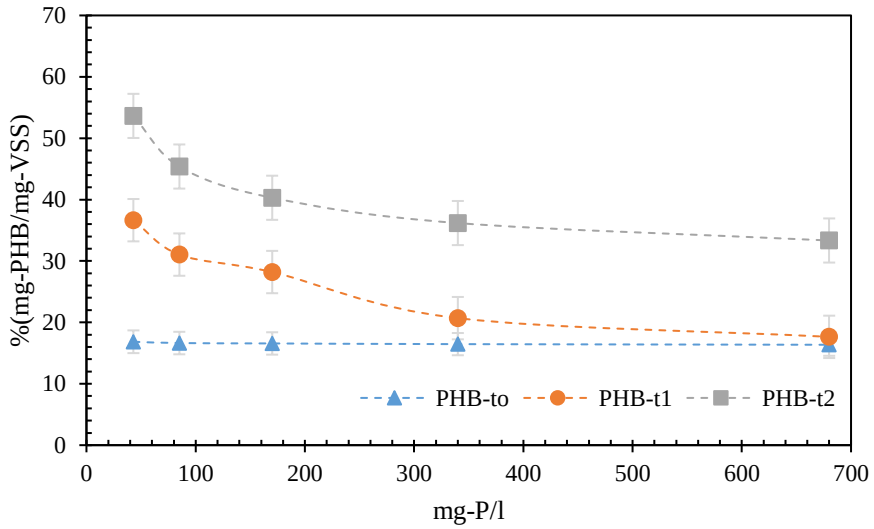


Figure 8.2: Change in a) TP contents and b) PHB contents during different phases where t_o is the start of the experiment, t_1 is the end of the anaerobic phase and t_2 is the end of the PHB accumulation phase

As the previous experiment examined the effect of phosphorus concentration under oxygen limitation, another set of experiments explored their effects under aerobic conditions. During this experiment, cells were harvested after the PHB accumulation phase of the repeated cycles where the TP content was 1.61±0.09% and the PHB was 40±4%. As shown in **Table 8.5**, the highest microbial activity was observed between 43-170 mg-P/l where the methane uptake rate was 1.61±0.1 mg-CH_4/hr and the biomass yield was 0.64±0.09 mg-VSS/mg-CH_4. Further increase in the phosphorus concentration had a negative effect on the growth of *Methylocystis hirsuta* where the methane uptake rate and biomass yield decreased to 0.84±0.08 mg-CH_4/hr and 0.12±0.03 mg-VSS/mg-CH_4, respectively at 680 mg-P/l.

Table 8.5: Carbon and Phosphorus mass balances under different PO_4 concentrations in the presence of oxygen

PO4 concentration (mg-p/l)	**43**	**170**	**340**	**680**
Harvest cycle	PHB			
Oxygen	aerobic			
Carbon-balance (mg-C)				
Biomass (mg-C)	15.2±0.36	11.26±0.29	5.55±0.51	1.23±0.86
PHB (mg-C)	-4.03±0.46	-4.64±1	-3.35±0.45	-2.11±0.04
CH_4 (mg-C)	-30.42±0.4	-27.63±1.12	-16.38±0.13	-15.07±0.13
CO_2 (mg-C)	16.62±0.95	20.23±0.32	15.77±1.23	13.72±0.1
Closure	-2.68±1.47	-0.77±1.55	1.59±1.41	-2.23±1.1
PHB_i (%)	40±2			
PHB_f (%)	13±1	14±3	23±2	32±2
PHB yield (mg-PHB/mg-CH_4)	-	-	-	-
Phosphorus balance (mg-P)				
PO4 (mg-P)	-0.8±0.2	-1±0.11	-0.34±0.19	-0.128±0.38
TP (mg-P)	0.87±0.02	0.85±0.02	0.26±0.015	0.038±0.014
Closure	0.073±0.31	-0.22±0.08	-0.082±0.17	-0.09±0.39
TP_i (%)	1.61±0.03			
TP_f (%)	2.41±0.01	2.75±0.09	1.91±0.02	1.6±0.04

In addition, the results show that the cells were still able to increase their TP content at lower phosphorus concentrations where the TP content increased from 1.61±0.08% to 2.41±0.06% at 43 mg-P/l. Further increase in the phosphorus concentration increased the TP content to 2.75±0.09% at 170 mg-P/l then the TP content declined at higher concentrations due to methane oxidation inhibition where it decreased to 1.61±0.04% at 680 mg-P/l. The growth observed under

aerobic conditions was accompanied with PHB consumption and also the cells grown at concentrations between 85-170 mg-P/l exhibited the highest PHB consumption where it decreased from 40±4% to 15±2% while it declined to only 32±3% at 680 mg-p/l at the end of the experiment as shown in **Figure 8.3a**

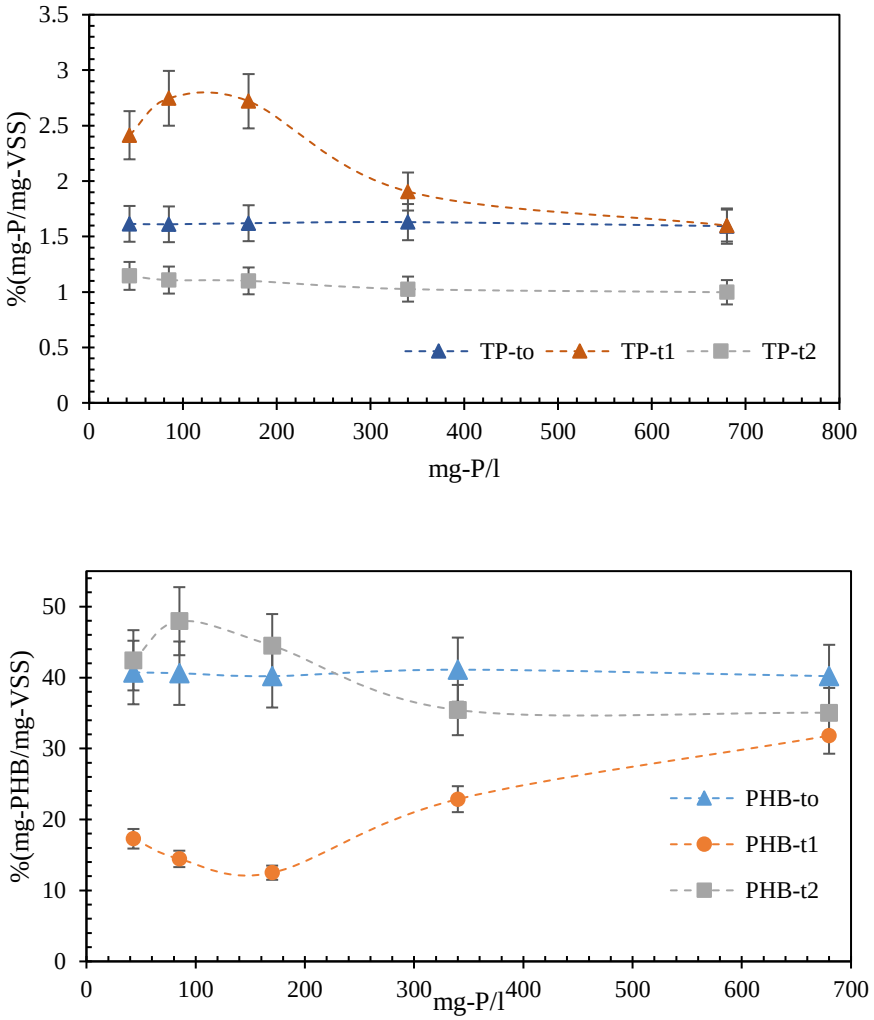


Figure 8.3: Change in a) TP contents and b) PHB contents during different phases where t_o is the start of the experiment, t_1 is the end of the anaerobic phase and t_2 is the end of the PHB accumulation phase

When the harvested cells grown at different P-concentrations subjected to P-free conditions, PHB accumulation was restored. The PHB accumulation was again affected by the biomass activity in the previous phase. As shown in **Figure 8.3b**, the highest PHB accumulation was associated with

the biomass having the highest TP content and both the PHB accumulation and TP consumption occurred simultaneously. The PHB accumulation for the cells harvested from the 43 mg-P/l experiment elevated from 13±3% to 42±2% while the TP content decreased from 2.41±0.08% to 1.17±0.04% at the end of the experiment. On the other hand, the PHB accumulation of the 680 mg-P/l harvested cells slightly increased from 32±3% to 35±1% and the TP decreased from 1.38±0.07% to 1±0.12%. it's worthy to mention the behavior during the PHB accumulation phase for the cells harvested from the oxygen and oxygen-limited applied conditions were completely different. The cells harvested from the oxygen-limited conditions had almost the same increase in the amount of PHB accumulated and the decrease in the TP content unlike the cells harvested from the aerobic conditions. However, the maximum PHB accumulation was almost the same for biomass which require further investigations to address the exact function of phosphate metabolism in methanotrophs as previously mentioned.

8.3.5 Strategies to maintain the high PHB accumulation by *Methylocystis hirsuta* under phosphorus limited conditions

Based on the collected information from the previous experiments, two operational cycles were tested during this experiment to maintain high PHB production depending on the changes in the TP levels observed. In the first strategy, when the cells were subjected to consecutive periods of phosphorus availability and limitation under aerobic conditions, a stable PHB accumulation was achieved at the end of each cycle. The methane uptake rate was 1.82±0.22mg-CH_4/hr during the aerobic growth phase while the biomass yield was 0.52±0.06 mg-VSS/mg-CH_4. As shown in **Figure 8.4**, The PHB accumulation reached 50±2% at the end of the P-limited phase and the yield was 0.51±0.035 mg-PHB/mg-CH_4. In addition, the TP content was increasing during the growth phase where it reached 2.66±0.05% before declining back to 1.18±0.06% under P-free conditions during PHB accumulation. Previous studies on other PHB-producing methanotrophic strains

subjected to continuous cycles of nitrogen-sufficient and limited phases reported a decrease in PHB production over time and the proteins synthesis (nitrogen storage) increased instead [76]. Contrarily, as shown in this study, phosphorus limitation and relying on the cellular TP resulted in a stable performance and PHB accumulation overtime.

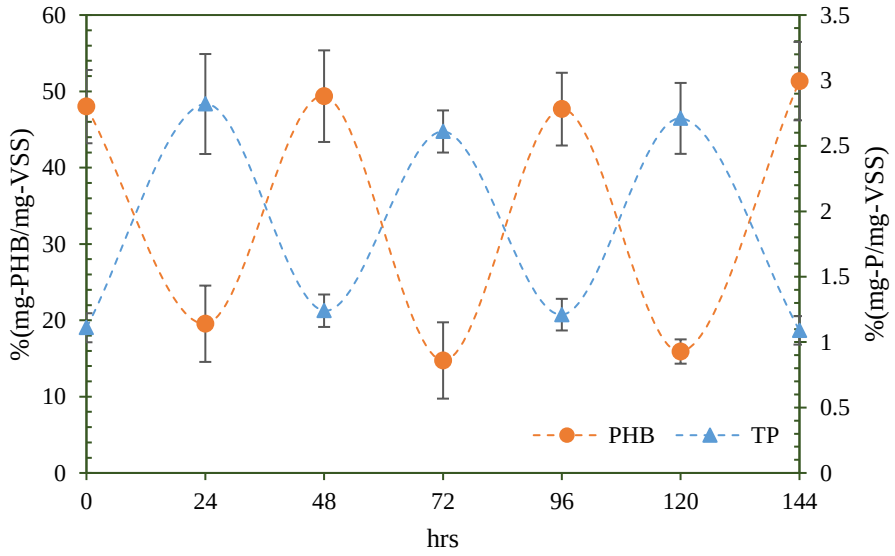


Figure 8.4: Changes in TP content and PHB levels during the operation of four consecutive cycles in strategy 1

On the other hand, the second operation strategy was based on the observed ability of *Methylocystis hirsuta* to accumulate PHB under oxygen limited condition relying on TP consumption. Nonetheless, the cycles were initiated by *Methylocystis hirsuta* cells having high TP content and low PHB, and their values were 2.99±0.08% and 15±1%, respectively. At the start of the oxygen-limited phase, the TP contents decreased to 1.79±0.07% while the PHB increased to 26±2% with an average yield of 0.55±0.05 mg-PHB/mg-CH_4. During the next phase, when oxygen was supplied under P-free conditions, PHB accumulation resumed with the support of the continued TP consumption and it reached 49±2% with a yield of 0.44±0.04 mg-PHB/mg-CH4 while TP was 0.84±0.09% at the end of this phase as shown in **Figure 8.5**. During the growth phase of this cycle

strategy the methane uptake rate was similar to the first one and the uptake was 1.7±0.1 mg-CH_4/hr and the biomass yield was 0.49±0.06 mg-VSS/mg-CH_4. both strategies achieved almost the same amount of PHB eventually, in addition, the values and yields of PHB are in agreement with the values reported for *Methylocystis hirsuta* in the literature [40], [83]. However, the implication of such strategies would be of great importance as a selective pressure for PHB-accumulating methanotrophs in a mixed culture or if a methane-based nutrient removal technology is desired.

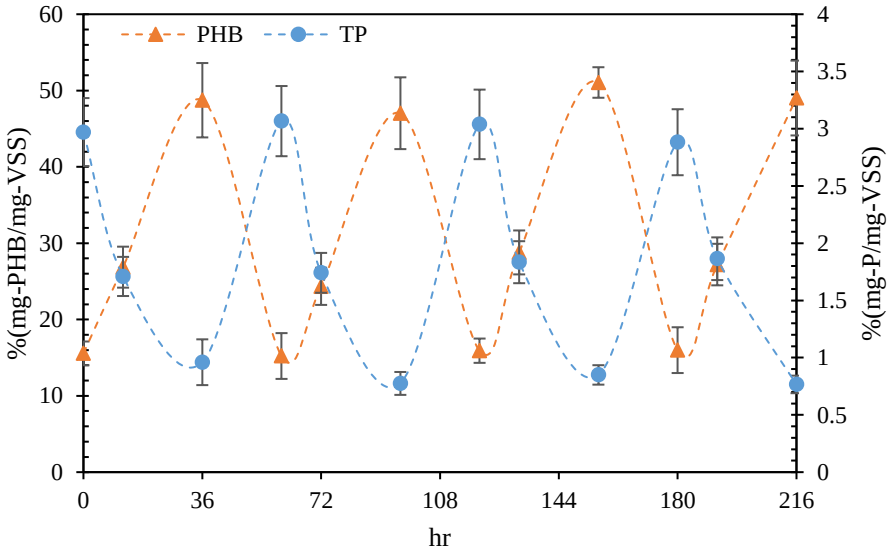


Figure 8.5: Changes in TP content and PHB levels during the operation of four consecutive cycles in strategy 2

8.4 Conclusions

The conducted experiments illustrated the impact of cellular phosphorus content on the Growth and PHB accumulation capacity of *Methylocystis hirsuta* with or without oxygen. In addition, the ability of *Methylocystis hirsuta* to uptake or release phosphates to support their PHB accumulation was demonstrated for the first time. The effect of initial TP and PHB contents was obvious and the choice of nitrogen source had a significant effect on the TP consumption especially under oxygen-limited conditions where only the consumption was observed when ammonium was supplied as

the nitrogen source. Regardless the nitrogen sources *Methylocystis hirsuta* was still able to accumulate PHB in the absence of oxygen. Increasing the phosphorus concentration had a negative effect on the methane uptake rate and the activity of *Methylocystis hirsuta*, however, at concentrations as low as 43 mg-P/l the cells were still able to uptake relatively high amounts of phosphorus. The developed strategies were successful to develop cycles relying on both the PHB and TP to have a stable PHB production overtime.

