Synthesis and degradation of polyhydroxybutyrate (PHB) under different nutrient combinations in the alphaproteobacterial methanotroph, *Methylocystis* sp. Rockwell

by
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ABSTRACT

Methane emissions and plastic pollution are two distinct environmental issues that arise from human activities. The ongoing increases in methane emissions and its atmospheric concentration pose a major environmental threat as methane is a potent greenhouse gas, with a warming effect 28 times greater than carbon dioxide over a 100-year period. On the other hand, plastic pollution is a worrisome environmental concern because plastic waste can remain in the environment for hundreds of years and harm wildlife, ecosystems, and human health. Although methane emissions and plastic pollution are separate problems, addressing both issues requires a comprehensive strategy to minimize their harmful effects. One of the promising solutions to these problems involves the application of methane-oxidizing bacteria called methanotrophs. These microorganisms use methane as their energy and carbon source for cellular functions and can be used in biotechnology, bioremediation, and bioconversion. In addition to methane, methanotrophic bacteria can also utilize and convert methanol, a common industrial waste by-product, into high-value products such as biofuels and bioplastics. However, despite years of research in the methanotroph field, the paucity of knowledge on species-specific nutritional requirements hinders the scaling up of industrial methanotroph technologies. Critical questions concerning optimal nutrient (carbon, nitrogen and oxygen) combinations for faster growth and product yield remain unexplored for industrializing specific methanotrophic strains.

To address this, the present work investigated the impact of nutrient combinations, culture properties and processing strategies for the bioconversion of methane and methanol into poly-3-hydroxybutyrate (PHB), a biodegradable thermoplastic polyester, by the alphaproteobacterial methanotroph *Methylocystis* sp. Rockwell.

Firstly, combinations of methane (as carbon source) and ammonium mineral salts (AMS) or nitrate mineral salts (NMS) media (as sources of nitrogen) were investigated to find nutrient combinations favoring the optimal, simultaneous, production of biomass and PHB by *Methylocystis* sp. Rockwell using Response Surface Methodology (RSM). While it is generally recognized that high N:C ratios favor biomass production and low ratios are necessary for PHB production, a multi-objective approach was used to determine the optimal N:C ratio of 0.016 to yield the maximum combined biomass and PHB.

Secondly, the impact of the metabolic state of the inocula (well-fed vs starved) on growth and PHB management with different levels and combinations of nutrients (methane, ammonium, and oxygen) was investigated. These experiments showed that well-fed inocula can grow even under nitrogen limitation and accumulate PHB in subsequent culture. Both biomass and PHB production were higher in an oxygen rich environment. However, recurrent nitrogen limitation when using starved inocula limited subsequence growth and PHB production. These findings offer valuable insights into inoculum preparation, media formulation and process design to improve biomass and PHB production in an industrial setting.

Thirdly, bioreactor experiments were conducted to demonstrate the potential of *Methylocystis* sp. Rockwell to consume methane and methanol to produce PHB and determine effective processing strategies for fed-batch cultivation. This study found that a methanol concentration of 22.5 mM exceeded the toxicity threshold, and confirmed that lower nitrogen concentrations were favorable for higher PHB accumulation. Adapted pulse-feeding of methanol and nitrogen in a fed-batch bioreactor led to a maximum PHB concentration of 654.56 ± 47.04 mg/L with a PHB dry cell weight content of 66.21 ± 6.64 % and a PHB productivity rate of 45.05 ± 5.77 mg/L/d after two weeks of operation. This work expanded the scope of methanol-based bioprocesses for optimizing biomass and PHB production in *Methylocystis* sp. Rockwell.

PREFACE

This thesis is an original research work performed by Hem Kanta Sharma. It has three research chapters, for which I contributed as primary author. One research chapter has been published and the remaining chapters will be submitted for publication.

Chapter 3 was published as Sharma, H.K., Sauvageau, D., Stein, L.Y. (2022) "Optimization of methane feed and N:C ratio for biomass and polyhydroxybutyrate production by the alphaproteobacterial methanotroph *Methylocystis* sp. Rockwell". *Methane*, *1*:355-364. https://doi.org/10.3390/methane1040026. As the primary author, I designed and carried out the experiments, analyzed experimental data and drafted the manuscript. Dr. Dominic Sauvageau and Dr. Lisa Y. Stein were the supervisory authors who envisioned the study, provided advice, provided funding, and contributed to experimental design, data interpretation and manuscript composition.

Chapters 4 and 5 will be submitted for publication as Sharma, H.K., Sauvageau, D., and Stein, L.Y. ": Impact of culture history on growth and fate of polyhydroxybutyrate in *Methylocystis* sp. Rockwell", and Sharma, H.K., Sauvageau, D., Stein, L.Y. "Improving growth and polyhydroxybutyrate production of *Methylocystis* sp. Rockwell using methanol fed-batch bioreactors", respectively. As the primary author, I designed and carried out the experiments, analyzed experimental data and drafted the manuscripts. Dr. Dominic Sauvageau and Dr. Lisa Y. Stein were the supervisory authors who envisioned the study, provided advice, provided funding, and contributed to experimental design, data interpretation and manuscript composition.

DEDICATION

I would like to dedicate this thesis work to my dear parents and loving wife. I am very lucky to have you in my life. Your understanding, thoughtfulness, motivation, love and caring have changed my life from a simple man to a wise person. "Thank you for everything."

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"May God grant us wisdom and strength to cope the predicament; may God exalt and expand the magnitude of this research work beneficial to humankind and environment."

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List of abbreviations

ACR : Acetyl-CoA reductase

AMS : Ammonium mineral salts

ANME : Anaerobic methane-oxidizing archaea

ANOVA : One-way analysis of variance

AOB : Ammonia-oxidizing bacteria

ATP : Adenosine triphosphate

BCB : Bubble-column bioreactors

BKT : β-ketothiolase

CFR : Continuous-flow reactors

CoA : Coenzyme A

CSTR : Continuously stirred tank reactors

DHAP : Dihydroxyacetone phosphate

DO : Dissolved oxygen

DOE : Design of experiments

dPHA : Denatured PHA

DW : Dry weight

ePhaZ : Extracellular PHA depolymerase

FADH : Formaldehyde dehydrogenase

FBP : Fructose 1,6-bisphosphate

FDH : Formate dehydrogenase

FMP : Fructose 6-phosphate

GAP : Glyceraldehyde 3-phosphate

GC-FID : Gas chromatography-Flame ionization detection

GC-TCD: Gas-chromatograph-Thermal conductivity detector

H6PI : Hexulose-6-phosphate isomerase

HAO : Hydroxylamine dehydrogenase

HPLC : High-performance liquid chromatography

HPS : Hexulose-phosphate-synthase

HURM : Hydroxylamine ubiquinone redox module

ICI : Imperial Chemical Industry Biological

iPhaZ : Intracellular PHA depolymerase

JGI : Joint Genome Institute

KDPG : 2-keto-3-deoxy-6-phosphogluconate

MDH : Methanol dehydrogenase

MMO : Methane monooxygenase

MOOC : Multi-objective optimal conditions

MTF : Methylene tetrahydrofolate

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

NADH: Nicotinamide adenine dinucleotide

NMS : Nitrate mineral salts

nPHA : Intracellular PHA granules

OD : Optical density

OMeGA: Methanotroph Genome Analysis

OVAT : One-variable-at-a-time

PHA : Polyhydroxyalkanoates

PhaZ : PHB depolymerases

PHB : Poly-3-hydroxybutyrate

PHBV : Poly-3-hydroxybutyrate-co-3-hydroxyvalerate

pMMO : Particulate methane monooxygenase

ppGpp : Guanosine tetraphosphate

(p)ppGpp: Guanosine pentaphosphate

RiMP : Ribose-5-phosphate

RPM : Rotation per minute

RSM : Response surface methodology

RuMP : Ribulose monophosphate

SCF : Self cycling fermentation

slpm : Standard liter per minute

sMMO : Soluble methane monooxygenase

TCA : Tricarboxylic acid

XuMP : Xylulose-5-phosphate

CHAPTER 1: Introduction

1.1. Global environmental concerns and PHAs from alphaproteobacterial methanotrophs

As nature is the foundation and support for life, environmental protection has indeed become an important worldwide concern. Global increase in atmospheric methane – a driver of global warming and climate change (Dlugokencky et al., 2011) – and escalating petroleum-based plastic pollution are two undeniable environmental problems stemming from human activities. While comprehending the sources and sinks of methane is intricate, it is essential to develop and implement effective approaches to mitigate the harmful methane emissions that otherwise negatively impact the environment (Mar et al., 2022). Moreover, the accumulation and persistence of non-degradable plastics and their often toxic additives on land and in waterbodies not only deteriorate the quality of environments but also threaten many lifeforms (Hahladakis et al., 2018; Teuten et al., 2009). These issues create a state of planetary emergency and thus require urgent attention.