Chapter 9

Conclusions and Future directions

9.1 Conclusions

Methanotrophs-based biopolymers production can contribute to overcoming major concerns threatening the environment. Firstly, the feedstock i.e. methane employed for the proposed technology is a major greenhouse gas contributing to the global warming phenomena. Secondly, the produced biopolymers from this process can substitute the petroleum-based plastics in different applications with the advantage of being biodegradable at their end-life. Furthermore, these biopolymers can be anaerobically degraded back to biogas to create a circular lifecycle. Finally, WWTPs can provide all the requirements for developing biogas-based biopolymers technologies and support resources recovery from waste streams.

The success of employing methane for PHB production will mainly depend on maintaining the selection of PHB accumulating methanotrophs on a long-term basis. Applying selective pressures should consider minimizing any inhibition that would hamper the PHB accumulation capacity of methanotrophic bacteria. The main conclusions from the conducted work were as following:

- Increasing ammonium concentration had a negative effect on both the growth rate and the biomass yields of type II methanotrophs enrichments and controlling the biomass density can mitigate this negative effect.
- Three factors should be taken into consideration while designing a methanotrophic bioreactor targeting type II methanotrophs which are F/M, C/N and N/M ratios. The F/M ratio mainly affected the specific growth rate of type II methanotrophs while the effect on the observed biomass yield was lower. Both the C/N and the N/M affected the growth rate

and biomass yield as well as the competition between methane and ammonium for MMO oxidation.

- Combining the adjustment of the C/N, F/M and N/M rations with the SRT control resulted in a stable mixed culture with high PHB accumulation capacity. However, switching the nitrogen source from ammonium to nitrate resulted in the invasion of non-producing PHB methanotrophs.
- Changing the seed source from WAS to AD sludge affected the lag phase as well as the microbial community composition.
- Despite the similar PHB accumulation achieved by WAS and AD enrichment, the later had the advantage of higher methane biodegradation kinetics due to the higher abundance of type II methanotrophs within the mixed culture.
- PHBV content is strongly affected by the concentration of VFAs (co-substrate concentration) while the methane to oxygen ratio has an obvious effect on the biopolymer composition (HV fraction).
- The potential of integrating methanotrophs into WWTPs to recover PHB from biogas was demonstrated All other growth requirements can be supplied from WWTPs to develop a sustainable microbial culture and feedstock within the wastewater and waste stream. without having a significant inhibition on their activity.
- Ammonium concentration in the AD centrate can be diluted to support the growth of type II methanotrophs in the mixed cultures.
- Biogas successfully supported the growth of methanotrophs during both the growth and PHB phases.

- The FST effluent increase the final biomass density and establish the conditions required to induce PHB accumulation.
- Methanotrophs growth and PHB accumulation on AD centrate or FST effluent was associated with complete nitrogen removal from the wastewater used.
- A novel concept using the stored PHB inside the cells of *Methylocystis hirsuta* to support methanol production was demonstrated.
- PHB consumption can mediate the methane to methanol conversion step and can reduce the reliance on external carbon source by almost 75% while achieving the same methanol concentrations.
- the linkage between the intracellular Phosphorus consumption and PHB production was investigated for the first time.
- Intracellular-P storage supported PHB accumulation under anaerobic conditions for *M.hirsuta*.
- Increasing PO4 concentration (above 100mg-P/l) had a negative effect on the methane uptake rate and the activity of *M.hirsuta*.
- At PO_4 concentrations as low as 43 mg-P/l the cells were still able to uptake relatively high amounts of phosphorus (3%).
- The developed strategies were successful to develop cycles relying on both the PHB and TP to have a stable PHB accumulation overtime.

9.2 Future work directions

- Employing the deduced ammonium-based selection strategies for type II methanotrophs at a larger scale bioreactor.

- Deep molecular studies on the existing microorganisms within the mixed culture and the syntrophic activities among them.
- Studying the long-term effect of employing biogas, AD centrate and FST effluent tank on maintaining the selection of type II methanotrophs as well as their PHB accumulation capacity.
- Exploring the effect of supplementing methanotrophs with waste fermentation products i.e.VFAs to produce different types of biopolymers from the PHA family.
- Studying the effect of the aforementioned conditions on the quality of the produced biopolymer.
- Optimizing the PHB to methanol conversion to improve the process productivity and studying the capability of other methanotrophic strains or mixed cultures.
- Investigating the effect of other inhibitors as well as their concentration on the PHB-mediated methanol production.
- Applying the conditions studied during the phosphorus experiment on other methanotrophic strains and mixed cultures.
- Performing molecular studies to confirm the activity of the enzymes responsible for polyphosphates synthesis and degradation and the response of PHB related enzymes to establish a metabolic model for the observation of this study.

10 List of References

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Appendix

A.1 Significance and correlation of the ammonium effect study

The significance of the effects of ammonium concentration, copper, F/M, C/N, N/Mi ratios on the specific growth rate, biomass yield, CH4 utilization rate, O/M and N/Mo ratios are summarized in **Table A.1**

Table A.1: Results of Pearson correlation test for the batch studies in Chapter 3

	Ammonium		**C/N**		**F/M**		**N/M_i**		**Copper**	
	P	**ρ**	**P**	**ρ**	**P**	**ρ**	**P**	**ρ**	**P**	**ρ**
C/N	0.139	-0.756			0.041	0.551	0.006	-0.574		
F/M	0.116	-0.785	0.099	0.413			0.440	0.178		
N/M_i	0.007	0.968	0.020	-0.558	0.232	0.341				
μ	0.012	-0.952	0.042	0.499	0.008	0.677	0.063	-0.413	0.005	-0.798
Y	0.014	-0.949	0.037	0.509	0.153	0.403	0.028	-0.480	0.036	-0.284
q	0.002	-0.987	0.106	0.406	0.001	0.798	0.274	-0.250	0.102	-0.803
O/M	0.040	0.896	0.233	-0.306	0.496	0.199	0.004	0.601	0.105	0.902
fs	0.013	-0.952	0.036	0.512	0.149	0.407	0.027	-0.481	0.646	-0.282
fe	0.013	0.952	0.036	-0.512	0.149	-0.41	0.027	0.481	0.646	0.282
N/M_o	0.003	0.980	0.060	-0.466	0.025	-0.59	0.000	0.895	0.643	0.973

A.2 Waste Activated Sludge characteristics

Waste activated sludge was obtained from Humber wastewater treatment plant located in Toronto, Canada and used for methanotrophs enrichment in this study with the following characteristics summarized in **Table A.2**

Table A.2: Characteristics of the waste activated sludge

Parameter	Value
TCOD (mg/l)	2192
sCOD (mg/l)	398
TS (mg/l)	6380
VS (mg/l)	4660
TSS (mg/l)	5964
VSS (mg/l)	4554
Nitrate (mg-N/l)	0.7
Ammonia (mg-N/l)	27.6
Total Phosphorus (mg/l)	66.1
pH	6.67

A.3 Molecular Biology analysis

The RNA extraction was performed using RNeasy Mini Kit QIAGEN from kit's protocol in "Purification of Total RNA from Bacterial Lysate." The steps are demonstrated as following, 10 ml of RNA protect Bacteria Reagent was added to 5 ml of bacterial suspension then mixed by vortexing for 5 seconds. The mix was incubated at room temperature at 22 °C for 5 minutes, after that the mix was centrifuged for 10 mins at 5000 rpm. The resulting supernatant was decanted and then, 200 µl of TE Buffer was added containing Lysozyme to lyse all of the cell debris and cell wall. Next, 10 seconds of vortex every 2 mins during incubation at room temperature for 5 mins. Followed by the addition of 700 µl of RLT Buffer with β-mercaptoethanol for RNA stabilization, then the mixture was vortexed for 10 seconds. 500 µL of 96% ethanol was then added and mixed. For RNA purification, 700 µl of Lysate was transferred to an RNeasy Mini spin column placed in a 2-ml collection tube then centrifuged for 15 seconds at 10000 rpm. The flow through was then discarded. Then, 700 of µl RW1 Buffer was added to the RNeasy Mini spin column followed by 15 seconds centrifugation at 10000rpm, the flow through was then discarded and collected in a tube. The RNeasy mini spin column was placed in a new collection tube where 500 µl of RPE buffer was added to the column and centrifuged for another 15 seconds afterwards the flow through was discarded then the collection tube was reused and 500 µl of RPE Buffer was added to the

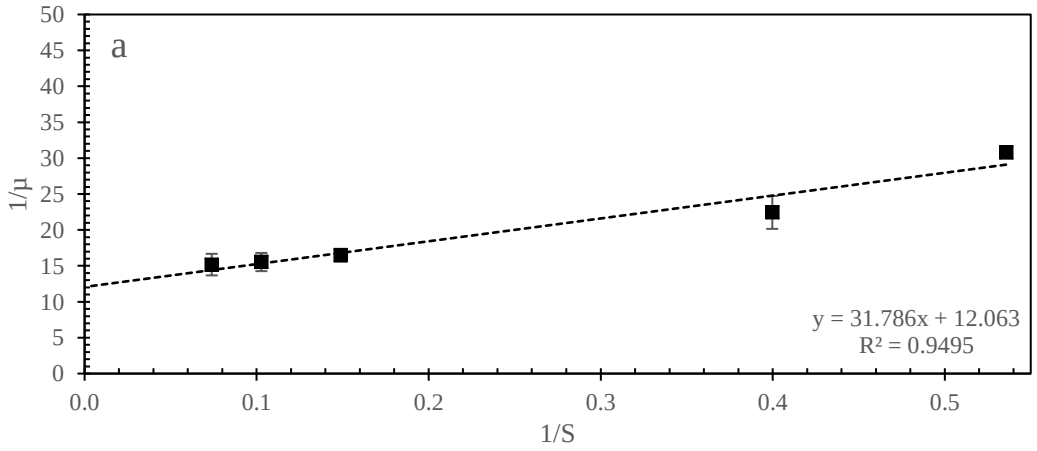
column then centrifuged for 2 mins. To wash the spin column membrane at the same speed. The RNeasy mini spin column was placed in a new 1.5 ml collection tube. Then 40 µl of RNase free water was placed directly to the spin column membrane then subjected to 1 min centrifugation at the same speed to elute the RNA.

Samples preparation was performed using one step RT-PCR QIAGEN kit following the instructor's protocol. The reaction mix was prepared and contained the following components added in the following order; 22 µl RNase-free water, 10 µl QIAGEN OneStep RT-PCR Buffer (5x), 10 µl 5x of Q-Solution, 2 µl dNTP mix, 1.5 µl Type I Primer, 1.5 µl Type II Primer, 2 µl QIAGEN One Step RT-PCR Enzyme Mix except for the 1 µg template RNA which was added directly to the PCR tubes. the thermal cycler was programmed according to the manufacturer's instructions with the melting temperature (T_m=50ºC) adjusted according to the employed primers containing the 50 µL master mix PCR tubes.

The target DNA was amplified using RT-PCR which confirms the presence of Type I, Type II Methanotrophs then products were separated for visualization using gel electrophoresis.

Briefly, 150 ml of 1% TAE buffer was prepared. Then, 1.5 g of agarose was dissolved in the TAE buffer with 3 µl of ethidium bromide before decanting the mix into the gel mold for 20 mins to dry. The gels were examined for fluorescent bands using an alpha imager amended with 2 µl dye (Eva green). In the first lane, 12 µl of RNA sample including 2 µl dye were injected 10 µl of 1 kb ladder then a current of 100 A and 50 v was implied for 1 hour at 60ºC before taking the images.

A.4 Methane biodegradation Kinetics



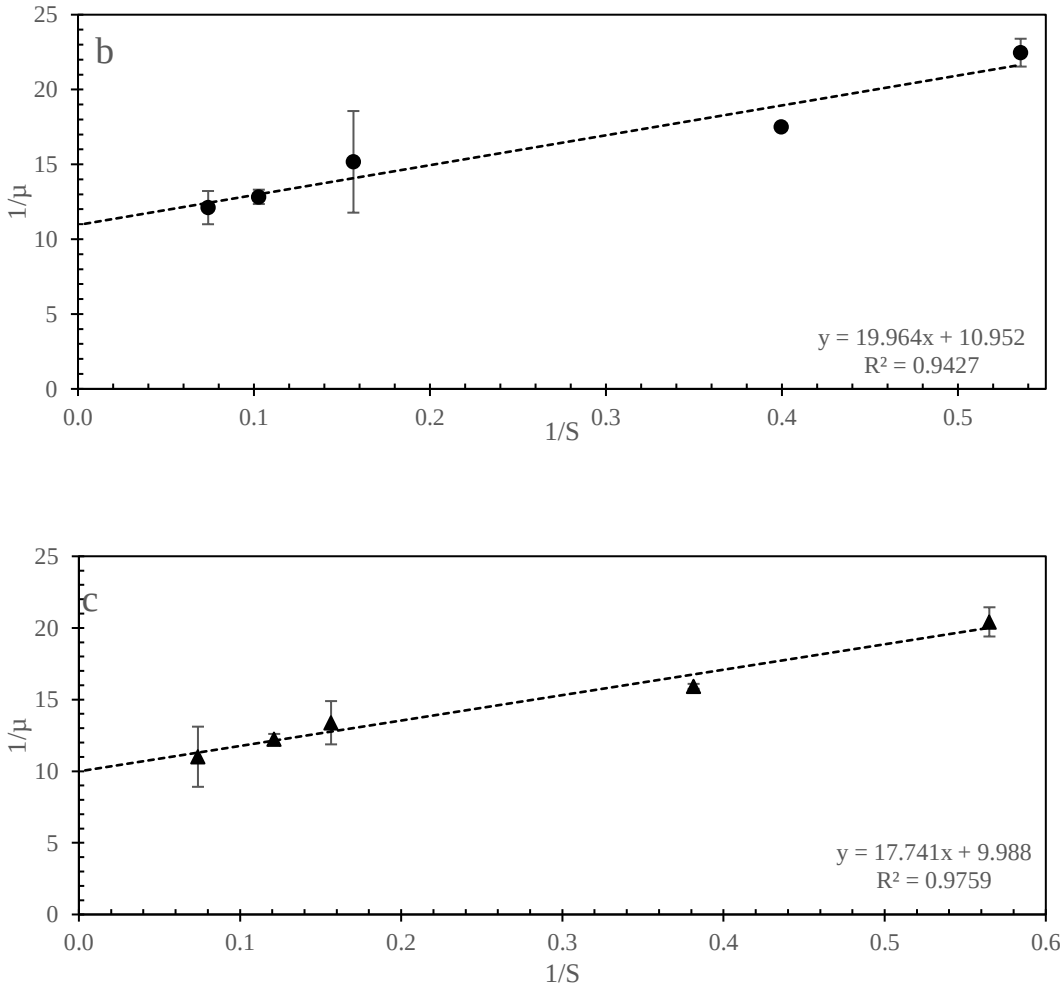


Figure A.1: Lineweaver-Burk correlation plot for a) cycle 8, b) cycle 23 and c) cycle 32