One of the most promising biological approaches to address the aforementioned issues is through the adoption of methanotroph-based biotechnology. Methanotrophs are a unique group of microorganisms that can use methane as their sole carbon and energy source. As a result, they have the potential to mitigate greenhouse gas emissions by converting methane into various bioproducts, including biodegradable plastics like polyhydroxyalkanoates (PHAs). Non-degradable petroleum plastics can be replaced with functionally equivalent, nontoxic, biodegradable and biocompatible polymers such as PHAs. Over the last few decades, there has been increasing scientific attention placed on identifying and producing such biopolymers. Many PHAs, such as poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), have been shown to be produced by microorganisms (Loureiro dos Santos, 2017), and it has been established that these microbes accumulate intracellular PHA granules under growth-limiting conditions.

Among these biopolymers, PHB is the most commonly used biopolymer for the manufacturing of bioplastics. Moreover, the replacement of conventional nondegradable plastics by bioplastics has the potential to minimize the effect of plastic pollution globally. A 6B's approach, consisting of "Bacteria-Biogas-Bioreactor-Biopolymers-Bioplastics-Biodegradation", could be an effective way to minimize both methane emission and plastic pollution, thereby reducing their respective carbon footprint (Fig. 1.1).

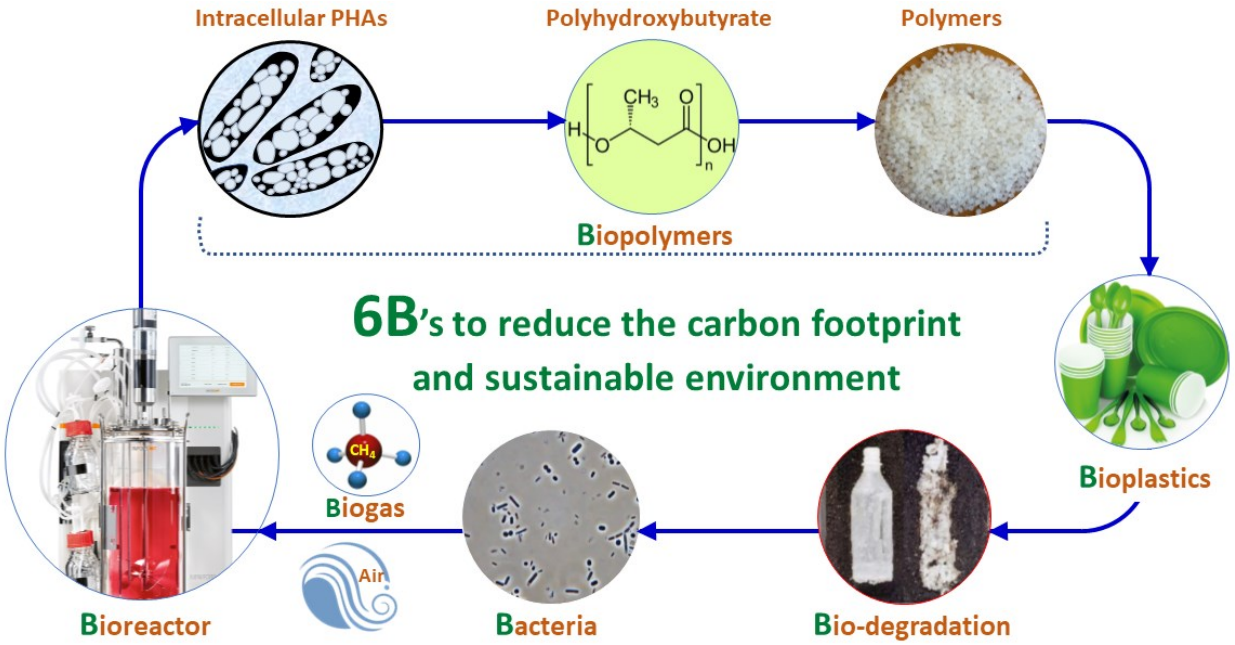

Fig. 1.1. 6B's approach (Bacteria-Biogas-Bioreactor-Biopolymers-Bioplastics-Biodegradation) of methanotroph cultivation for production of biodegradable plastic, reduce carbon footprint and create sustainable environment.

1.2. Project outline and scope of study

Although PHB production from sugar-based carbon sources have been studied more commonly among different bacteria (Sen et al., 2019; Jiang et al., 2016; El-Kadi et al., 2021), bioconversion of single-carbon compounds such as methane and methanol using methanotrophic

bacteria for PHB production is a promising alternative because the methanotrophic bioconversion approach is devoid of sugar based feed stocks hence, there is no competition for food and land use to produce this biopolymer. Moreover, it is beneficial in terms of carbon sequestration, mitigation of methane emissions and plastics pollution. The alphaproteobacterial methanotrophs have been extensively studied for PHB production from methane or methanol because of their enzymatic machinery for metabolic conversion of single-carbon substrates to PHB. This approach relies on the efficient growth and bioconversion abilities of methanotrophs (Tays et al., 2018) and has the potential to offer a nontoxic, biodegradable and biocompatible polymer with a reduced carbon footprint as a sustainable alternative to synthetic polymers. As this approach does not rely on sugar-based fermentation, it has the potential to be a cost-effective, environmentally friendly, and socially acceptable biotechnological approach. There is a clear understanding that PHB production in alphaproteobacterial methanotrophs can be achieved by providing them with excess carbon followed by a starvation signal (Pieja, Rostkowski, et al., 2011; Rostkowski et al., 2013). Many alphaproteobacterial methanotrophs, including species of *Methylocystis* and *Methylosinus*, can utilize single-carbon compounds such as methane or methanol as a sole source of carbon and energy for their growth and PHB accumulation. They assimilate carbon via the serine pathway and incorporate it into intracellular PHB granules via the PHB biosynthetic pathway (Strong et al., 2016). While all alphaproteobacterial methanotrophs use the same metabolic pathways for PHB production, there is significant variability in preferred carbon and nitrogen sources that enhance PHB synthesis among different species (Zaldívar Carrillo et al., 2018; Zhang et al., 2017).

Despite evidence of different nitrogen source preference to support growth of methanotrophic isolates (Rostkowski et al., 2013; Tays et al., 2018; Zhang et al., 2017) the impact of nutrients on PHB production by alphaproteobacterial methanotrophs has not been thoroughly studied. While *Methylosinus trichosporium* OB3b (Pieja, Rostkowski, et al., 2011; Zaldívar

Carrillo et al., 2018; Zhang et al., 2019), *Methylocystis parvus* OBBP (Asenjo & Suk, 1986; Pieja, Rostkowski, et al., 2011; Pieja, Sundstrom, et al., 2011; Sundstrom & Criddle, 2015) and *Methylocystis hirsute* (García-Pérez et al., 2018; Pieja, Rostkowski, et al., 2011) have been extensively studied for their growth and PHB production, there has not yet been similar study conducted on *Methylocystis* sp. Rockwell for optimizing nutrient combinations towards biomass and PHB production. As the alphaproteobacterial methanotrophs are known to have PHB production capacity under a wide set of conditions (Pieja, Rostkowski, et al., 2011; Stein et al., 2011), preference of *Methylocystis* sp. Rockwell to grow on ammonium (Tays et al., 2018) and its ability to produce moderate amount of PHB even under non-optimized conditions, comparable to that of optimized conditions for *M. trichosporium* OB3b (Lazic et al., 2022; Whiddon, 2018), indicates that *Methylocystis* sp. Rockwell is a promising candidate for further investigation and industrialization.

The work in this thesis is divided into three research chapters. The first study (Chapter 3) employed response surface methodology (RSM) using a full factorial experimental design simultaneously (de Baun, 1959) to optimize the supply of methane and nitrogen (ammonium and nitrate) sources with the aim to improve biomass and PHB production simultaneously. In the second study (Chapter 4), the growth of *Methylocystis* sp. Rockwell in batch cultures from well-fed versus starved inoculum and the fate of its PHB content under various nutrient combinations were investigated. The third study (Chapter 5) focused on the development of a methanol-based fed-batch bioreactor operation to achieve high cell density and improved PHB production.

1.3. Knowledge gap and rationale of study

Methane is not only an abundant natural gas that methanotrophs prefer to utilize as a growth substrate, but also an inexpensive feedstock for large scale production of PHB. Utilization of

effluent or waste methane can reduce greenhouse gas emissions and is an effective means of carbon sequestration (Pieja, Sundstrom, et al., 2011). The development and implementation of efficient methane bioconversion technologies for PHB production should be accomplished to mitigate both methane emissions and plastic pollution. However, creating processes that result in high bacterial biomass levels containing high amounts of PHB is a major bottleneck for industrial implementation. Despite considerable progress towards understanding the roles of different enzymes involved in PHB accumulation and degradation within microorganisms, it is unclear how growth conditions and cultivation influence PHB accumulation and consumption in methanotrophs. To resolve these knowledge gaps, a clear understanding of suitable culture conditions leading to biomass production and how cultivation affects the fate of intracellular PHB are important towards optimizing production and recovery of PHB in an industrial context. Although many studies have focused on PHB production by methanotrophs, challenges such as variability in nutritional preference, inadequate knowledge on appropriate cultivation methods, and economic viability of using methanotrophs for PHB production still exist. Further research is required to comprehend the potential of methanotrophs as sustainable producers of bioplastics, particularly on the effect of nutrient preferences on their growth and PHB production (Tays et al., 2018; Zaldívar Carrillo et al., 2018; Rostkowski et al., 2013; Sundstrom & Criddle, 2015).

In the present study, the methane-consuming bacterium *Methylocystis* sp. strain Rockwell was used as experimental organism. *Methylocystis* sp. Rockwell is an aerobic, Gram-negative, alphaproteobacterial methanotroph. Recent studies have been conducted in the Stein and Sauvageau laboratories to assess the effects of carbon and nitrogen sources on growth and metabolite production in *Methylocystis* sp. Rockwell (Tays et al., 2018; Lazic et al., 2021). While it was established that *Methylocystis* sp. Rockwell prefers ammonium instead of nitrate as a N-source (Tays et al., 2018), there has yet to be a study aimed at optimizing methane feed and N:C

ratio for simultaneous biomass and PHB production. Additionally, there have been no previous attempts to cultivate *Methylocystis* sp. Rockwell in bioreactors to achieve high cell density and product titer. However, to design a suitable bioreactor, it is essential to understand how growth and PHB accumulation changes with inoculum history and various nutrient combinations. All of these factors have been explored in this thesis to develop more effective scale-up strategies for biomass and PHB production by *Methylocystis* sp. Rockwell that are applicable towards its industrialization.

1.4. Hypothesis

Although each research chapter is organized around a specific hypothesis, a general hypothesis for the unified work is that a bioprocess based on nutrient supply strategies and multi-objective optimization approaches can simultaneously yield high biomass and high PHB cell content of *Methylocystis* sp. Rockwell (Deb & Deb, 2014). This result can be achieved despite biomass and PHB accumulation having opposing responses to nitrogen limitation; however, both are necessary to achieve for industrial scale adoption of a methanotroph based bioindustry.

In Chapter 3, I hypothesized that a multi-objective strategy will identify an optimal N:C ratio to enhance intracellular PHB accumulation in *Methylocystis* sp. Rockwell while maintaining high growth. To achieve these goals, an effective strategy based on the level of nitrogen to methane supply enabled the accumulation of PHB during active growth of *Methylocystis* sp. Rockwell, which is a similar strategy used in other methanotrophic bacteria (D'Alessio et al., 2017).

In Chapter 4, as bacteria can utilize PHB to maintain cellular integrity and sustain metabolic processes under nutrient starvation by balancing carbon and energy levels (Pieja, Sundstrom, et al., 2011; Sadykov et al., 2017; Müller-Santos et al., 2020), I hypothesized that metabolic state of a batch culture inoculum (well-fed versus starved) would influence bacterial growth and the fate of intracellular PHB in subsequent cultures. An experimental approach involving 16 different nutrient

combinations with varying levels of carbon, nitrogen and oxygen was employed to compare the growth and PHB content of *Methylocystis* sp. Rockwell for batch cultures initiated with either well-fed or starved inocula.

Finally, based on the results from Chapters 3 and 4, *Methylocystis* sp. Rockwell demonstrated PHB accumulation during growth, particularly under low concentration of ammonium and high levels of oxygen. Thus, I hypothesized that extending the exponential growth phase while controlling carbon source and its supply, and also maintaining a low N:C ratio, could enhance biomass and PHB production in a fed-batch bioreactor. I also hypothesized based on experiments by Lazic et al. (2021) that methanol can induce a stress condition that enhances PHB production over growth on methane alone. To test these hypotheses, a 3-L benchtop bioreactor was used to conduct fed-batch experiments by selecting and configuring processing parameters to best optimize growth and PHB production.

1.5. Objectives of this study

To optimize the production of PHB, achieving a high cell density is crucial, which requires the optimization of variables and a clear understanding of intracellular PHB synthesis and degradation cycles. Therefore, the long-term goals of this collection of studies is to comprehend the combined effect of nutrients, inoculation, and feeding strategies on growth and PHB production. This understanding will ultimately assist in developing an effective scale-up strategy for biomass and PHB production by *Methylocystis* sp. Rockwell.

To achieve this long-term goal, the thesis is designed around three main objectives for *Methylocystis* sp. Rockwell: to identify the optimal N:C ratio required to balance its biomass and PHB production, to assess the impact of culture history on its growth and fate of PHB under

different nutrient combinations, and to improve its biomass and PHB production in a methanol fed-batch bioreactor operation. Each objective represents an individual research chapter of this thesis.

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CHAPTER 2: Literature review

2.1. Methane as a greenhouse gas

Methane (CH_4) is the simplest alkane (hydrocarbon) and a major component of natural gas. It is a reduced organic compound abundantly found in nature and plays a vital role in atmospheric chemistry and biogeochemical cycles (Keppler et al., 2009). It is formed naturally in anoxic environments, biologically through microbial decomposition of organic matter, and geochemically when layers of decomposing organic matter undergo thermal breakdown processes due to intense heat and pressure below the Earth surface or seafloor (Barnes & Goldberg, 1976). Methane formation takes place on upland soil, below ground, in wetlands, under the seafloor, in permafrost or in deep ocean sediments (in the form of methane hydrates), among other locations (O'Connor et al., 2010).

The anaerobic decomposition of submerged vegetation/organic matter results in the release of methane; a process commonly called methanogenesis. The microorganisms belonging to the archaeal domain that perform methanogenesis are known as methanogens (Zabranska & Pokorna, 2018). These archaea can use substrates such as hydrogen, carbon dioxide, acetate, formate, and methanol to produce methane (Enzmann et al., 2018), and they are mainly found in anoxic environments, such as marshes, wetlands, the ocean floor, and the digestive tract of animals (including termites, ruminant, human). Methane from agriculture, solid and liquid waste, and fossil fuel production constitute major sources of anthropogenic emissions; however, methanogenesis from wetlands is the greatest contributor to atmospheric methane at about 167 Mt per year (Fig. 2.1) (Saunois et al., 2016). Enteric methane emissions, mainly from ruminant livestock, produce about 80 Mt of methane annually, representing a third of all anthropogenic methane emissions (Eckard et al., 2010). Similarly, the world population of termites is estimated to produce 1 – 3% of global methane emissions (Nauer et al., 2018). Methane hydrates, a frozen mixture of water and

concentrated natural gas formed at high pressures and low temperatures in the deep ocean floor (Zhang et al., 2017), have an estimated reservoir of about 1146 Gt of methane. These hydrates naturally release methane to the atmosphere as they undergo freeze-thaw cycles (Kretschmer et al., 2015), a process that is expected to accelerate with permafrost thaw associated with global warming.