Table A.3: Calculated kinetic parameters for the biomass collected at cycles 8, 23 and 32

Parameter	**Cycle 8**	**Cycle 23**	**Cycle 32**
μ_{max} (hr^{-1})	0.083±0.009	0.091±0.004	0.1±0.01
q_{max} (mg-CH_4/mg-VSS-hr)	0.1±0.01	0.13±0.01	0.14±0.03
Ks (mg-CH_4/l)	2.63±0.3	1.82±0.2	1.78±0.2
Y (mg-VSS/mg-CH_4)	0.81±0.03	0.71±0.02	0.7±0.05

A.5 Significance and correlation of the O.D and M/O effect on PHB accumulation

The significance of the effects of O.D and M/O ratio on the PHB content and yieldare summarized in **Table A.4**

Table A.4: Results of Pearson correlation test for the batch studies on PHB in Chapter 5

		P-value	**ρ**
O.D	**PHB**	0.006	-0.714
	Yield	0.274	-0.328
M/O	**PHB**	0.0033	-0.772
	Yield	0.0026	-0.782

A.6 Significance and correlation of the valeric acid concentration and M/O effect on PHBV accumulation

The significance of the effects of Valeric acid concentration and M/O ratio on the PHBV content and yieldare summarized in **Table A.5**

Table A.5: Results of Pearson correlation test for the batch studies on PHBV in Chapter 5

		P-Value	**ρ**
Valeric acid	**PHBV**	0.004	-0.645
	HV	0.005	0.632
	Yield	0.0001	-0.789
M/O	**PHBV**	0.023	0.453
	HV	0.021	-0.458

A.7 Nutrients concentration in AD centrate

Table A.6: Concentration of nutrients in AD centrate

Chemical	Concentration
sCOD (mg/l)	300
NH_4 (mg-N/l)	532
NO_3 (mg-N/l)	10
NO_2 (mg-N/l)	0.2
PO_4 (mg /l)	120
pH	7.2

A.8 Significance and correlation of the Biogas and AD centrate experiment

The significance of the effects of using Biogas, AD centrate or both of them on different growth parameters are summarized in **Table A.7**

Table A.7: Results of Pearson correlation test for the batch studies on the effect of biogas and AD centrate in Chapter 6

	P-value	**ρ**
μ	0.125	-0.588
Y	0.120	-0.594
q	0.914	0.046
Biomass density	0.088	-0.640
CH_4 uptake	0.292	0.427
O_2 uptake	0.153	0.555

A.9 Significance and correlation of the AD centrate content experiment

The significance of the effect of AD centrate content on different growth parameters are summarized in **Table A.8**

Table A.8: Results of Pearson correlation test for the batch studies on the effect of AD centrate content in Chapter 6

	P-value	**ρ**
μ	3.64E-06	-0.970
Y	0.00019	-0.917
q	0.009389	-0.769
Biomass density	0.000461	-0.895
CH_4 uptake	7.1E-05	-0.935
O_2 uptake	9.27E-05	-0.931

A.10 Calculations used for the estimation of different parameters

$$\frac{F}{M} = \frac{CH4i}{VSSi} \quad \textbf{(1)}$$

Where F/M is the food to microorganisms ratio (mg-CH_4/mg-VSS), CH_{4i} is the initial amount of methane in the headspace (mg) and VSS_i is the initial amount of biomass in the liquid phase (mg).

$$\frac{C}{N} = \frac{CH4i}{NH4i} \quad \textbf{(2)}$$

The C/N is the carbon to nitrogen ratio (mg-CH4/mg-N), CH_{4i} is the initial amount of methane in the headspace (mg) and NH_{4i} is the amount of ammonium in the liquid phase (mg-N).

$$(\frac{N}{M})i = \frac{NH4i}{VSSi} \quad \textbf{(3)}$$

N/M_i is the initial nitrogen to microorganisms ratio (mg-N/mg-VSS), NH_{4i} is the amount of ammonium in the liquid phase (mg-N) and VSS_i is the initial amount of biomass in the liquid phase (mg).

$$\frac{M}{O} = \frac{CH4i}{O2i} \quad \textbf{(4)}$$

M/O is the methane to oxygen ratio (mg-CH_4/mg-O_2), CH_{4o} and O_{2o} are the initial amounts of methane and oxygen in the headspace (mg) respectively.

$$\mu = \frac{(\boldsymbol{O.Df - O.Di})/\boldsymbol{t}}{\boldsymbol{O.Di}} \quad \textbf{(5)}$$

Where μ is the specific growth rate (hr^{-1}), $O.D_f$ is the optical density of the biomass at the end of the experiment, $O.D_i$ is the optical density of the biomass at the start of the experiment and t is the experiment duration in hours.

$$\boldsymbol{Yob = \frac{VSSf - VSSi}{CH4f - CH4i}} \quad \textbf{(6)}$$

Y_{ob} is the observed biomass yield (mg-VSS/mg-CH_4), VSSf and VSSi are the initial and final biomass densities respectively, CH_{4i} and CH_{4f} are the initial and final amounts of methane in the head space respectively.

$$\boldsymbol{q = \frac{\mu}{Yobs}} \quad \textbf{(7)}$$

Where q is the methane utilization rate (mg-CH_4/mg-VSS.hr), μ is the specific growth rate (hr^{-1}) and Y_{obs} is the observed biomass yield (mg-VSS/mg-CH_4).

$$\boldsymbol{\left(\frac{N}{M}\right)o = \frac{NH4f - NH4i}{VSSf - VSSi}} \quad \textbf{(8)}$$

N/M_o is the observed ratio between nitrogen consumption and biomass generation (mg-N/mg-VSS), NH_{4i} and NH_{4f} are the ammonium concentrations at the start and the end of experiment respectively, VSS_i and VSS_f are the biomass densities at the start and the end of the experiment respectively.

$$\boldsymbol{\frac{O}{M} = \frac{O2f - O2i}{CH4f - CH4i}} \quad \textbf{(9)}$$

O/M is the molar oxygen to methane consumption ratio, O_{2i} and O_{2f} are the initial and final mass of oxygen in the headspace (mmol), CH_{4i} and CH_{4f} are the initial and final mass of methane in the headspace respectively.

$$\boldsymbol{CH4\ (O2)uptake\ rate = \frac{CH4f - CH4i\ or(O2f - O2i)}{t}} \quad \textbf{(10)}$$

CH4 or O2 uptake rate is the methane or oxygen uptake rate (mg/hr), t is the duration of the experiment in hours.

$$\boldsymbol{PHB\ content = \frac{PHB}{VSSt} x\ 100} \quad \textbf{(11)}$$

PHB content (%) is the amount of PHB in the cellular biomass, PHB is the concentration in mg/l and VSS_t is the total concentration of biomass and PHB (mg/l)

$$\boldsymbol{PHB\ yield = \frac{PHB}{CH4f - CH4i}} \quad \textbf{(12)}$$

PHB yield (mg-PHB/mg-CH_4) is the amount of PHB produced (mg-PHB) per amount of substrate (methane consumed) where CH_{4i} and CH_{4f} are the masses of methane (mg) in the headspace at the start and the end of the experiment respectively.

$$\boldsymbol{methanol\ conversion\ efficiency = \frac{CH3OH}{CH4f - CH4i} x100} \quad \textbf{(13)}$$

The methanol conversion efficiency (%) is calculated by dividing the amount of methanol produced (mmol) by the amount of methane consumed (mmol), CH_{4i} and CH_{4f} are the masses of methane (mg) in the headspace at the start and the end of the experiment respectively.

$$\boldsymbol{PHB\ mineralization = \frac{PHBf - PHBi}{CO2}} \quad \textbf{(14)}$$

PHB mineralization is amount of PHB used for CO_2 production (mg-C/mg-C), PHB_i and PHB_f are the masses (mg-C) at the start and the end of the experiment respectively. CO_2 is the total mass of C-CO_2 produced during the experiment duration and the chemical formula of PHB is $C_4H_6O_2$.

$$\boldsymbol{TP\ content = \frac{TP}{VSS} x100} \quad \textbf{(15)}$$

Where TP is the total phosphorus concentration (mg-P/l) and VSS is the biomass density (mg-VSS/l).

A.11 Raw data for the conducted experiments

Table A.9: Raw Data for Chapter 3 experiments

Exp.	Duration (hrs)	Ni (mM)	Ni (mg-N)	Copperi (µM)	CH_{4i} (mg-CH4)	CH_{4i} (mM CH4)	O_{2i}(mg-O2)	O_2i (mmol-O2)	Biomassi (mg-VSS)	C/N (mg-CH4/mg-N)	F/M (mg-CH4/mg-VSS)	N/Mi(mg-N/mg-VSS)	OD (in)	OD (f)	µ (hr-1)
AM-5	44	5	3.5	5	49.11	3.07	116.7	3.65	10.2	14.1	4.79	0.34	0.43	1.34	0.048
AM-10	44	10	7	5	48.99	3.06	116.0	3.63	8.21	7.00	5.96	0.85	0.34	1.02	0.045
AM-20	44	20	14	5	50.56	3.16	119.7	3.74	9.83	3.61	5.14	1.42	0.41	1.04	0.035
AM-40	44	40	28	5	50.86	3.18	113.8	3.56	9.64	1.82	5.27	2.90	0.40	0.7	0.017
AM-80	44	80	54	5	49.26	3.08	112.2	3.51	13.8	0.91	3.55	3.89	0.58	0.78	0.008
AM-5	40	5	3.5	5	42.33	2.65	104.7	3.27	16.4	12.0	2.57	0.21	0.69	1.18	0.018
AM-10	40	10	7	5	43.83	2.74	105.6	3.30	15.3	6.26	2.85	0.46	0.64	1.13	0.01
AM-20	40	20	14	5	43.43	2.71	106.4	3.33	15.4	3.10	2.81	0.91	0.64	1.12	0.01
AM5-0	41	5	3.5	0	39.11	2.44	91.98	2.87	9.55	11.1	4.10	0.37	0.40	1.49	0.06
A20-0	41	5	3.5	0	47.87	2.99	109.9	3.44	11.6	13.6	4.11	0.30	0.48	1.66	0.05
M/O -1	47	5	3.5	10	51.48	3.22	117.7	3.68	14.2	14.7	3.60	0.25	0.6	1.31	0.02
M/O -2	24	5	3.5	10	21.78	1.36	93.62	2.93	15.4	6.22	1.41	0.23	0.65	0.97	0.02
M/O -3	24	5	3.5	10	9.44	0.59	79.71	2.49	13.1	2.70	0.72	0.27	0.55	0.69	0.01
M/O -4	22	5	3.5	10	38.32	2.40	30.62	0.96	14.3	10.9	2.68	0.24	0.60	1.09	0.03
N/M-1	47	5	3.5	10	51.48	3.22	117.7	3.68	14.2	14.7	3.60	0.25	0.6	1.31	0.02
N/M-2	47	2.5	1.75	5	52.36	3.27	117.9	3.69	8.05	29.9	6.51	0.22	0.33	1.08	0.04
Cu-0	41	0	3.5	0	39.11	2.44	91.98	2.87	9.55	11.1	4.10	0.37	0.40	1.49	0.06
Cu-5	44	5	3.5	5	49.11	3.07	116.7	3.65	10.2	14.0	4.79	0.34	0.43	1.34	0.04
Cu-10	41	10	3.5	10	40.89	2.56	98.06	3.06	9.38	11.6	4.36	0.37	0.39	1.59	0.07
Cu-20	41	20	3.5	20	42.16	2.63	101.1	3.16	11.1	12.0	3.78	0.31	0.46	1.61	0.05
Cu-40	44	40	3.5	40	43.09	2.69	103.1	3.22	11.6	12.3	3.72	0.30	0.48	1.28	0.03