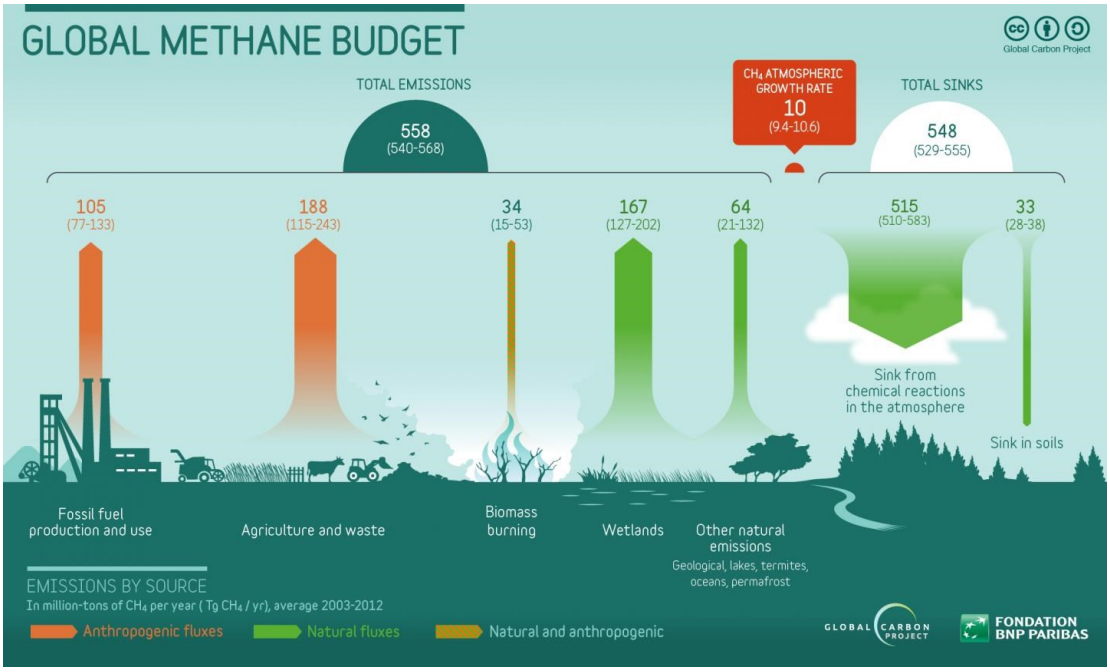

Fig. 2.1. Global Methane Budget. Methane emission vs sink analysis has estimated an annual growth rate of 10 million tons of atmospheric methane (Saunois et al., 2016). Copyright, CC Attribution 3.0 Unported.

Atmospheric methane, which is methane released from the biosphere to the atmosphere, is primarily emitted during production, transportation, and use of fossil fuels as well as through agricultural activities such as livestock farming, rice cultivation, and manure management. The global atmospheric methane concentration has more than doubled when compared to pre-industrial levels (1819 ppb compared to 722 ppb) and continues to accumulate at a rapid pace (Abdalla et al., 2016). Methane emission/sink analysis has estimated an annual growth rate of 10 million tons of

atmospheric methane (Saunois et al., 2016), with contributions from both anthropogenic and natural emissions. Anthropogenic methane emissions account for 60% of atmospheric methane and drive at least 25% of global warming (Saunois et al., 2016). The largest anthropogenic methane emitters are China (from coal production), the United States (from oil and gas, livestock, and landfills) and Russia (from natural gas and oil). India, Brazil and Mexico are also large emitters of atmospheric methane. As methane is a highly potent greenhouse gas with a global warming potential about 28 times greater than carbon dioxide (CO_2) over a period of 100 years, its increasing concentration in the atmosphere inhibits the escape of solar radiation from the Earth's surface, driving global warming, climate change and a range of environmental problems (MacCarty et al., 2008; Dlugokencky et al., 2011). Capturing of methane from oil and industrial waste seems unfeasible because it requires a large amount of air and energy. For this reason, the gaseous hydrocarbons from oil and industrial waste consisting of– natural gas, propane, ethylene, propylene, butane, etc. is usually flared with atmospheric oxygen results in the production of carbon dioxide. The World Bank has estimated that about 150 billion m^3 of natural gas is flared annually around the world to reduce the harmful effect of atmospheric methane (Emam, 2015).

2.2. Petroleum-based plastic pollution

Petroleum-based plastic pollution is another environmental concern caused by the accumulation of plastic waste in the environment. Manufacturing of conventional plastics involves extraction, transportation, and processing of fossil fuels or petroleum. Nondegradable petroleum-based synthetic polymers, such as polyethylene, polystyrene, polypropylene, polyvinyl chloride, polyurethanes etc., are most commonly used for the production of plastics materials (Gewert et al., 2015) that are aimed to improve our quality of life, with a wide range of applications from packaging materials to life-saving medical devices. However, the widespread accumulation of

plastic materials in the environment, from oceans to landfills, is a rapidly growing environmental concern (DiGregorio, 2009). The overwhelming increase in global plastics production reached 460 million metric tons in 2019, and nearly 55 % (4600 million of tons) of plastic materials are dumped to landfill or discarded annually (Ritchie & Roser, 2018). Although conventional plastics have some useful properties – such as being inexpensive, durable, lightweight, corrosion-resistant and electrical insulators (Thompson et al., 2009) – their recalcitrant nature hinders the natural degradation processes. When plastics are discarded, they can persist for hundreds of years in the natural environment and generally break down into smaller particles, release chemical residue (such as dioxins, sulfur oxides, hydrogen chloride, cadmium) during incineration and leach harmful additives like plasticizers (phthalates), flame retardants, and antioxidants, in landfill sites (Hahladakis et al., 2018; Teuten et al., 2009; Keshavarz & Roy, 2010; Harding et al., 2007). Plastic pollution can have negative impacts on the environment, including harming wildlife and humans, polluting waterbodies, and contributing to climate change. The impact of plastic pollution on wildlife is particularly significant, with marine animals (including fish, birds, turtles and other animals) frequently ingesting plastic waste or becoming entangled in it, leading to injury or death. This creates a critical situation in terms of threatening various life forms (Gewert et al., 2015; Krueger et al., 2015). Moreover, plastic waste management, including incineration and landfilling, releases greenhouse gases such as methane and carbon dioxide, contributing to further climate change. This justifies the need for the development and adoption of biodegradable, sustainable alternatives like bioplastics.

2.3. Methanotrophs and biochemistry of methane oxidation

Methanotrophs belong to a broader class of bacteria known as methylotrophs which grow on reduced single-carbon substrates (such as methane and/or methanol) and multi-carbon

substrates without carbon-carbon bonds (such as dimethyl ether, dimethylamine, trimethylamine). Proteobacterial methanotrophs are aerobic prokaryotes capable of oxidizing methane and methanol as their sole carbon source for growth and energy production. They are generally slow growers due to a reliance on low energy metabolic pathways (Murrell, 2010; Trotsenko & Murrell, 2008). Methanotrophs are found in a wide range of habitats from upland soils of grasslands and forests to sediments in wetlands and the ocean floor, and generally ecosystems where methanogenesis is taking place. Methanotrophs present in these habitats oxidize methane before it is released to the atmosphere and thus represent the biological methane sink of the global carbon cycle (Topp & Pattey, 1997).

Methanotrophs are broadly classified into four phyla: *Proteobacteria, Verrucomicrobia*, NC10 and anaerobic methane-oxidizing archaea (ANME). The proteobacterial methanotrophs are further classified as *Gammaproteobacteria* and *Alphaproteobacteria*. These proteobacterial methanotrophs have distinct physiological traits such as carbon assimilation pathways, inner membrane structure and lipid composition (Hanson & Hanson, 1996). Gammaproteobacterial methanotrophs – e.g. *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus*, *Methylosarcina* – possess uniform intracytoplasmic membranes with a series of flat disc-shaped vesicles, whereas alphaproteobacterial methanotrophs – such as *Methylosinus* and *Methylocystis* – possess paired membranes consisting of vesicles running parallel mainly to the cell margin (Higgins, I. J., Best, D. J., Hammond, R. C., & Scott, 1981) except the *Methylocapsa* and *Methylocella* of *Beijerinckiaceae* family which lack extensive internal membrane system (Dedysh et al., 2005).

The bioconversion of methane to CO_2 is a multistep pathway which takes place in aerobic methanotrophs under ambient conditions. Despite their taxonomic diversity, aerobic and NC10 methanotrophs share a unique physiological capability of methane oxidation initiated by catalytic

action of methane monooxygenase (MMO). There are two forms of MMO: the cytoplasmic soluble methane monooxygenase (sMMO) complex is found mainly in alphaproteobacterial methanotrophs, and the membrane-bound, particulate methane monooxygenase (pMMO) is found in most aerobic and NC10 methanotrophs. Both MMOs oxidize the C-H bond of methane to methanol in the presence of oxygen and required reducing equivalents (Hanson & Hanson, 1996). sMMO requires NAD(P)H, whereas pMMO requires cytochromes and NADH to oxidize methane into methanol (Lipscomb, 1994). Methane oxidation in the gammaproteobacterial methanotrophs use a direct coupling mechanism in which methanol oxidation provides electrons for methane oxidation and cytochrome-c proteins directly supply reducing power from methanol dehydrogenase to MMO (Bordel et al., 2019). Whereas alphaproteobacterial methanotrophs use a redox arm mechanism in which ubiquinone acts as an electron supplier (electrons come from NADH produced by formate oxidation) to the MMO (Bordel et al., 2019). Methane oxidation requires two reduction equivalents (NADH) and an oxygen molecule to oxidize methane to methanol. This oxidation process is first initiated by splitting of O-O by the action of NADH. One O-atom is reduced to water and the second O-atom is incorporated in methane to form methanol.

Subsequent oxidation of methanol to CO_2 is catalyzed by dehydrogenase enzymes producing formaldehyde and formate as respective intermediates. Methanol dehydrogenase (MDH) further oxidizes methanol to formaldehyde (HCHO). Formaldehyde is the central metabolite of methane oxidation which can enter either anabolic or catabolic pathways. In the catabolic pathway, formaldehyde is oxidized to formate (HCOOH) via tetrahydromethanopterin (H_4MPT) and tetrahydrofolate (H_4F) pathways involving $NAD(P)^+$ dependent dehydrogenase enzymes, which is then further oxidized to CO_2 by formate dehydrogenase (FDH), along with the regeneration of two NADH molecules (an important reducing equivalent to initiate the methane oxidation reaction) (Fig. 2.2) (Lipscomb, 1994). The reducing equivalents so formed in

the sequential oxidation of methanol, formaldehyde, and formate conserve the energy by producing electrons necessary for oxidative phosphorylation. Two of the six electrons formed during the methanol oxidation are necessary for methane oxidation (first step), while the remaining four make their way towards the membrane-bound electron transport chain to reduce the terminal electron acceptor, oxygen (Fig. 2.2) and create a proton motive force to generate ATP (Trotsenko & Murrell, 2008; AlSayed et al., 2018).

However, the formaldehyde assimilation pathway is distinct among proteobacterial methanotrophs: gammaproteobacterial methanotrophs assimilate formaldehyde via the ribulose monophosphate (RuMP) pathway, whereas alphaproteobacterial methanotrophs assimilate formaldehyde via the serine pathway (Hanson & Hanson, 1996; Smith & Murrell, 2009).

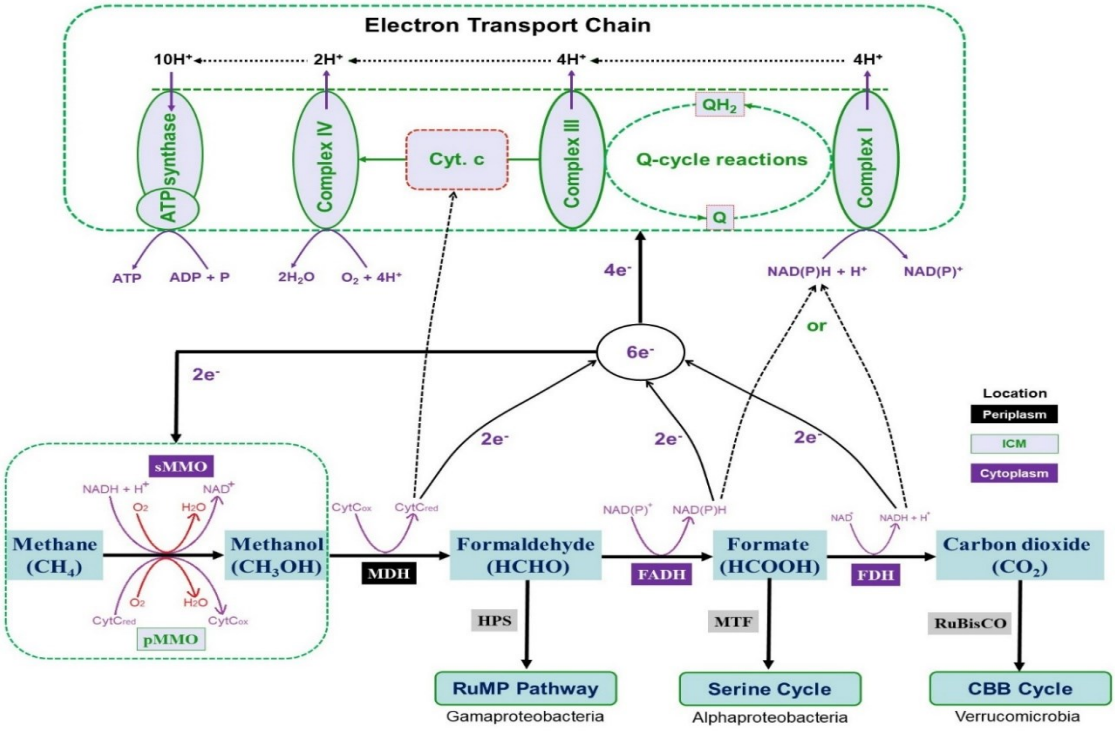

Fig. 2.2. Methane oxidation, carbon assimilation and oxidative phosphorylation in methanotrophs, adapted from (Hanson & Hanson, 1996; AlSayed et al., 2018; Tanthachoon et al., 2008) with modifications. Font colour represent the locations of enzymes and their biochemical reactions occurring inside the cell.

2.3.1. Ribulose monophosphate (RuMP) pathway

Gammaproteobacterial methanotrophs typically assimilate formaldehyde through the RuMP pathway (Fig. 2.3.) in three main steps: fixation, cleavage and rearrangement (Fei et al., 2014). Assimilation (i.e. fixation) begins with aldol condensation of HCHO with D-ribulose-5-phosphate catalysed by hexulose-phosphate-synthase (HPS) to form 3-hexulose 6-phosphate, which is further isomerized into fructose 6-phosphate by hexulose-6-phosphate isomerase (H6PI) (Clarke, 1982). The second step (i.e. cleavage) involves further breakdown of fructose-6-phosphate (FMP) to either fructose 1,6-bisphosphate (FBP) by phosphofructokinase, or 2-keto-3-deoxy-6-phosphogluconate (KDPG) by Entner–Doudoroff enzymes (White et al., 2007). Both FBP and KDPG are further cleaved by aldolases to give different products. The cleavage of FBP results in the formation of glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), while KDPG cleavage results in the formation of pyruvate (Fei et al., 2014). The final step (i.e. rearrangement) involves a series of reactions in which the remaining GAP and FMP molecules are catalyzed by transketolase, transaldolase or sedoheptulose-1,7-bisphosphate aldolase to produce two molecules of xylulose-5-phosphate (XuMP) and one molecule of ribose-5-phosphate (RiMP), which finally produce RuMP by ribulose-phosphate 3-epimerase and ribose-5-phosphate isomerase (Fei et al., 2014; White et al., 2007).