Cu-80	44	80	3.5	80	48.95	3.06	114.8	3.59	10.2	13.9	4.76	0.34	0.43	1.01	0.03
Exp.	**CH4 uptake (mg-CH4)**	**CH4 uptake (mM-CH4)**	**O2 uptake (mg-O2)**	**O2 uptake (mM-O2)**	**CH4 rate (mg-CH4/hr)**	**O2 rate (mg-O2/hr)**	**Y (mg-VSS/mg-CH4)**	**q(mg-CH4/mg-VSS.hr)**	**O/M (mg-O2/mg-CH4)**	**O/M (mMO2/mM-CH4)**	**fs**	**fe**	**N- uptake (mg-N)**	**N/Mo (mg-N/mg-TSS)**	**VSS (inc) (mg-VSS)**
AM-5	36.34	2.27	108.69	3.40	0.83	2.47	0.60	0.08	2.99	1.50	0.49	0.51	2.5	0.11	21.8
AM-10	29.34	1.83	103.86	3.25	0.67	2.36	0.55	0.081	3.54	1.77	0.45	0.55	2.6	0.16	16.3
AM-20	28.66	1.79	107.45	3.36	0.65	2.44	0.52	0.066	3.75	1.87	0.42	0.58	3.1	0.21	14.9
AM-40	21.46	1.34	94.63	2.96	0.49	2.15	0.33	0.051	4.41	2.20	0.27	0.73	4.6	0.65	7.0
AM-80	18.35	1.15	83.93	2.62	0.42	1.91	0.26	0.030	4.57	2.29	0.21	0.79	9.4	1.97	4.8
AM-5	24.03	1.50	98.59	3.08	0.60	2.46	0.49	0.037	4.10	2.05	0.40	0.60	1.7	0.14	11.8
AM-10	27.27	1.70	102.46	3.20	0.68	2.56	0.43	0.044	3.76	1.88	0.35	0.65	2.1	0.18	11.7
AM-20	26.75	1.67	101.85	3.18	0.67	2.55	0.42	0.043	3.81	1.90	0.35	0.65	2.7	0.24	11.4
AM5-0	26.98	1.69	84.66	2.65	0.66		0.96	0.069	3.14	1.57	0.78	0.22	2.8	0.11	26.0
A20-0	29.58	1.85	106.20	3.32	0.72		0.95	0.062	3.59	1.80	0.77	0.23	3.9	0.14	28.0
M/O -1	40.69	2.54	111.83	3.49	0.87	2.38	0.42	0.060	2.75	1.37	0.34	0.66	2.0	0.12	16.9
M/O -2	18.55	1.16	59.07	1.85	0.77	2.46	0.42	0.050	3.18	1.59	0.34	0.66	1.9	0.24	7.8
M/O -3	8.17	0.51	27.05	0.85	0.34	1.13	0.41	0.027	3.31	1.66	0.33	0.67	1.0	0.30	3.3
M/O -4	14.28	0.89	35.95	1.12	0.65	1.63	0.82	0.045	2.52	1.26	0.67	0.33	1.4	0.12	11.7
N/M-1	40.69	2.54	113.75	3.55	0.87	2.42	0.42	0.060	2.80	1.40	0.34	0.66	2.0	0.12	16.9
N/M-2	23.02	1.44	89.90	2.81	0.49	1.91	0.77	0.061	3.91	1.95	0.63	0.37	2.0	0.11	17.7
Cu-0	26.98	1.69	84.66	2.65	0.66	2.06	0.96	0.067	3.14	1.57	0.78	0.22	2.8	0.11	26.0
Cu-5	36.34	2.27	108.69	3.40	0.83	2.47	0.60	0.080	2.99	1.50	0.49	0.51	2.5	0.11	21.8
Cu-10	27.28	1.71	96.13	3.00	0.67	2.34	1.05	0.069	3.52	1.76	0.85	0.15	2.8	0.10	28.6
Cu-20	25.29	1.58	92.41	2.89	0.62	2.25	1.08	0.054	3.65	1.83	0.88	0.12	2.9	0.11	27.3
Cu-40	21.51	1.34	83.82	2.62	0.49	1.91	0.88	0.042	3.90	1.95	0.72	0.28	2.6	0.14	19.0
Cu-80	19.88	1.24	84.40	2.64	0.45	1.92	0.70	0.044	4.24	2.12	0.57	0.43	2.3	0.17	13.9

Table A.10: Raw Data for Chapter 4 experiments

Exp.	O.D in	TSS in (mg)	O.D out	VSS out (mg)	Growth rate hr-1	CH4 in (mg)	CH4 Out (mg)	CH4 consumed (mg)	CH4 in (mmol)	CH4 consumed (mmol)	CH4 uptake rate (mg/hr)	O2 in (mg)	O2 out (mg)
Set 2	0.70	16.55	2.26	53.83	0.047	65.16	18.31	46.85	4.07	2.93	0.98	187.12	42.74
	0.66	15.76	1.76	41.81	0.034	72.43	28.97	43.46	4.53	2.72	0.91	185.71	34.24
	0.77	18.21	2.08	49.62	0.036	63.04	22.78	40.26	3.94	2.52	0.84	174.87	31.20
Set3	0.39	9.29	1.67	39.67	0.068	67.28	27.84	39.44	4.21	2.46	0.82	194.95	61.30
	0.32	7.62	1.16	27.64	0.055	69.03	31.26	37.77	4.31	2.36	0.79	202.57	71.44
	0.39	9.17	1.58	37.52	0.064	67.06	25.29	41.78	4.19	2.61	0.87	204.73	56.45
Set 2	0.88	20.83	2.34	55.62	0.035	66.07	20.49	45.58	4.13	2.85	0.95	181.56	31.14
	0.58	13.79	1.54	36.55	0.034	64.69	13.97	50.72	4.04	3.17	1.06	170.72	43.03
	0.71	16.83	1.68	39.90	0.029	68.45	22.72	45.73	4.28	2.86	0.95	198.71	35.79
Set3	0.39	9.19	1.30	30.95	0.049	71.26	31.60	39.66	4.45	2.48	0.83	195.99	57.12
	0.39	9.29	0.91	21.69	0.028	68.33	30.91	37.42	4.27	2.34	0.78	187.01	41.15
	0.37	8.79	1.11	26.40	0.042	70.79	32.27	38.52	4.42	2.41	0.80	186.96	50.40
Set 2	0.90	21.40	1.20	28.50	0.007	76.00	27.51	48.48	4.75	3.03	1.01	198.43	55.85
	0.80	19.05	2.06	49.05	0.033	69.60	29.98	39.62	4.35	2.48	0.83	178.48	29.49
	0.63	14.88	0.98	23.36	0.012	75.58	30.88	44.70	4.72	2.79	0.93	201.78	48.39
Set3	0.28	6.62	1.01	23.95	0.055	78.28	39.70	38.58	4.89	2.41	0.80	206.26	57.14
	0.25	5.88	0.82	19.45	0.048	78.64	35.89	42.75	4.92	2.67	0.89	212.63	64.44
	0.37	8.86	0.75	17.86	0.021	80.88	40.17	40.71	5.05	2.54	0.85	187.84	50.31
Set 2	0.67	15.93	1.34	31.79	0.021	74.21	40.91	33.30	4.64	2.08	0.69	205.94	67.82
	0.62	14.71	0.98	23.36	0.012	70.83	45.57	25.26	4.43	1.58	0.53	182.01	81.18
	0.56	13.40	1.11	26.45	0.020	76.72	46.16	30.56	4.79	1.91	0.64	188.93	63.32

Set3	0.25	6.05	1.30	30.83	0.085	80.98	52.89	28.09	5.06	1.76	0.59	185.18	80.57
	0.29	6.88	1.15	27.43	0.062	79.87	57.90	21.97	4.99	1.37	0.46	184.04	83.23
	0.23	5.52	0.82	19.48	0.053	78.67	52.77	25.90	4.92	1.62	0.54	182.51	85.14
Set 2	0.70	16.55	1.75	41.57	0.032	86.56	31.40	55.17	5.41	3.45	1.15	208.14	49.66
	0.70	16.55	1.75	41.57	0.032	76.33	27.42	48.91	4.77	3.06	1.02	185.82	43.37
	0.45	10.74	1.84	43.79	0.064	75.93	42.39	33.54	4.75	2.10	0.70	201.08	74.99
Set3	0.37	8.88	1.96	46.76	0.089	78.07	47.93	30.14	4.88	1.88	0.63	197.14	55.83
	0.34	8.19	1.38	32.81	0.063	87.85	43.60	44.25	5.49	2.77	0.92	212.65	67.96
	0.31	7.36	0.94	22.43	0.043	82.73	62.94	19.78	5.17	1.24	0.41	188.29	165.5
Set 2	0.72	17.10	1.81	42.98	0.032	85.70	40.69	45.01	5.36	2.81	0.94	231.36	88.57
	0.70	16.67	2.29	54.60	0.047	92.71	40.14	52.58	5.79	3.29	1.10	224.70	60.24
	0.66	15.64	2.55	60.62	0.060	86.69	32.20	54.49	5.42	3.41	1.14	230.01	56.58
Set3	0.32	7.71	1.60	38.00	0.082	86.99	56.43	30.56	5.44	1.91	0.64	201.52	87.01
	0.30	7.21	1.51	35.95	0.083	82.43	52.13	30.30	5.15	1.89	0.63	204.11	94.12
	0.30	7.07	1.34	31.98	0.073	86.67	59.24	27.44	5.42	1.71	0.57	210.81	119.2
Set 2	0.63	14.95	2.07	49.33	0.048	92.82	37.05	55.77	5.80	3.49	1.16	245.44	58.79
	0.74	17.67	2.62	62.29	0.053	85.91	28.48	57.43	5.37	3.59	1.20	227.22	48.64
	0.70	16.69	2.65	63.02	0.058	87.27	31.10	56.17	5.45	3.51	1.17	223.82	42.09
Set3	0.28	6.74	1.73	41.17	0.106	100.0	60.78	39.27	6.25	2.45	0.82	253.00	104.2
	0.34	8.17	1.64	39.14	0.079	93.10	37.51	55.59	5.82	3.47	1.16	261.10	87.33
	0.31	7.26	1.83	43.57	0.104	85.87	45.96	39.92	5.37	2.49	0.83	237.69	101.3
Set 2	0.75	17.81	1.83	43.62	0.030	83.18	28.51	54.68	5.20	3.42	1.14	235.92	76.29
	0.73	17.45	2.55	60.71	0.052	85.11	26.54	58.57	5.32	3.66	1.22	225.96	40.95
	0.81	19.17	2.53	60.31	0.045	82.95	24.01	58.95	5.18	3.68	1.23	229.52	33.70
Set3	0.39	9.19	2.02	48.14	0.088	77.87	24.98	52.89	4.87	3.31	1.10	198.81	39.83
	0.37	8.86	1.86	44.24	0.083	81.00	31.44	49.56	5.06	3.10	1.03	221.69	59.16

	0.41	9.64	2.20	52.43	0.092	82.17	26.55	55.62	5.14	3.48	1.16	218.30	42.54
Set 2	0.70	16.62	2.52	59.90	0.054	91.77	21.45	70.33	5.74	4.40	1.47	247.45	60.70
	0.67	16.05	2.56	60.86	0.058	91.65	30.80	60.86	5.73	3.80	1.27	240.73	40.19
	0.67	16.00	2.62	62.48	0.061	97.95	37.76	60.20	6.12	3.76	1.25	263.82	49.81
Set3	0.41	9.76	1.25	29.76	0.043	91.08	45.08	46.00	5.69	2.87	0.96	251.81	109.6
	0.31	7.38	1.40	33.33	0.073	84.69	35.34	49.35	5.29	3.08	1.03	236.52	67.41
	0.37	8.90	2.25	53.48	0.104	73.64	23.29	50.36	4.60	3.15	1.05	247.41	68.47
Set 2	0.72	17.21	2.54	60.48	0.052	103.4	44.01	59.45	6.47	3.72	1.24	237.65	71.43
	0.75	17.81	2.62	62.48	0.052	95.68	45.07	50.62	5.98	3.16	1.05	227.56	45.71
	0.74	17.55	2.12	50.50	0.039	93.70	37.21	56.49	5.86	3.53	1.18	230.19	40.29
Set3	0.37	8.81	2.14	50.83	0.099	96.58	44.51	52.07	6.04	3.25	1.08	234.61	58.57
	0.33	7.76	2.20	52.31	0.120	100.8	46.71	54.12	6.30	3.38	1.13	242.69	68.21
	0.36	8.67	1.69	40.33	0.076	97.82	66.74	31.08	6.11	1.94	0.65	221.64	109.7
Set 2	0.76	18.10	2.52	59.90	0.048	80.35	31.65	48.70	5.02	3.04	1.01	179.95	38.71
	0.68	16.26	1.98	47.24	0.040	80.21	37.85	42.37	5.01	2.65	0.88	171.52	34.68
	0.69	16.40	2.07	49.38	0.042	82.73	44.38	38.34	5.17	2.40	0.80	181.74	62.21
Set3	0.38	9.10	2.12	50.43	0.095	81.67	47.38	34.29	5.10	2.14	0.71	187.48	76.58
	0.41	9.83	2.29	54.60	0.095	82.64	46.81	35.84	5.17	2.24	0.75	183.78	68.22
	0.38	8.93	2.18	51.93	0.100	80.79	36.91	43.87	5.05	2.74	0.91	173.80	41.68
Set 2	0.81	19.21	2.06	49.00	0.032	70.41	28.38	42.03	4.40	2.63	0.88	174.03	34.52
	0.59	14.10	2.20	52.29	0.056	65.84	27.27	38.57	4.11	2.41	0.80	165.90	46.72
	0.63	15.02	2.51	59.79	0.062	69.72	26.74	42.98	4.36	2.69	0.90	163.39	23.13
Set3	0.38	9.14	1.99	47.48	0.087	69.98	28.52	41.46	4.37	2.59	0.86	171.70	43.93
	0.42	9.95	2.30	54.74	0.094	67.20	25.09	42.11	4.20	2.63	0.88	157.05	29.24
	0.39	9.38	2.30	54.81	0.101	70.49	24.32	46.17	4.41	2.89	0.96	175.30	33.04
Set 2	0.71	17.00	2.03	48.40	0.038	65.29	23.10	42.19	4.08	2.64	0.88	156.76	45.66

	0.72	17.10	2.49	59.21	0.051	64.89	14.31	50.59	4.06	3.16	1.05	154.98	29.41
	0.68	16.26	2.59	61.55	0.058	72.46	19.65	52.81	4.53	3.30	1.10	186.93	37.70
Set3	0.29	6.90	2.05	48.88	0.127	74.57	27.78	46.79	4.66	2.92	0.97	179.89	57.86
	0.35	8.43	1.85	43.98	0.088	70.99	28.37	42.62	4.44	2.66	0.89	166.94	57.11
	0.35	8.31	2.00	47.55	0.098	70.76	24.89	45.87	4.42	2.87	0.96	168.77	47.15
Set 2	0.66	15.69	2.49	59.19	0.058	81.32	26.61	54.71	5.08	3.42	1.14	189.01	33.74
	0.72	17.19	2.59	61.60	0.054	77.61	25.98	51.63	4.85	3.23	1.08	179.20	19.53
	0.70	16.64	2.30	54.69	0.048	78.96	24.98	53.98	4.93	3.37	1.12	186.17	22.56
Set3	0.34	8.19	2.26	53.76	0.116	80.20	25.16	55.04	5.01	3.44	1.15	180.44	33.04
	0.33	7.88	2.27	54.10	0.122	76.57	29.91	46.67	4.79	2.92	0.97	182.30	31.03
	0.38	8.95	2.10	49.90	0.095	73.90	25.19	48.71	4.62	3.04	1.01	186.74	32.31
Set 2	0.73	17.33	2.23	52.98	0.043	83.59	20.66	62.93	5.22	3.93	1.31	199.33	37.10
	0.69	16.50	2.25	53.48	0.047	85.27	26.63	58.64	5.33	3.67	1.22	202.42	25.58
	0.68	16.21	1.88	44.67	0.037	81.42	27.47	53.95	5.09	3.37	1.12	203.14	36.21
Set3	0.31	7.33	2.03	48.38	0.117	92.86	39.23	53.64	5.80	3.35	1.12	217.92	47.51
	0.34	8.07	1.93	45.86	0.098	87.21	32.06	55.15	5.45	3.45	1.15	204.40	41.72
	0.28	6.57	2.12	50.43	0.139	86.07	25.72	60.35	5.38	3.77	1.26	206.07	36.23
Set 2	0.64	15.24	2.14	50.95	0.051	68.33	38.94	29.38	4.27	1.84	0.64	171.71	64.80
	0.66	15.79	2.15	51.21	0.049	70.03	35.71	34.32	4.38	2.15	0.75	172.48	64.75
	0.53	12.62	2.30	54.86	0.073	71.77	28.31	43.46	4.49	2.72	0.94	172.70	50.68
Set3	0.34	7.98	1.74	41.52	0.091	69.49	40.45	29.04	4.34	1.81	0.63	175.38	88.78
	0.32	7.52	2.05	48.90	0.120	76.41	37.95	38.46	4.78	2.40	0.84	177.34	72.83
	0.33	7.90	1.83	43.52	0.098	70.68	30.39	40.30	4.42	2.52	0.88	160.66	73.09
Set 2	0.68	16.10	2.48	58.95	0.055	64.21	18.12	46.10	4.01	2.88	0.96	160.27	29.65
	0.64	15.17	2.53	60.17	0.062	63.81	20.09	43.72	3.99	2.73	0.91	155.24	23.59
	0.64	15.14	2.52	59.88	0.062	58.83	22.68	36.15	3.68	2.26	0.75	148.89	22.38