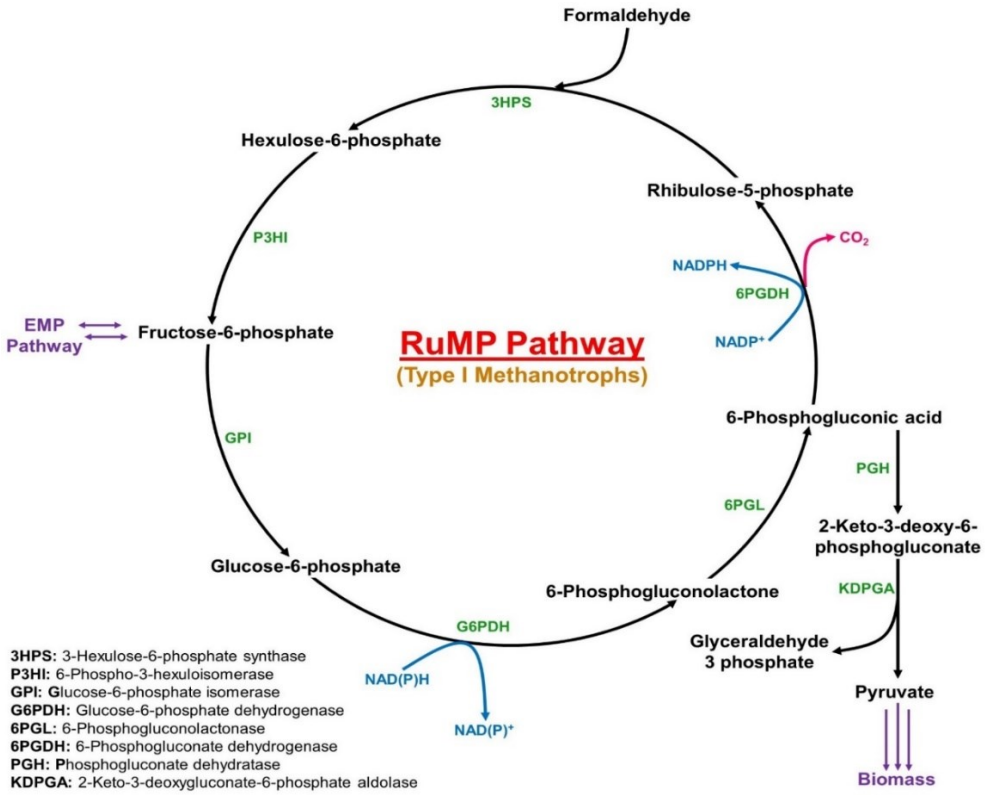

Fig. 2.3. RuMP pathway of gammaproteobacterial methanotrophs, adapted from (Fei et al., 2014; White et al., 2007).

2.3.2. Serine cycle

Alphaproteobacterial methanotrophs assimilate formaldehyde using the serine pathway (Fig. 2.4). This is initiated by H_4MPT and H_4F where formaldehyde first convert into methylene tetrahydrofolate then combines with glycine to form serine. Serine undergoes transamination with glyoxylate catalysed by serine glyoxylate aminotransferase to produce hydroxypyruvate and glycine. Hydroxypyruvate undergoes reduction to glycerate by hydroxypyruvate reductase. Glycerate, in turn, is transformed into 3-P-glycerate with the aid of glycerate kinase. Subsequently, 3-P-glycerate is catalysed by enolase into phosphoenol-pyruvate which undergoes carboxylation to form oxaloacetate. Malate dehydrogenase reduces the oxaloacetate into malate and then further converted into malyl-coA by action of malate thiokinase. Through the catalysis of malyl-CoA

lyase, malyl-CoA is cleaved into acetyl-coA and glyoxylate. Once acetyl-CoA is formed during nutrient-rich conditions, it can either contribute to fatty acid and phospholipid biosynthesis or enter the tricarboxylic acid (TCA) cycle to produce energy or the ethylmalonyl-CoA (EMC) pathway to produce glyoxylate (Trotsenko & Murrell, 2008). Otherwise, under nutrient deficiency, acetyl-CoA enters the PHB biosynthetic pathway (Karthikeyan et al., 2015). The serine cycle utilizes two ATP and two NADH for the formation of each molecule of acetyl-CoA (Erik Kristensen & Bo Thamdrup, 2005).

2.3.3. PHB cycle

As mentioned above, alphaproteobacterial methanotrophs synthesize PHB from acetyl-CoA under nutrient deficient conditions (Fig. 2.4). This is promoted when there is reduced TCA activity, higher NAD(P)H concentration, and induction of the genes encoding enzymes for PHB formation (Gillmaier et al., 2016). PHB biosynthesis begins with the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA by the β-ketothiolase (BKT). The enzyme acetyl-CoA reductase (ACR) further acts on acetoacetyl-CoA to form hydroxybutyryl-CoA. Finally, polyhydroxybutyrate synthetase catalyzes the polymerisation of hydroxybutyryl-CoA to form PHB (Karthikeyan et al., 2015). The PHB so formed can be utilized for energy storage, a mobilizable carbon repository, or as a reducing equivalent (Sipkema et al., 2000) to initiate methane oxidation. At this point, PHB can also be extracted as a biopolymer to manufacture bioplastic (Getachew & Woldesenbet, 2016).

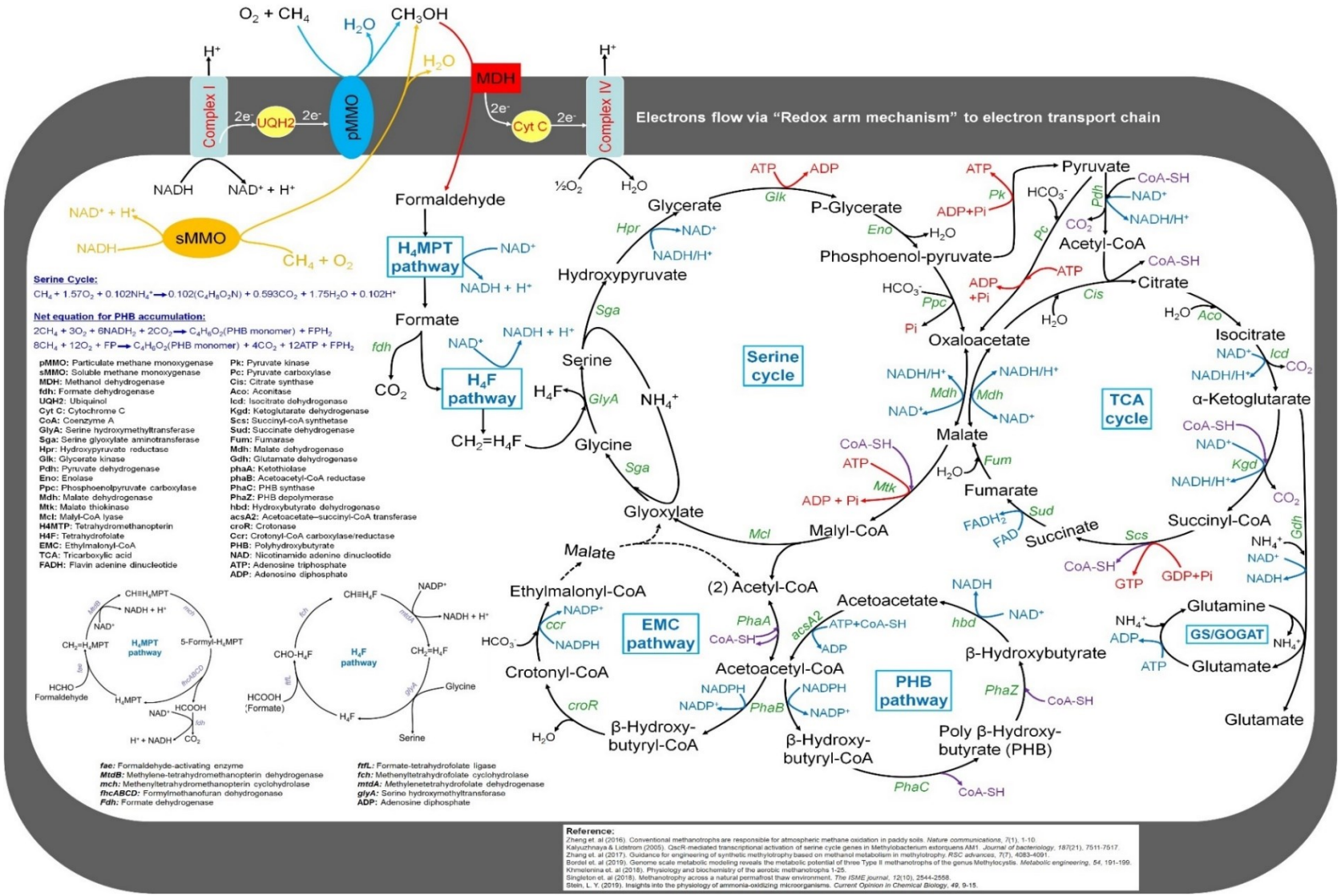

Fig. 2.4. Metabolic pathways of methane oxidation and carbon assimilation, and electron flow via Redox arm mechanism in alphaproteobacterial methanotrophs adapted from (Bordel et al., 2019; Cai et al., 2016; Kalyuzhnaya & Lidstrom, 2005; W. Zhang et al., 2017; Khmelenina et al., 2018; Singleton et al., 2018; Stein, 2019), with necessary modifications.