Set3	0.29	6.79	2.29	54.55	0.147	72.53	34.90	37.63	4.53	2.35	0.78	166.34	50.42
	0.33	7.86	1.76	41.83	0.090	74.74	35.42	39.32	4.67	2.46	0.82	170.29	44.75
	0.32	7.60	1.60	37.98	0.083	50.36	19.54	30.83	3.15	1.93	0.64	126.72	32.74
Set 2	0.61	14.62	2.26	53.69	0.056	93.40	38.65	54.75	5.84	3.42	1.14	230.78	50.71
	0.77	18.21	2.62	62.40	0.051	91.40	22.56	68.84	5.71	4.30	1.43	217.77	25.40
	0.73	17.33	2.43	57.74	0.049	91.52	26.24	65.27	5.72	4.08	1.36	221.46	45.09
Set3	0.37	8.69	2.10	50.07	0.099	89.74	37.63	52.11	5.61	3.26	1.09	215.08	48.04
	0.35	8.31	2.25	53.48	0.113	91.98	21.04	70.94	5.75	4.43	1.48	220.84	37.99
	0.35	8.24	2.16	51.38	0.109	91.36	28.46	62.90	5.71	3.93	1.31	222.41	52.39
Set 2	0.61	14.45	2.11	50.14	0.051	84.34	39.44	44.91	5.27	2.81	0.94	212.43	61.05
	0.75	17.74	2.17	51.69	0.040	76.54	37.41	39.13	4.78	2.45	0.82	199.48	61.31
	0.66	15.71	2.22	52.95	0.049	70.10	25.97	44.13	4.38	2.76	0.92	184.65	33.21
Set3	0.40	9.60	1.72	41.00	0.068	66.58	30.90	35.68	4.16	2.23	0.74	179.04	77.05
	0.36	8.48	2.18	51.90	0.107	72.28	31.91	40.37	4.52	2.52	0.84	175.04	42.06
	0.28	6.71	1.94	46.12	0.122	73.51	31.64	41.87	4.59	2.62	0.87	180.67	53.08
Set 2	0.47	11.29	2.21	52.60	0.076	80.21	36.42	43.79	5.01	2.74	0.91	203.58	58.62
	0.55	13.19	2.18	51.98	0.061	81.24	33.18	48.06	5.08	3.00	1.00	199.16	35.33
	0.56	13.38	2.08	49.40	0.056	82.23	42.70	39.54	5.14	2.47	0.82	203.30	61.86
Set3	0.17	4.02	1.89	44.90	0.212	82.26	44.03	38.22	5.14	2.39	0.80	198.61	71.94
	0.23	5.38	2.08	49.40	0.170	80.34	32.86	47.48	5.02	2.97	0.99	194.82	46.66
	0.20	4.83	1.91	45.36	0.175	87.31	46.20	41.11	5.46	2.57	0.86	201.64	66.52
Set 2	0.71	16.88	2.10	50.07	0.041	86.38	35.26	51.13	5.40	3.20	1.07	223.09	77.66
	0.60	14.36	2.17	51.69	0.054	82.74	35.16	47.58	5.17	2.97	0.99	197.88	61.99
	0.70	16.57	2.38	56.74	0.050	81.88	31.64	50.24	5.12	3.14	1.05	202.82	48.27
Set3	0.36	8.52	1.93	46.00	0.092	85.09	39.81	45.29	5.32	2.83	0.94	208.84	74.06
	0.28	6.74	1.78	42.45	0.110	83.18	44.90	38.28	5.20	2.39	0.80	220.16	102.2

	0.24	5.76	1.70	40.52	0.126	85.27	51.90	33.38	5.33	2.09	0.70	192.63	91.43
Set 2	0.52	12.38	2.07	49.38	0.062	81.84	30.62	51.22	5.11	3.20	1.07	215.67	81.84
	0.59	14.07	2.29	54.55	0.060	88.74	37.76	50.98	5.55	3.19	1.06	229.99	88.74
	0.58	13.74	2.33	55.57	0.063	90.04	39.33	50.72	5.63	3.17	1.06	232.55	90.04
Set3	0.30	7.21	2.20	52.33	0.130	86.45	43.48	42.98	5.40	2.69	0.90	212.82	86.45
	0.29	6.98	1.90	45.19	0.114	91.65	40.22	51.43	5.73	3.21	1.07	234.98	91.65
	0.32	7.60	1.98	47.21	0.109	83.27	29.94	53.33	5.20	3.33	1.11	200.66	83.27
Set 2	0.68	16.19	2.58	61.52	0.058	84.15	26.74	57.41	5.26	3.59	1.20	226.62	53.45
	0.56	13.31	2.14	50.88	0.059	85.08	35.83	49.25	5.32	3.08	1.03	226.72	77.27
	0.59	14.02	2.23	53.19	0.058	88.65	43.68	44.96	5.54	2.81	0.94	233.36	78.69
Set3	0.33	7.81	1.85	44.02	0.097	93.56	52.46	41.09	5.85	2.57	0.86	234.35	114.5
	0.34	8.02	2.14	50.90	0.111	93.09	42.51	50.58	5.82	3.16	1.05	235.44	88.24
	0.34	8.12	1.86	44.19	0.093	96.60	55.36	41.24	6.04	2.58	0.86	242.83	106.0
Set 2	0.64	15.31	2.47	58.79	0.059	93.02	36.84	56.18	5.81	3.51	1.17	234.89	67.20
	0.59	14.07	2.20	52.31	0.057	86.50	36.74	49.77	5.41	3.11	1.04	236.31	75.72
	0.56	13.40	2.46	58.60	0.070	88.80	31.67	57.13	5.55	3.57	1.19	222.06	52.01
Set3	0.26	6.17	2.04	48.48	0.143	86.87	36.49	50.39	5.43	3.15	1.05	214.46	65.80
	0.26	6.10	2.06	49.07	0.147	86.50	35.83	50.67	5.41	3.17	1.06	217.40	68.87
	0.25	5.83	1.81	43.02	0.133	84.42	38.82	45.60	5.28	2.85	0.95	206.58	76.87
Set 2	0.61	14.60	2.15	51.12	0.052	71.91	37.55	34.37	4.49	2.15	0.72	212.22	91.46
	0.61	14.50	2.19	52.05	0.054	71.97	27.92	44.05	4.50	2.75	0.92	203.10	56.36
	0.57	13.67	2.11	50.21	0.056	74.49	26.14	48.35	4.66	3.02	1.01	221.24	67.10
Set3	0.31	7.40	2.09	49.76	0.119	72.09	27.31	44.78	4.51	2.80	0.93	205.65	61.68
	0.33	7.83	2.06	49.14	0.110	78.48	27.80	50.68	4.90	3.17	1.06	198.13	53.30
	0.28	6.76	1.92	45.69	0.120	80.24	35.55	44.69	5.02	2.79	0.93	211.59	66.26
Set 2	0.68	16.17	1.92	45.79	0.038	90.37	33.63	56.74	5.65	3.55	1.18	211.69	46.88

	0.58	13.90	2.20	52.40	0.058	93.69	64.67	29.01	5.86	1.81	0.60	224.44	41.66
	0.58	13.88	2.08	49.62	0.054	92.72	37.58	55.14	5.80	3.45	1.15	219.26	39.86
Set3	0.36	8.52	1.98	47.21	0.095	100.0	41.18	58.91	6.26	3.68	1.23	231.94	66.85
	0.34	8.05	2.05	48.69	0.105	98.47	39.37	59.10	6.15	3.69	1.23	236.01	56.54
	0.32	7.50	1.85	43.98	0.101	98.58	47.27	51.31	6.16	3.21	1.07	242.89	63.41
Set 2	0.68	16.17	1.92	45.79	0.038	47.58	20.73	26.85	2.97	1.68	0.56	91.92	16.71
	0.58	13.90	2.20	52.40	0.058	52.27	25.72	26.54	3.27	1.66	0.55	104.82	13.87
	0.58	13.88	2.08	49.62	0.054	52.79	23.60	29.19	3.30	1.82	0.61	110.62	15.27
Set3	0.36	8.52	1.98	47.21	0.095	56.07	21.56	34.50	3.50	2.16	0.72	113.20	18.57
	0.34	8.05	2.05	48.69	0.105	53.70	23.75	29.95	3.36	1.87	0.62	114.75	20.67
	0.32	7.50	1.85	43.98	0.101	52.32	28.78	23.54	3.27	1.47	0.49	115.48	19.65
Experiment	**O2 consumed (mg)**	**O2 in (mmol)**	**O2 uptake rate (mg/hr)**	**F/M g-CH4/gVSS**	**C/N g-CH4/g-N**	**N/Mi g-N/gVSS**	**Y (g-VSS/g-CH4)**	**q (mg/mg/hr)**	**NH4 in (mg-N)**	**NH4 in (mg-N/l)**	**NH4 out (mg-N)**	**NH4 Consumed (mg-N)**	**N/M (mg-N/mg-VSS)**
	144.37	5.85	3.01	3.94	18.62	0.21	0.80	0.026	3.50	70.00	7.00	3.15	0.08
Set 2	151.46	5.80	3.16	4.60	20.69	0.22	0.60	0.035	3.50	70.00	15.40	2.73	0.10
	143.68	5.46	2.99	3.46	18.01	0.19	0.78	0.027	3.50	70.00	11.20	2.94	0.09
	133.65	6.09	2.78	7.25	19.22	0.38	0.77	0.027	3.50	70.00	16.50	2.68	0.09
Set3	131.14	6.33	2.73	9.06	19.72	0.46	0.53	0.039	3.50	70.00	14.40	2.78	0.14
	148.28	6.40	3.09	7.32	19.16	0.38	0.68	0.031	3.50	70.00	13.40	2.83	0.10
	150.42	5.67	3.13	3.17	18.88	0.17	0.76	0.027	3.50	70.00	3.30	3.34	0.10
Set 2	127.68	5.33	2.66	4.69	18.48	0.25	0.45	0.046	3.50	70.00	8.50	3.08	0.14
	162.93	6.21	3.39	4.07	19.56	0.21	0.50	0.041	3.50	70.00	8.00	3.10	0.13
	138.87	6.12	2.89	7.75	20.36	0.38	0.55	0.038	3.50	70.00	14.90	2.76	0.13
Set3	145.86	5.84	3.04	7.36	19.52	0.38	0.33	0.063	3.50	70.00	10.70	2.97	0.24
	136.56	5.84	2.85	8.06	20.23	0.40	0.46	0.046	3.50	70.00	13.70	2.82	0.16

Set 2	142.57	6.20	2.97	3.55	21.71	0.16	0.15	0.142	3.50	70.00	14.10	2.80	0.39
	149.00	5.58	3.10	3.65	19.89	0.18	0.76	0.028	3.50	70.00	7.30	3.14	0.10
	153.39	6.31	3.20	5.08	21.59	0.24	0.19	0.110	3.50	70.00	7.70	3.12	0.37
Set3	149.12	6.45	3.11	11.83	22.37	0.53	0.45	0.046	3.50	70.00	9.20	3.04	0.18
	148.19	6.64	3.09	13.37	22.47	0.60	0.32	0.066	3.50	70.00	7.40	3.13	0.23
	137.53	5.87	2.87	9.13	23.11	0.40	0.22	0.094	3.50	70.00	9.60	3.02	0.34
Set 2	138.12	6.44	2.88	4.66	21.20	0.22	0.48	0.044	3.50	70.00	5.80	3.21	0.20
	100.82	5.69	2.10	4.81	20.24	0.24	0.34	0.061	3.50	70.00	17.00	2.65	0.31
	125.61	5.90	2.62	5.72	21.92	0.26	0.43	0.049	3.50	70.00	15.60	2.72	0.21
Set3	104.61	5.79	2.18	13.39	23.14	0.58	0.88	0.024	3.50	70.00	16.60	2.67	0.11
	100.81	5.75	2.10	11.61	22.82	0.51	0.94	0.022	3.50	70.00	19.00	2.55	0.12
	97.37	5.70	2.03	14.24	22.48	0.63	0.54	0.039	3.50	70.00	19.90	2.51	0.18
Set 2	158.49	6.50	3.30	5.23	24.73	0.21	0.45	0.046	3.50	70.00	2.80	3.36	0.13
	142.45	5.81	2.97	4.61	21.81	0.21	0.51	0.041	3.50	70.00	2.80	3.36	0.13
	126.09	6.28	2.63	7.07	21.69	0.33	0.99	0.021	3.50	70.00	6.10	3.20	0.10
Set3	141.31	6.16	2.94	8.79	22.31	0.39	1.26	0.017	3.50	70.00	9.00	3.05	0.08
	144.69	6.65	3.01	10.73	25.10	0.43	0.56	0.037	3.50	70.00	11.80	2.91	0.12
	22.73	5.88	0.47	11.24	23.64	0.48	0.76	0.027	3.50	70.00	21.60	2.42	0.16
Set 2	142.79	7.23	2.97	5.01	20.41	0.25	0.57	0.036	4.20	82.00	9.70	3.62	0.14
	164.47	7.02	3.43	5.56	22.07	0.25	0.72	0.029	4.20	82.00	4.40	3.88	0.10
	173.42	7.19	3.61	5.54	20.64	0.27	0.83	0.025	4.20	82.00	2.70	3.97	0.09
Set3	114.51	6.30	2.39	11.28	20.71	0.54	0.99	0.021	4.20	82.00	18.60	3.17	0.10
	109.98	6.38	2.29	11.43	19.63	0.58	0.95	0.022	4.20	82.00	20.40	3.08	0.11
	91.53	6.59	1.91	12.26	20.64	0.59	0.91	0.023	4.20	82.00	29.60	2.62	0.11
Set 2	186.65	7.67	3.89	6.21	22.10	0.28	0.62	0.034	4.20	82.00	6.00	3.80	0.11
	178.59	7.10	3.72	4.86	20.45	0.24	0.78	0.027	4.20	82.00	3.10	3.95	0.09