2.4. Polyhydroxyalkanoates (PHAs)

Microbial biopolymers in the PHA family are widely found in Gram-negative bacteria and function as storage compounds of carbon and energy. The most common PHAs are polyhydroxybutyrate (PHB) and polyhydroxybutyrate co-hydroxyvalerate (PHBV) which can be used as matrices for biodegradable plastic (bioplastics) (Ansari & Fatma, 2014; Paula et al., 2018).

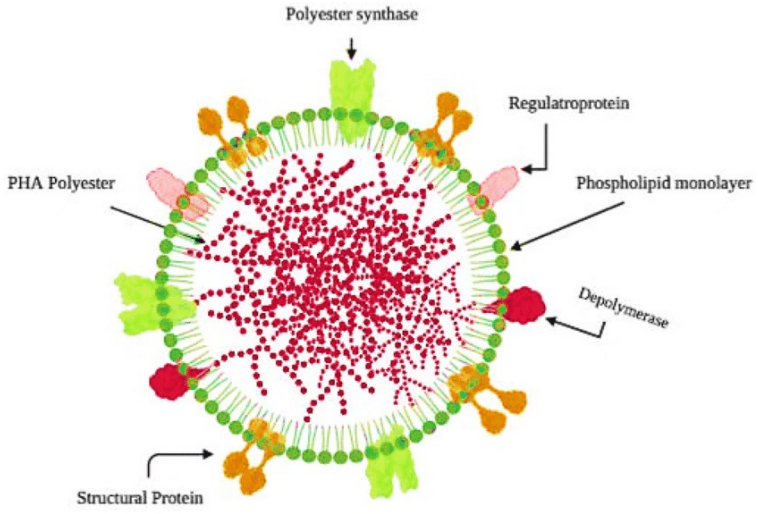

Fig. 2.5. Schematic demonstration of a native PHA granule, adapted with permission from (Muneer et al., 2020), Copyright 2023, Springer Nature.

Intracellular PHA granules (nPHA) are found in an amorphous elastomeric state enclosed by a phospholipid layer, that also harbors some proteins, to form a granule (Fig. 2.5). While nPHA granules are mobile and sensitive to physical and chemical stress, the denatured polymer (dPHA) is partially crystalline, similar to synthetic PHB (Jendrossek & Handrick, 2002). Generally, cell disruption denatures the surface layer of nPHA, leading to the rapid solidification of granules and crystallization of the polymer. The surface layer of nPHA granules can be denatured physically by freeze-thaw cycles or repeated centrifugation, and chemically by solvents, detergents, and alkali. However, amorphous nPHA can be recovered by carefully disrupting the cells using a French press

or enzymatic lysis method followed by density gradient centrifugation (Jendrossek & Handrick, 2002; Merrick & Doudoroff, 1964). nPHA has attracted commercial and academic interest due to its non-toxic, biocompatible, biodegradable, renewable and eco-friendly nature. It has wider application as PHA polymer latexes (for use in paints and paper coating), biomedical application (for use in protein purification, drug delivery, molecular imaging, and biosensors), and production of biodegradable plastic materials (Bonartsev et al., 2019; Philip et al., 2007; Ray & Kalia, 2017).

2.4.1. PHB metabolism and regulating mechanism

PHB metabolism involves synthesis and degradation catalysed by PHB synthase and PHB depolymerase, respectively. As described in section 2.3.3, PHB synthesis occurs when PHB synthase along with other enzymes catalyze the polymerization of acetyl-CoA into PHB when the organism undergoes a stress. As for PHB degradation, it is catalyzed by PHB depolymerases (PhaZs). There are two main types of PhaZs – intracellular PHA depolymerase (i-PhaZ) and extracellular PHA depolymerase (e-PhaZ). I-PhaZs actively hydrolyze the amorphous form of native PHB granules inside the cell, also known as mobilization, but it cannot hydrolyze semicrystalline or crystalline PHB, except for i-PhaZ from *Bacillus megaterium* which can degrade both native PHB granules and semicrystalline PHB into its monomeric forms (Tseng et al., 2006; Chen et al., 2009). E-PhaZs are generally composed of a signal peptide, a large N-terminal catalytic domain, a short linker region, and a substrate-binding domain at the C-terminal. The signal peptide is generally composed of 22 to 58 amino acids responsible for secreting the mature PhaZ through the membrane (Jendrossek & Handrick, 2002). While most PHB-producing bacteria derive carbon and energy from PHB metabolism during periods of carbon starvation, this is not the case for methanotrophs. Methanotrophic bacteria cannot grow by utilizing stored PHB granules (Pieja, Sundstrom, et al., 2011). As methanotrophic bacteria mostly rely on C1 compounds (methane

and/or methanol) for carbon and energy, the role of intracellular PHB granules is not well understood, except as a general energy storage and reducing equivalent.

Generally, PHB accumulation in bacteria is an effective adaptive mechanism associated with stress response (called stringent response) and cell survival strategy (Obruca et al., 2016). Nitrogen limitation is associated with nitrogen regulation, (Ntr-) mediated, stringent response which activates the ppGpp synthetase, RelA, resulting in elevation of intracellular nucleotides – guanosine tetraphosphate and guanosine pentaphosphate, collectively referred to as (p)ppGpp, or the24ffecttor alarmone (Brown et al., 2014). Such increased (p)ppGpp levels have positive correlation with PHB accumulation in PHB-producing bacteria (Juengert et al., 2017).

2.4.2. Degradation of PHAs

PHA biopolymers are susceptible to chemical and thermal degradation; however, the work in this thesis mainly focused on PHB biodegradation. As PHAs are synthesized and stored in the form of intracellular cytoplasmic granules by bacteria (Anderson & Dawes, 1990), the biodegradation of PHAs takes place through enzymatic hydrolysis under a range of environmental conditions. In fact, both environmental conditions and PHA properties affect the rate of its degradation. PHA degradation through the microbial activity of PHA-degrading enzymes can take place in natural environments such as soil, sludge, and seawater. The rate of biodegradation by PHA depolymerase enzymes (PhaZs) is highly dependent on the crystallinity of the polymer. Depending upon the secretion and mode of action there are two types of PHA depolymerases: intracellular PhaZs that degrade amorphous PHA, and extracellular PhaZ which are secreted outside the cell and degrade crystalline PHA. Both intracellular and extracellular PhaZs, are classified under the hydrolase enzyme categories (EC: 3.1.1.75) (Knoll et al., 2009), and can degrade PHAs (mostly PHB) into monomeric or dimeric units (Williams, 1988), which are further

metabolized into carbon dioxide, water, methane, inorganic compounds and biomass (Folino et al., 2020; Meereboer et al., 2020). While degrading the biopolymer, PhaZs easily hydrolyze the amorphous regions first followed by subsequent slower degradation of crystalline regions (Kumagai et al., 1992). Most PhaZs act on the polymer by initially binding to the crystalline regions of polymer followed by its slower degradation (Abe et al., 1995). This implies that PhaZs generally favour degradation of lower crystallinity PHA. Usually, the aerobic mineralization of biopolymers produces carbon dioxide while anaerobic degradation produces biogas (carbon dioxide and methane) (Meereboer et al., 2020).

A (2009) study showed that, out of 587 PHA depolymerases compiled in the PHA depolymerase engineering database (http://www.ded.uni-stuttgart.de), only about 30 had been experimentally validated for PHA depolymerase activity (Knoll et al., 2009), and most of them were from non-methanotrophic microorganisms. Among non-methanotrophic microbes, several PHB-degrading extracellular PhaZs (Handrick et al., 2000; Juengert et al., 2017; Jung et al., 2018; Martínez-Tobón et al., 2018; A. Shah et al., 2008) and very few intracellular PhaZs (Saegusa et al., 2001; Tseng et al., 2006; Huisman et al., 1991) have been characterized. Vecherskaya et al. have observed intracellular PHB degradation in *Methylocystis parvus* under anaerobic condition when cells were deprived of exogenous carbon sources (Vecherskaya et al., 2009).