	181.73	6.99	3.79	5.23	20.78	0.25	0.82	0.025	4.20	82.00	1.70	4.02	0.09
Set3	148.74	7.91	3.10	14.85	23.82	0.62	0.88	0.024	4.20	82.00	11.40	3.53	0.10
	173.77	8.16	3.62	11.40	22.17	0.51	0.56	0.037	4.20	82.00	11.60	3.52	0.11
	136.32	7.43	2.84	11.82	20.45	0.58	0.91	0.023	4.20	82.00	11.50	3.53	0.10
Set 2	159.63	7.37	3.33	4.67	19.81	0.24	0.47	0.044	4.20	82.00	6.00	3.80	0.15
	185.01	7.06	3.85	4.88	20.27	0.24	0.74	0.028	4.20	82.00	1.70	4.02	0.09
	195.82	7.17	4.08	4.33	19.75	0.22	0.70	0.030	4.20	82.00	1.30	4.04	0.10
Set3	158.97	6.21	3.31	8.47	18.54	0.46	0.74	0.028	4.20	82.00	7.10	3.75	0.10
	162.54	6.93	3.39	9.14	19.29	0.47	0.71	0.029	4.20	82.00	8.30	3.69	0.10
	175.75	6.82	3.66	8.52	19.56	0.44	0.77	0.027	4.20	82.00	3.00	3.95	0.09
Set 2	186.75	7.73	3.89	5.52	21.85	0.25	0.62	0.034	4.20	82.00	0.20	4.09	0.09
	200.55	7.52	4.18	5.71	21.82	0.26	0.74	0.028	4.20	82.00	1.40	4.03	0.09
	214.01	8.24	4.46	6.12	23.32	0.26	0.77	0.027	4.20	82.00	1.10	4.05	0.09
Set3	142.12	7.87	2.96	9.33	21.68	0.43	0.43	0.048	4.20	82.00	14.80	3.36	0.17
	169.11	7.39	3.52	11.47	20.16	0.57	0.53	0.040	4.20	82.00	7.90	3.71	0.14
	178.94	7.73	3.73	8.27	17.53	0.47	0.89	0.024	4.20	82.00	5.50	3.83	0.09
Set 2	166.22	7.43	3.46	6.01	24.63	0.24	0.73	0.029	4.20	82.00	2.90	3.96	0.09
	181.85	7.11	3.79	5.37	22.78	0.24	0.88	0.024	4.20	82.00	3.20	3.94	0.09
	189.90	7.19	3.96	5.34	22.31	0.24	0.58	0.036	4.20	82.00	11.20	3.54	0.11
Set3	176.04	7.33	3.67	10.96	23.00	0.48	0.81	0.026	4.20	82.00	7.20	3.74	0.09
	174.49	7.58	3.64	12.99	24.01	0.54	0.82	0.025	4.20	82.00	9.30	3.64	0.08
	111.86	6.93	2.33	11.29	23.29	0.48	1.02	0.020	4.20	82.00	17.20	3.24	0.10
Set 2	141.24	5.62	2.94	4.44	19.13	0.23	0.86	0.024	4.20	82.00	11.20	3.54	0.08
	136.84	5.36	2.85	4.93	19.10	0.26	0.73	0.028	4.20	82.00	25.00	2.85	0.09
	119.53	5.68	2.49	5.04	19.70	0.26	0.86	0.024	4.20	82.00	23.70	2.92	0.09
Set3	110.90	5.86	2.31	8.98	19.45	0.46	1.21	0.017	4.20	82.00	19.30	3.14	0.08

	115.57	5.74	2.41	8.40	19.68	0.43	1.25	0.017	4.20	82.00	20.20	3.09	0.07
	132.12	5.43	2.75	9.05	19.23	0.47	0.98	0.021	4.20	82.00	14.10	3.40	0.08
Set 2	139.51	5.44	2.91	3.66	16.76	0.22	0.71	0.029	4.20	82.00	20.60	3.07	0.10
	119.18	5.18	2.48	4.67	15.68	0.30	0.99	0.021	4.20	82.00	21.40	3.03	0.08
	140.26	5.11	2.92	4.64	16.60	0.28	1.04	0.020	4.20	82.00	14.70	3.37	0.08
Set3	127.76	5.37	2.66	7.65	16.66	0.46	0.92	0.023	4.20	82.00	25.60	2.82	0.07
	127.81	4.91	2.66	6.75	16.00	0.42	1.06	0.020	4.20	82.00	21.20	3.04	0.07
	142.26	5.48	2.96	7.51	16.78	0.45	0.98	0.021	4.20	82.00	18.70	3.17	0.07
Set 2	111.10	4.90	2.31	3.84	15.54	0.25	0.74	0.028	4.20	82.00	26.60	2.77	0.09
	125.57	4.84	2.62	3.80	15.45	0.25	0.83	0.025	4.20	82.00	16.30	3.29	0.08
	149.23	5.84	3.11	4.46	17.25	0.26	0.86	0.024	4.20	82.00	9.40	3.63	0.08
Set3	122.03	5.62	2.54	10.80	17.76	0.61	0.90	0.023	4.20	82.00	21.30	3.04	0.07
	109.83	5.22	2.29	8.42	16.90	0.50	0.83	0.025	4.20	82.00	27.60	2.72	0.08
	121.62	5.27	2.53	8.52	16.85	0.51	0.86	0.024	4.20	82.00	26.60	2.77	0.07
Set 2	155.27	5.91	3.23	5.18	19.36	0.27	0.80	0.026	4.20	82.00	5.10	3.85	0.09
	159.67	5.60	3.33	4.51	18.48	0.24	0.86	0.024	4.20	82.00	6.10	3.80	0.09
	163.61	5.82	3.41	4.74	18.80	0.25	0.70	0.030	4.20	82.00	17.30	3.24	0.09
Set3	147.40	5.64	3.07	9.79	19.10	0.51	0.83	0.025	4.20	82.00	10.60	3.57	0.08
	151.27	5.70	3.15	9.72	18.23	0.53	0.99	0.021	4.20	82.00	9.20	3.64	0.08
	154.43	5.84	3.22	8.26	17.60	0.47	0.84	0.025	4.20	82.00	16.70	3.27	0.08
Set 2	162.23	6.23	3.38	4.82	19.90	0.24	0.57	0.037	4.20	82.00	17.70	3.22	0.09
	176.83	6.33	3.68	5.17	20.30	0.25	0.63	0.033	4.20	82.00	17.70	3.22	0.09
	166.93	6.35	3.48	5.02	19.39	0.26	0.53	0.040	4.20	82.00	15.30	3.34	0.12
Set3	170.41	6.81	3.55	12.66	22.11	0.57	0.77	0.027	4.20	82.00	18.50	3.18	0.08
	162.67	6.39	3.39	10.80	20.76	0.52	0.69	0.030	4.20	82.00	16.90	3.26	0.09
	169.84	6.44	3.54	13.10	20.49	0.64	0.73	0.029	4.20	82.00	13.90	3.41	0.08

Set 2	106.92	5.37	2.32	4.48	16.27	0.28	1.22	0.017	4.20	82.00	9.60	3.62	0.10
	107.72	5.39	2.34	4.44	16.67	0.27	1.03	0.020	4.20	82.00	15.30	3.34	0.09
	122.02	5.40	2.65	5.69	17.09	0.33	0.97	0.021	4.20	82.00	3.40	3.93	0.09
Set3	86.60	5.48	1.88	8.71	16.54	0.53	1.16	0.018	4.20	82.00	13.70	3.42	0.10
	104.51	5.54	2.27	10.16	18.19	0.56	1.08	0.019	4.20	82.00	8.80	3.66	0.09
	87.57	5.02	1.90	8.94	16.83	0.53	0.88	0.024	4.20	82.00	12.70	3.47	0.10
Set 2	130.63	5.01	2.72	3.99	15.29	0.26	0.93	0.022	4.20	82.00	2.00	4.00	0.09
	131.64	4.85	2.74	4.21	15.19	0.28	1.03	0.020	4.20	82.00	1.20	4.04	0.09
	126.51	4.65	2.64	3.89	14.01	0.28	1.24	0.017	4.20	82.00	3.30	3.94	0.09
Set3	115.92	5.20	2.42	10.69	17.27	0.62	1.27	0.016	4.20	82.00	6.80	3.76	0.08
	125.54	5.32	2.62	9.51	17.80	0.53	0.86	0.024	4.20	82.00	8.40	3.68	0.11
	93.98	3.96	1.96	6.63	11.99	0.55	0.99	0.021	4.20	82.00	9.00	3.65	0.12
Set 2	180.07	7.21	3.75	6.39	22.24	0.29	0.71	0.029	4.20	82.00	5.40	3.83	0.10
	192.37	6.81	4.01	5.02	21.76	0.23	0.64	0.032	4.20	82.00	2.10	4.00	0.09
	176.37	6.92	3.67	5.28	21.79	0.24	0.62	0.034	4.20	82.00	8.80	3.66	0.09
Set3	167.05	6.72	3.48	10.33	21.37	0.48	0.79	0.026	4.20	82.00	13.30	3.44	0.08
	182.85	6.90	3.81	11.07	21.90	0.51	0.64	0.033	4.20	82.00	7.70	3.72	0.08
	170.02	6.95	3.54	11.09	21.75	0.51	0.69	0.030	4.20	82.00	9.70	3.62	0.08
Set 2	151.38	6.64	3.15	5.84	20.08	0.29	0.79	0.026	4.20	82.00	4.30	3.89	0.11
	138.16	6.23	2.88	4.31	18.22	0.24	0.87	0.024	4.20	82.00	9.20	3.64	0.11
	151.44	5.77	3.16	4.46	16.69	0.27	0.84	0.025	4.20	82.00	10.00	3.60	0.10
Set3	101.99	5.60	2.12	6.94	15.85	0.44	0.88	0.024	4.20	82.00	8.70	3.67	0.12
	132.98	5.47	2.77	8.53	17.21	0.50	1.08	0.019	4.20	82.00	8.70	3.67	0.08
	127.59	5.65	2.66	10.95	17.50	0.63	0.94	0.022	4.20	82.00	9.70	3.62	0.09
Set 2	144.95	6.36	3.02	7.11	19.10	0.37	0.94	0.022	4.20	82.00	6.30	3.79	0.09
	163.82	6.22	3.41	6.16	19.34	0.32	0.81	0.026	4.20	82.00	7.10	3.75	0.10

	141.44	6.35	2.95	6.15	19.58	0.31	0.91	0.023	4.20	82.00	8.50	3.68	0.10
Set3	126.67	6.21	2.64	20.44	19.58	1.04	1.07	0.019	4.20	82.00	12.30	3.49	0.09
	148.16	6.09	3.09	14.93	19.13	0.78	0.93	0.022	4.20	82.00	9.40	3.63	0.08
	135.12	6.30	2.82	18.06	20.79	0.87	0.99	0.021	4.20	82.00	25.20	2.84	0.07
Set 2	145.43	6.97	3.03	5.12	20.57	0.25	0.65	0.032	4.20	82.00	8.60	3.67	0.11
	135.89	6.18	2.83	5.76	19.70	0.29	0.78	0.027	4.20	82.00	11.60	3.52	0.09
	154.56	6.34	3.22	4.94	19.49	0.25	0.80	0.026	4.20	82.00	5.40	3.83	0.10
Set3	134.78	6.53	2.81	9.98	20.26	0.49	0.83	0.025	4.20	82.00	9.20	3.64	0.10
	117.94	6.88	2.46	12.34	19.80	0.62	0.93	0.022	4.20	82.00	14.60	3.37	0.09
	101.20	6.02	2.11	14.80	20.30	0.73	1.04	0.020	4.20	82.00	16.20	3.29	0.09
Set 2	133.84	6.74	2.79	6.61	19.48	0.34	0.72	0.029	4.20	82.00	0.40	4.08	0.11
	141.25	7.19	2.94	6.31	21.13	0.30	0.79	0.026	4.20	82.00	3.30	3.94	0.10
	142.51	7.27	2.97	6.55	21.44	0.31	0.82	0.025	4.20	82.00	7.20	3.74	0.09
Set3	126.37	6.65	2.63	11.98	20.58	0.58	1.05	0.020	4.20	82.00	10.60	3.57	0.08
	143.33	7.34	2.99	13.14	21.82	0.60	0.74	0.028	4.20	82.00	8.80	3.66	0.10
	117.39	6.27	2.45	10.96	19.83	0.55	0.74	0.028	4.20	82.00	10.30	3.59	0.09
Set 2	173.16	7.08	3.61	5.20	20.04	0.26	0.79	0.026	4.20	82.00	2.40	3.98	0.09
	149.45	7.08	3.11	6.39	20.26	0.32	0.76	0.027	4.20	82.00	18.60	3.17	0.08
	154.67	7.29	3.22	6.32	21.11	0.30	0.87	0.024	4.20	82.00	10.60	3.57	0.09
Set3	119.85	7.32	2.50	11.98	22.28	0.54	0.88	0.024	4.20	82.00	18.40	3.18	0.09
	147.20	7.36	3.07	11.60	22.17	0.52	0.85	0.025	4.20	82.00	14.90	3.36	0.08
	136.79	7.59	2.85	11.90	23.00	0.52	0.87	0.024	4.20	82.00	22.80	2.96	0.08
Set 2	167.69	7.34	3.49	6.08	22.15	0.27	0.77	0.027	4.20	82.00	8.20	3.69	0.08
	160.59	7.38	3.35	6.15	20.60	0.30	0.77	0.027	4.20	82.00	11.20	3.54	0.09
	170.05	6.94	3.54	6.62	21.14	0.31	0.79	0.026	4.20	82.00	10.80	3.56	0.08
Set3	148.66	6.70	3.10	14.09	20.68	0.68	0.84	0.025	4.20	82.00	14.60	3.37	0.08

	148.53	6.79	3.09	14.19	20.60	0.69	0.85	0.025	4.20	82.00	13.70	3.42	0.08
	129.72	6.46	2.70	14.47	20.10	0.72	0.82	0.026	4.20	82.00	16.70	3.27	0.09
Set 2	120.76	6.63	2.52	4.93	17.12	0.29	1.06	0.020	4.20	82.00	16.90	3.26	0.09
	146.74	6.35	3.06	4.96	17.14	0.29	0.85	0.024	4.20	82.00	12.30	3.49	0.09
	154.14	6.91	3.21	5.45	17.74	0.31	0.76	0.028	4.20	82.00	13.90	3.41	0.09
Set3	143.97	6.43	3.00	9.74	17.16	0.57	0.95	0.022	4.20	82.00	12.50	3.48	0.08
	144.82	6.19	3.02	10.02	18.68	0.54	0.82	0.026	4.20	82.00	13.00	3.45	0.08
	145.33	6.61	3.03	11.87	19.11	0.62	0.87	0.024	4.20	82.00	14.60	3.37	0.09
Set 2	164.80	6.62	3.43	5.59	21.52	0.26	0.52	0.040	4.20	82.00	11.10	3.55	0.12
	182.79	7.01	3.81	6.74	22.31	0.30	1.33	0.016	4.20	82.00	23.10	2.95	0.08
	179.40	6.85	3.74	6.68	22.08	0.30	0.65	0.032	4.20	82.00	7.50	3.73	0.10
Set3	165.09	7.25	3.44	11.74	23.83	0.49	0.66	0.032	4.20	82.00	7.40	3.73	0.10
	179.47	7.38	3.74	12.24	23.45	0.52	0.69	0.030	4.20	82.00	6.70	3.77	0.09
	179.48	7.59	3.74	13.14	23.47	0.56	0.71	0.029	4.20	82.00	6.10	3.80	0.10
Set 2	75.21	2.87	1.57	2.94	11.33	0.26	1.10	0.019	4.20	82.00	11.10	3.55	0.12
	90.95	3.28	1.89	3.76	12.44	0.30	1.45	0.014	4.20	82.00	23.10	2.95	0.08
	95.35	3.46	1.99	3.80	12.57	0.30	1.22	0.017	4.20	82.00	7.50	3.73	0.10
Set3	94.63	3.54	1.97	6.58	13.35	0.49	1.12	0.019	4.20	82.00	7.40	3.73	0.10
	94.07	3.59	1.96	6.67	12.79	0.52	1.36	0.015	4.20	82.00	6.70	3.77	0.09
	95.83	3.61	2.00	6.98	12.46	0.56	1.55	0.013	4.20	82.00	6.10	3.80	0.10

Table A.11: Raw Data for Chapter 5 experiments

Experiment	VSS (mg/l)	VSS (mg)	CH4 consumed (mg)	PHB (mg)	PHB (%)	PHB (mg/l)	Y_{PHB} (mgPHB/mgCH4)
O.D=2	1076.67	26.92	22.77	13.85	51.46	554.00	0.61
	965.71	24.14	19.30	11.98	49.62	479.20	0.62
	1137.62	28.44	19.92	14.07	49.47	562.80	0.71
O.D=1.5	784.29	19.61	18.17	9.57	48.81	382.80	0.53
	939.52	23.49	17.33	11.57	49.26	462.80	0.67
	907.62	22.69	19.52	10.36	45.66	414.40	0.53
O.D=1	688.57	17.21	14.71	8.55	49.67	342.00	0.58
	723.81	18.10	16.59	9.75	53.88	390.00	0.59
	715.71	17.89	16.30	9.16	51.19	366.40	0.56
O.D=0.5	440.48	11.01	15.52	5.83	52.94	233.20	0.38
	451.43	11.29	16.41	6.28	55.65	251.20	0.38
	421.90	10.55	12.61	5.13	48.64	205.20	0.41
Experiment	**VSS (mg/l)**	**CH4 consumed (mg)**	**PHB (mg)**	**PHB (%)**		**PHB (mg/l)**	**Y_{PHB} (mgPHB/mgCH4)**
M(1);O(1)	824.76	18.50	8.46	41.03		338.40	0.46
	818.10	19.20	8.56	41.85		342.40	0.45
	851.90	17.93	9.65	45.31		386.00	0.54
M(2);O(1)	840.00	21.79	9.06	43.14		362.40	0.42
	715.71	18.63	7.50	41.92		300.00	0.40
	862.86	24.69	10.29	47.70		411.60	0.42
M(1);O(2)	814.76	19.40	6.25	30.67		249.91	0.32
	798.10	20.76	6.94	34.79		277.68	0.33

	829.05		20.24		7.50		36.20		300.11		0.37
M(4);O(1)	626.67		12.96		1.82		11.59		72.62		0.14
	617.62		7.44		0.96		6.23		38.45		0.13
	626.67		14.61		1.60		10.23		64.08		0.11
Experiment	**VSS (mg/l)**	**VSS (mg)**	**CH4 initial (mg)**	**CH4 consumed (mg)**	**HB (mg)**	**HV (mg)**	**PHBV (mg)**	**PHBV (%)**	**HV (%)**	**PHBV (mg/l)**	**Y_{PHB} (mgPHBV/mgCH4)**
Valeric acid 50 mg/l	864.76	21.62	114.59	21.64	5.64	1.96	7.60	35.15	25.79	304.00	0.70
	870.48	21.76	118.93	22.48	5.46	1.92	7.38	33.91	26.02	295.20	0.66
	839.52	20.99	107.66	19.76	5.08	1.50	6.58	31.35	22.80	263.20	0.67
Valeric acid 100 mg/l	752.86	18.82	104.33	27.97	5.37	3.43	8.80	46.76	38.98	352.00	0.63
	858.57	21.46	96.14	38.82	7.12	3.89	11.02	51.32	35.34	440.64	0.57
	725.71	18.14	93.62	29.32	5.04	2.44	7.48	41.21	32.58	299.04	0.51
Valeric acid 200 mg/l	878.57	21.96	98.98	27.50	3.60	1.73	5.33	24.27	32.46	213.20	0.39
	832.38	20.81	104.70	32.12	2.76	1.82	4.58	22.01	39.74	183.20	0.29
	843.81	21.10	106.10	19.40	2.33	1.97	4.30	20.36	45.81	171.84	0.44
Valeric acid 500 mg/l	800.48	20.01	100.85	25.91	1.82	1.88	3.70	18.49	50.81	148.00	0.29
	754.76	18.87	100.83	22.29	2.11	1.40	3.51	18.60	39.81	140.36	0.31
	836.19	20.90	101.09	27.06	1.69	1.70	3.39	16.22	50.15	135.60	0.25
Valeric acid 1000 mg/l	782.86	19.57	98.04	26.88	1.81	1.92	3.73	19.06	51.47	149.20	0.28
	739.05	18.48	94.94	25.85	1.89	1.99	3.88	21.00	51.29	155.20	0.30
	739.05	18.48	94.94	28.05	1.70	1.79	3.49	18.90	51.29	139.68	0.25
Valeric acid 2000 mg/l	851.43	21.29	101.81	40.86	1.56	1.45	3.01	14.14	48.17	120.40	0.15
	725.71	18.14	88.51	34.25	1.64	1.20	2.84	15.65	42.25	113.60	0.17
	709.52	17.74	101.18	31.47	1.36	1.52	2.88	16.23	52.80	115.16	0.18

Experiment	Valeric acid (mg/l)	VSS (mg/l)	VSS (mg)	HB (mg)	HV (mg)	PHBV (mg)	PHBV (%)	HV (%)	PHBV (mg/l)
M(1):O(1)	50	334.29	16.71	3.32	0.72	4.04	24.17	17.82	80.80
		320.00	16.00	2.75	0.79	3.54	22.13	22.32	70.80
M(2):O(1)		351.90	17.60	4.65	0.40	5.05	28.71	7.96	101.04
		355.71	17.79	3.85	0.43	4.28	24.08	10.09	85.64
M(1):O(2)		306.67	15.33	2.49	0.84	3.33	21.70	25.16	66.54
		316.19	15.81	2.06	0.90	2.96	18.74	30.38	59.25
M(1):O(3)		321.90	16.10	1.99	0.84	2.83	17.58	29.61	56.60
		340.48	17.02	1.65	0.94	2.59	15.21	36.29	51.80
M(1):O(1)	100	867.14	21.68	6.12	3.39	9.50	43.84	35.65	172.80
		865.24	21.63	3.78	1.83	5.61	25.94	32.55	102.00
		888.57	22.21	6.12	3.39	9.50	42.78	35.65	172.80
M(2):O(1)		944.29	23.61	7.58	4.12	11.70	49.56	35.21	234.00
		877.14	21.93	6.52	3.62	10.14	46.24	35.70	202.80
		900.95	22.52	6.02	3.52	9.54	42.36	36.90	190.80
M(1):O(2)		907.62	22.69	6.14	3.52	9.66	42.57	36.44	193.20
		888.10	22.20	5.58	3.36	8.94	40.27	37.58	178.80
		839.52	20.99	5.82	3.64	9.46	45.07	38.48	189.20
M(1):O(1)	200	338.57	16.93	3.10	1.73	4.83	28.53	35.82	96.60
		354.76	17.74	2.76	1.82	4.58	25.82	39.74	91.60
M(2):O(1)		319.52	15.98	3.23	1.63	4.86	30.39	33.57	97.10
		325.24	16.26	2.88	1.72	4.60	28.29	37.39	92.00
M(1):O(2)		376.19	18.81	2.10	2.03	4.13	21.96	49.15	82.60
		424.76	21.24	1.98	2.12	4.10	19.30	51.71	82.00
M(1):O(3)		297.14	14.86	0.85	1.24	2.09	14.07	59.33	41.80
		326.67	16.33	0.92	1.25	2.17	13.29	57.60	43.40

Table A.12: Raw data for Chapter 6 experiments

Exp.	O.D in	O.Dout	TSS inc	µ(hr-1)	O2 cons. (mg)	CH4cons. (mg)	O/M	O/M (molar)	fe	fs	Y	q	biomass density	F/M	C/N	N/M
CH4+ AMS	0.37	1.61	29.4	0.069	137	46	2.97	1.48	0.48	0.52	0.63	0.11	931	6.09	15	0.41
	0.39	1.68	30.7	0.069	105	38	2.72	1.36	0.36	0.64	0.79	0.09	972	4.39	11.3	0.39
CH4+ centrate	0.51	1.94	34.2	0.059	160	55	2.90	1.45	0.45	0.55	0.62	0.10	998	4.68	15.6	0.30
	0.47	1.84	32.7	0.062	164	57	2.87	1.44	0.44	0.57	0.57	0.11	971	4.78	14.7	0.32
Biogas+AMS	0.39	1.60	28.8	0.065	116	40	2.85	1.43	0.43	0.57	0.70	0.09	878	3.55	12.7	0.39
	0.35	1.55	28.7	0.072	153	50	3.07	1.53	0.53	0.47	0.57	0.13	938	4.00	9.19	0.44
Biogas+centrate	0.47	1.69	29.0	0.054	163	52	3.10	1.55	0.55	0.45	0.55	0.10	818	5.10	12.7	0.32
	0.48	1.83	32.1	0.058	174	56	3.08	1.54	0.54	0.46	0.57	0.10	930	5.72	11.1	0.31
centrate (%)	O.D in	O.D out	TSS inc	µ (hr-1)	O2 cons.(mg)	CH4 cons.(mg)	O/M	O/M (molar)	fe	fs	Y	q	CH4 (mg/hr)	O2 (mg/hr)	N/Mi	F/M
100	0.28	0.43	2.02	0.011	0	0	0	0	0	0	0	0	0	0	5.39	2.65
	0.27	0.41	1.90	0.011	0	0	0	0	0	0	0	0	0	0	5.67	3.69
50	0.39	1.18	16.67	0.043	50.18	14.83	3.38	1.69	0.69	0.31	0.38	0.11	1.24	4.18	1.98	2.51
	0.37	1.18	17.38	0.045	56.28	16.61	3.39	1.69	0.69	0.31	0.38	0.12	1.38	4.69	2.06	3.11
20	0.39	1.49	29.31	0.060	73.57	23.82	3.09	1.54	0.54	0.46	0.56	0.11	1.98	6.13	0.99	2.86
	0.38	1.49	30.36	0.062	80.07	27.59	2.90	1.45	0.45	0.55	0.67	0.09	2.30	6.67	1.02	3.25
10	0.46	2.05	50.71	0.072	98.27	35.34	2.78	1.39	0.39	0.61	0.75	0.10	2.95	8.19	0.33	4.34
	0.46	2.09	53.33	0.074	98.70	43.05	2.29	1.15	0.15	0.85	1.05	0.07	3.59	8.23	0.33	5.06
5	0.40	1.62	34.02	0.06	147.96	48.84	3.03	1.51	0.51	0.49	0.60	0.11	4.07	12.33	0.19	5.39
	0.40	1.57	32.05	0.06	147.27	50.25	2.93	1.47	0.47	0.53	0.66	0.09	4.19	12.27	0.19	5.75

Table A.13: Raw data for Chapter 7 experiments

Exp.	Growth (hrs)	PHB (hrs)	Format (mM)	VSS (mg/l)	PHBi (mg)	PHBi (%)	mg-CH4 consumed	CH4mg-C	CO2 produced	CO2mg-C	PHBf (mg)	PHBf (%)	PHBmg-C	Methanol (mg/l)	Methanol mg-C	Eff (%)	PHB conv (mg-C/mg-C)
1	12	0	100	652			12.6	9.49	30.9	8.44				378	7.09	74.7	0.00
				689			11.8	8.85	26.7	7.30				322	6.04	68.2	0.00
	24	0	100	1042			14.5	10.9	26.1	7.12				298	5.60	51.3	0.00
				1090			11.1	8.34	26.3	7.19				306	5.75	68.9	0.00
	48	0	100	1289			14.9	11.2	21.5	5.88				190	3.58	31.8	0.00
				1411			9.22	6.92	25.0	6.82				181	3.40	49.1	0.00
	72	0	100	1272			10.2	7.68	26.1	7.12				158	2.97	38.6	0.00
				1290			7.89	5.92	22.9	6.27				125	2.35	39.7	0.00
2	0	12	100	892	277	31.1	9.07	6.80	20.8	5.69	218	24.5	1.64	194	3.65	53.6	28.8
				867	324	37.4	8.50	6.37	21.5	5.87	256	29.6	1.89	172	3.23	50.6	32.2
	0	24	100	890	450	50.6	7.46	5.59	22.7	6.19	317	35.6	3.72	140	2.64	47.1	60.1
				930	509	54.7	8.22	6.16	23.2	6.34	379	40.8	3.61	150	2.82	45.7	56.9
	0	48	100	846	397	46.9	6.65	4.99	19.4	5.30	245	28.9	4.24	111	2.09	41.8	79.8
				833	425	51.0	6.79	5.09	21.4	5.86	280.	33.7	4.04	104	1.95	38.3	68.9
	0	72	100	840	409	48.7	5.20	3.90	19.0	5.19	248	29.5	4.50	86.	1.63	41.7	86.7
				803	385	47.9	5.19	3.89	19.6	5.36	222	27.6	4.54	90.	1.70	43.6	84.8
	0	12	0	794	207	26.1	7.16	5.37	18.4	5.03	91.4	11.5	3.24	128	2.41	44.8	64.3
				847	289	34.1	7.19	5.39	18.9	5.17	163.	19.2	3.51	119	2.25	41.7	67.9
	0	24	0	872	434	49.8	6.40	4.80	24.1	6.60	253	29.0	5.05	100	1.89	39.4	76.5
				880	440	50.0	5.42	4.07	27.8	7.60	241.	27.3	5.55	93.	1.76	43.3	73.0
	0	48	0	833	372	44.6	5.63	4.23	26.1	7.13	145	17.5	6.31	74	1.40	33.1	88.6
				833	397	47.7	4.39	3.29	30.0	8.19	185	22.2	5.94	61.3	1.15	34.9	72.4

Experiment	Growth phase(hrs)	PHB phase (hrs)	Format (mM)	Methanol production Duration (hrs)	VSS (mg/l)	PHBi (mg)	PHBi (%)	mg-CH4 consumed	CH4mg-C	CO2 produced	CO2mg-C	PHBf (mg)	PHBf (%)	PHBmg-C	Methanol (mg/l)	Methanol mg-C	Eff (%)	PHB conv (mg-C/mg-C)
3	24	0	100	6	635			13.5	10.1	6.84	1.87			0.00	271	5.08	50.0	0.0
					660			10.9	8.21	8.37	2.28			0.00	128	2.41	29.4	0.0
	24	0	100	12	692			14.4	10.8	15.4	4.22			0.00	337	6.32	58.2	0.0
					700			15.9	11.9	13.5	3.69			0.00	361	6.77	56.5	0.0
	24	0	100	24	620			18.7	14.1	17.1	4.67			0.00	434	8.14	57.8	0.0
					689			16.4	12.3	19.1	5.21			0.00	343	6.45	52.2	0.0
	24	0	100	48	704			22.5	16.9	17.7	4.83			0.00	302	5.67	33.5	0.0
					631			23.1	17.3	16.8	4.60			0.00	399	7.49	43.2	0.0
4	24	24	100	6	854	412	48.3	10.0	7.52	15.0	4.10	373	45.7	1.08	81.3	1.52	20.3	26.4
					878	412	47.0	10.4	7.86	17.0	4.66	357	44.3	1.53	58.0	1.09	13.8	32.8
	24	24	100	12	871	412	47.3	17.2	12.9	27.8	7.61	271	35.1	3.95	264	4.95	38.3	51.9
					950	412	43.4	17.8	13.4	32.1	8.76	241	29.8	4.76	218	4.10	30.6	54.4
	24	24	100	24	860	412	47.9	13.3	10.0	35.8	9.78	154	20.6	7.20	319	5.99	59.9	73.6
					872	412	47.3	13.7	10.2	36.6	9.99	170	22.7	6.75	347	6.51	63.4	67.5
	24	24	100	48	865	412	47.6	15.9	11.9	36.8	10.0	141	18.3	7.57	416	7.82	65.3	75.4
					885	412	46.6	13.1	9.83	39.8	10.8	103	13.6	8.62	382	7.17	72.9	79.4
5	24	0	25	6	654			11.1	8.33	25.8	7.06			18.2	154	2.90	34.8	0.0
					657			12.3	9.24	28.0	7.64			18.3	132	2.49	26.9	0.0
	24	0	25	12	66			13.9	10.4	18.8	5.14			1.83	232	4.35	41.6	0.0
					683			18.0	13.5	25.0	6.84			19.0	233	4.39	32.5	0.0
	24	0	25	24	687			20.0	15.0	32.6	8.91			19.1	224	4.21	28.0	0.0
					656			14.0	10.5	28.4	7.76			18.3	248	4.66	44.3	0.0
	24	0	25	48	646			19.9	14.9	37.1	10.1			18.0	138	2.59	17.3	0.0
					662			18.9	14.2	35.2	9.61			18.4	195	3.66	25.8	0.0
6	24	24	25	6	680	340	50	12.9	9.74	24.	6.71	201	31.9	3.88	117	2.21	22.7	57.8
					669	340	50.9	11.7	8.7	32.2	8.78	188	29.9	4.25	114	2.14	24.4	48.4

	24	24	25	12	660	340	51.6	15.6	11.7	27.7	7.56	163	27.6	4.93	250	4.70	40.1	65.2
					658	340	51.7	17.2	12.9	24.6	6.73	161	27.0	4.99	257	4.84	37.4	74.2
	24	24	25	24	669	340	50.8	17.0	12.8	25.2	6.89	129.	20.3	5.88	315	5.91	46.1	85.3
					695	340	49	16.5	12.4	24.	6.55	160.	24.8	5.03	337	6.32	51.0	76.9
	24	24	25	36	667	340	51	19.1	14.3	31.8	8.68	73.2	11.0	7.46	346	6.49	45.1	85.9
					660	340	51.5	17.1	12.8	28.4	7.77	93.1	13.2	6.90	366	6.87	53.4	88.9
7	24	24	0	6	571	306	53.6	11.5	8.28	9.61	2.62	251	39.6	1.54	97.5	1.83	22.1	58.6
					601	306	51.0	8.92	6.69	8.49	2.31	263	38.4	1.21	93.1	1.75	26.1	52.3
	24	24	0	12	572	306	53.6	9.87	7.41	13.7	3.74	212	27.9	2.62	132	2.48	33.5	70.0
					596	306	51.4	9.09	6.81	10.9	2.99	242	27.0	1.79	104	1.95	28.6	59.9
	24	24	0	24	567	306	54.0	10.9	8.21	15.8	4.33	194	18.9	3.13	166	3.13	38.1	72.2
					589	306	52.0	8.71	6.53	14.6	3.98	208.	20.5	2.75	147	2.76	42.3	69.1
	24	24	0	48	555	306	55.2	16.3	12.2	27.9	7.62	109.	10.7	5.50	320	6.01	49.1	72.1
					590	306	52.0	8.84	6.63	21.2	5.79	139	14.5	4.66	162	3.05	45.9	80.5

Table A.14: Raw data for Chapter 8 results

Harvest	media	oxygen	VSSin (mg/l)	PO4i (mg/l)	PO4i mg-p/l	Tpin (mg/l)	Tpin (mg-p/l)	TP (%)	PHB (mg)	PHB (%)
Growth	AMS	Aerobic	828.57	459.00	146.88	59.22	18.95	2.66	117.42	14.17
			870.48	459.00	146.88	59.69	19.10	2.54	117.42	13.49
Growth	AMS (P-free)	Aerobic	863.33	0.00	0.00	59.62	19.08	2.56	117.42	13.60
			850.95	0.00	0.00	59.48	19.03	2.59	117.42	13.80
Growth	AMS	Anoxic	867.62	459.00	146.88	59.66	19.09	2.54	117.42	13.53
			860.48	459.00	146.88	59.58	19.07	2.57	117.42	13.65
Growth	AMS (P-free)	Anoxic	854.29	0.00	0.00	59.52	19.05	2.58	117.42	13.74
			867.62	0.00	0.00	59.66	19.09	2.54	117.42	13.53
Growth	NMS	Aerobic	771.90	456.00	145.92	53.44	17.10	2.41	63.12	8.18
			750.48	462.00	147.84	53.30	17.06	2.48	63.12	8.41
Growth	NMS (P-free)	Aerobic	753.81	0.00	0.00	53.33	17.06	2.47	63.12	8.37
			788.10	0.00	0.00	53.54	17.13	2.36	63.12	8.01
Growth	NMS	Anoxic	787.62	456.00	145.92	53.54	17.13	2.36	63.12	8.01
			834.29	452.00	144.64	53.80	17.21	2.23	63.12	7.57
Growth	NMS (P-free)	Anoxic	642.86	0.00	0.00	52.49	16.80	2.90	63.12	9.82
			778.10	0.00	0.00	53.48	17.11	2.39	63.12	8.11
PHB	NMS	Aerobic	950.95	456.00	145.92	20.20	6.46	1.08	354.48	37.28
			994.76	456.00	145.92	20.73	6.63	1.04	354.48	35.63
PHB	NMS (P-free)	Aerobic	989.05	0.00	0.00	20.66	6.61	1.04	354.48	35.84
			969.52	0.00	0.00	20.43	6.54	1.06	354.48	36.56
PHB	NMS	Anoxic	956.67	456.00	145.92	20.27	6.49	1.08	354.48	37.05
			952.86	456.00	145.92	20.22	6.47	1.08	354.48	37.20

PHB	NMS (P-free)	Anoxic	1014.29	0.00	0.00	20.95	6.70	1.02	354.48	34.95
			972.86	0.00	0.00	20.47	6.55	1.06	354.48	36.44
PHB	0.25x-AMS	Aerobic	775.24	110.00	35.20	23.20	7.42	1.62	316.4	40.81
			779.05	114.00	36.48	23.28	7.45	1.61	316.4	40.61
PHB	0.5x-AMS	Aerobic	779.52	280.00	89.60	23.29	7.45	1.61	316.4	40.59
			778.57	250.00	80.00	23.27	7.45	1.61	316.4	40.64
PHB	2x-AMS	Aerobic	780.00	900.00	288.00	23.30	7.46	1.61	316.4	40.56
			759.52	950.00	304.00	22.87	7.32	1.65	316.4	41.66
PHB	4x-AMS	Aerobic	788.57	1800.00	576.00	23.47	7.51	1.59	316.4	40.12
			785.71	1750.00	560.00	23.41	7.49	1.60	316.4	40.27

Harvest	**media**	**oxygen**	**VSSf (mg/l)**	**CH4 con (mg)**	**CO2 prod (mg)**	**PO4f (mg/l)**	**PO4 mg-P/l**	**Tpf (mg/l)**	**Tpf (mg-p/l)**	**TPf (%)**	**PHBf (mg)**	**PHBf (%)**
Growth	AMS	Aerobic	1396	38.63	59.13	395.0	126.4	115.7	37.04	2.92	127.6	9.15
			1423	38.42	58.49	417.0	133.4	113	36.18	2.86	160.0	11.24
Growth	AMS (P-free)	Aerobic	1225	33.05	62.83	26.00	8.32	26.91	8.61	1.18	498.0	40.66
			1223	35.29	58.59	26.00	8.32	33.43	10.70	1.43	475.1	38.82
Growth	AMS	Anoxic	1011	11.98	18.60	505.0	161.6	24.15	7.73	0.98	223.7	22.12
			1000	12.11	15.39	498	159.3	19.38	6.20	0.85	274.3	27.43
Growth	AMS (P-free)	Anoxic	1012	11.56	16.64	40.00	12.80	26.10	8.35	1.20	316.7	31.29
			1043	13.72	14.69	35.00	11.20	25.68	8.22	1.07	273.3	26.20
Growth	NMS	Aerobic	1312	37.29	54.47	400.0	128.0	107.5	34.43	2.94	140.9	10.74
			1304	38.98	53.21	410.0	131.2	115.3	36.92	3.20	149.6	11.48
Growth	NMS (P-free)	Aerobic	1387	37.74	51.22	21.00	6.72	27.72	8.87	1.02	518.3	37.35
			1378	38.56	56.55	23.00	7.36	25.60	8.19	0.93	492.8	35.75
Growth	NMS	Anoxic	984.2	23.21	52.57	430.0	137.6	84.73	27.11	2.90	49.70	5.05
			1031	21.50	44.55	422.0	135.0	84.46	27.03	2.75	49.78	4.83

Growth	NMS (P-free)	Anoxic	777.8	10.76	16.74	29.00	9.28	28.86	9.24	1.44	136.9	17.60
			988.4	13.03	18.04	27.00	8.64	27.86	8.92	1.08	162.4	16.43
PHB	NMS	Aerobic	1518	46.03	71.18	350.0	112.0	112.7	36.09	2.78	220.2	14.50
			1513	41.94	62.26	355.0	113.6	109.7	35.12	2.77	246.0	16.26
PHB	NMS (P-free)	Aerobic	1420	40.86	58.78	5.00	1.60	15.03	4.81	0.72	754.8	53.14
			1461	37.69	56.29	4.00	1.28	14.69	4.70	0.65	733.4	50.18
PHB	NMS	Anoxic	1135	26.60	38.88	390	124.8	78.47	25.11	2.89	319.3	26.89
			1201	28.82	45.59	389.0	124.4	79.58	25.47	2.80	290.9	24.22
PHB	NMS (P-free)	Anoxic	1192	14.53	17.82	3.00	0.96	19.70	6.30	0.87	465.0	38.99
			1089	13.63	18.92	2.00	0.64	18.95	6.06	0.88	399.7	36.69
PHB	0.25x-AMS	Aerobic	1199	40.94	58.47	73.00	23.36	81.79	26.17	2.42	166.4	13.35
			1208	40.18	63.41	51.00	16.32	79.99	25.60	2.40	190.9	15.21
PHB	0.5x-AMS	Aerobic	1056	35.78	73.36	208.0	66.56	84.18	26.94	2.81	192.2	16.70
			1018	37.91	75.01	188.0	60.16	88.74	28.40	2.68	146.8	12.19
PHB	2x-AMS	Aerobic	867.1	21.97	54.65	887.0	283.8	43.26	13.84	1.89	230.8	23.99
			850.4	21.71	61.02	920.0	294.4	46.62	14.92	1.92	215.8	21.73
PHB	4x-AMS	Aerobic	736.6	20.22	50.00	1775	568.0	31.75	10.16	1.63	303.4	32.73
			779.5	19.98	50.61	1759.	562.8	31.25	10.00	1.57	284.9	30.90
Growth	0.25x-AMS	Anaerobic	843.6	15.47	27.10	132.0	42.24	19.76	6.32	1.23	277.0	35.02
			820.5	14.86	26.59	138.0	44.16	20.01	6.40	1.35	293.8	38.27
Growth	0.5x-AMS	Anaerobic	738.9	8.93	19.52	245.0	78.40	22.36	7.16	1.35	209.9	28.41
			766.8	9.64	19.73	259.0	82.88	20.77	6.65	1.40	240.5	33.68
Growth	2x-AMS	Anaerobic	593.6	4.62	18.45	869.0	278.0	30.28	9.69	1.92	141.2	21.86
			677.3	5.08	19.66	965.0	308.8	31.18	9.98	1.70	142.5	19.53
Growth	4x-AMS	Anaerobic	591.5	3.37	17.12	1812	579.8	44.73	14.31	2.44	109.2	15.68
			602.6	3.37	17.12	1945.	622.4	42.65	13.65	2.44	136.6	19.60