2.5. PHB production from alphaproteobacterial methanotrophs

Many bacteria can accumulate PHB intracellularly, up to 80% of their dry cell weight (Khosravi-Darani et al., 2013). Methanotrophs produce PHB under stress or nutrient deficiency (Getachew & Woldesenbet, 2016), often when there are limiting essential nutrients or under excess availability of carbon sources (Lee et al., 1994). While most alphaproteobacterial methanotrophs are known for their ability to produce PHB, members of the genera *Methylosinus* and *Methylocystis*

are predominantly used for the development of bioprocesses (Pieja, Rostkowski, et al., 2011). Zhang et al. have reported accumulation of 0.6 g PHB/L with PHB content of 30% from *Methylosinus trichosporium* IMV301 in a two-stage batch culture – growth phase using CH_4/air gas mixture (1:1, v/v) followed by PHB accumulation phase (Y. Zhang et al., 2008). In one study, 48.7 ± 8.3 mg PHB/L, corresponding to 52.5 ± 6.3% (dry cell weight), was obtained from a batch culture of *Methylosinus trichosporium* OB3b grown in a combination of methane + methanol (total of 20 mmol carbon) with nitrate as the N-source and optimal N:C ratio of 0.017 (Zaldívar Carrillo et al., 2018). A higher PHB concentration of 3.43 g/L was also observed in pure cultures of *Methylocystis parvus* OBBP in a continuous-flow bioreactor supplied with CH_4:O_2 gas mixture (1:1, v/v) (Sundstrom & Criddle, 2015). Table 2.1 highlights PHB production among studies using various strains of alphaproteobacterial methanotrophs cultivated under different conditions.

Table 2.1. Comparison of growth conditions and PHB production from alphaproteobacterial methanotrophs

Strain	Culture conditions	PHB production	References
M. trichosporium sp. OB3B	Carbon: 30% CH_4 + 70% CH_3OH & Nitrogen: KNO_3 with N:C ratio of 0.017. Batch culture with a total of 20 mmol carbon.	PHB content: 52.5 ± 6.3% PHB: 48.7 ± 8.3 mg/L	(Zaldívar Carrillo et al., 2018)
	CH_4:O_2 = 1:1 & vitamin solution. Culture grown to mid-exponential phase then transferred to nitrate-free medium and incubated for 24 h.	PHB content: 38 ± 4%	(Pieja, Rostkowski, et al., 2011)
	Initial headspace: 0.5 atm CH_4, 0.3 atm N_2 & 0.2 atm O_2. Headspace gas refreshed every 12 h. Harvested after 5 days (OD of 1.5±0.05).	PHB content: 55.5% PHB: 901.8 mg/L	(T. Zhang et al., 2019)
M. parvus OBBP	Supply CH_4 & O_2 mixture (50% each)/24 h for 2 min. Increased mass transfer of CH_4 and O_2 could produced 5 g/L of cells in batch culture.	PHB content: 68%	(Asenjo & Suk, 1986)
	CH_4:O_2 = 1:1 & vitamin solution. Culture grown to mid-exponential phase then transferred to nitrate-free medium and incubated for 24 h.	PHB content: 36 ± 8%	(Pieja, Rostkowski, et al., 2011)
	CH_4 & O_2 (55 ml each), no nitrogen in 125 ml serum bottles. Time: 18-66 h. O_2:CH_4 molar consumption= 1:1.4	PHB content: 50%, PHB: 0.28 mg/mL	(Pieja, Sundstrom, et al., 2011)
	Experiments performed in a continuous-flow reactor. CH_4:O_2 = 1:1 (v/v) with 5 µM Cu & 7.2 µM Ca. Biomass = 6.9 ± 0.3 g/liter	PHB content : 49.4 ± 1.6%, PHB : 3.43 ± 0.24 g/L	(Sundstrom & Criddle, 2015)
	5 strains of alphaproteobacterial methanotrophs in sequencing batch reactor operated under repeated nitrogen & CH_4 limitations, and feast–famine conditions were dominated by *M. parvus* OBPP.	Repeated nitrogen & CH_4 limitations induced higher PHB accumulation	(Pieja et al., 2012)
Methylocystis sp. 42/22	CH_4:O_2 = 1:1 & vitamin solution. Culture grown to mid-exponential phase then transferred to nitrate-free medium and incubated for 24 h.	PHB content: 25 ± 7%	(Pieja, Rostkowski, et al., 2011)
Methylocystis sp. SC2	CH_4:O_2 = 1:1 & vitamin solution. Culture grown to mid-exponential phase then transferred to nitrate-free medium and incubated for 24 h.	PHB content: 30±13%	(Pieja, Rostkowski, et al., 2011)
***Methylocystis* sp. Rockwell**	Optimization of growth and PHB production. Fate of PHB in different nutrient conditions. Batch and fed-batch operation in bioreactors.	See chapter 3, 4 and 5	**This study**
M. hirsute	CH_4 abatement in bubble column bioreactor. Biomass = 4.5 ± 0.6 g/L.	PHB content: 40%	(García-Pérez et al., 2018)
M. hirsute CSC1	CH_4:O_2 = 1:1 & vitamin solution. Culture grown to mid-exponential phase then transferred to nitrate-free medium and incubated for 24 h.	PHB content: 7 ± 2%	(Pieja, Rostkowski, et al., 2011)
M. hirsuta DSMZ 18500	High cell density cultivation with 1:1 ratio of methanol:ethanol in a loop bioreactor achieved 4.5 g/L of cell biomass.	PHB content of 85%	(Ghoddosi et al., 2019)
M. trichosporium IMV 3011	CH_4:air (v/v) = 1:1 (replenished 12 hourly)	PHB content: 38.6%	(Xin et al., 2007)
	Improved growth and PHB production when methanol was added to the culture grown with methane than those without methanol	PHB: 0.6 g/L PHB content: 40%	(Y. Zhang et al., 2008)
	Culture grown in methanol fed-batch achieved biomass of 2.91 g/L. Addition of malic acid increased biomass (3.32 g/L) and PHB yield.	PHB content increased from 47.6%. to 58.5%	(Song et al., 2011)
	Addition of copper in the culture grown on methane led to 2 folds increased in cells biomass (0.48 g/L) and increase in PHB content.	PHB content increased to 8.3%	(J. Dong, 2012)

2.6. Factors affecting growth of methanotrophs and PHB production

Microbes rely on carbon for both their structure and as an energy source, while nitrogen is crucial for the synthesis of amino acids, proteins, and nucleic acids. The growth and cellular functions of bacteria are determined by the balance of carbon and nitrogen in their surrounding environment. In conditions where there is a higher proportion of carbon to nitrogen (known as nitrogen limitation), bacteria need to seek out additional sources of nitrogen to balance out the excess carbon. Under such circumstance, bacteria may undergo several physiological and metabolic changes. One of the most common responses is a decrease in the rate of growth and division, as the bacteria conserve their resources and focus on survival rather than replication. They may scavenge for nitrogen from alternative sources in the environment, such as amino acids or organic nitrogen compounds. Alternatively, they may start accumulating storage compounds such as PHB, which are used to store carbon. Various physiochemical parameters such as carbon sources (methane, methanol, glucose, lignocellulosic biomass, etc.), nitrogen sources (nitrate and ammonia salts), oxygen concentration, temperature, pH, and chemical inhibitors like furan-based chemicals, organic acids, and some phenolic compounds, play an important role in growth and PHB production (Fergala et al., 2018). Moreover, the synergistic effect of carbon, nitrogen, and oxygen present in the media can impact PHB production in methanotrophs (Zaldívar Carrillo et al., 2018).

2.6.1 Carbon sources

As mentioned earlier, most methanotrophs can utilize either methane or methanol as a source of carbon for growth. Tays et al. (2018) studied growth preferences in five methanotrophic strains using either methane or methanol as a carbon source, and ammonium or nitrate as a nitrogen source. In that study, methane was preferred to methanol by most of the strains tested; in fact, growth inhibition was observed by the majority of strains when methanol (20 mM) was used as a

sole carbon source. Although methanol toxicity has been known to inhibit growth of methanotrophs (Whittenbury et al., 1970), the application of a suitable concentration of methanol offers a readily available carbon source, as methanol is water miscible, and require only a moderate amount oxygen to catabolize (Khosravi-Darani et al., 2013). Alphaproteobacterial methanotrophs store carbon in the form of PHB when there is an excess availability of carbon (methane and/or methanol) and oxygen but under nitrogen starvation (Getachew & Woldesenbet, 2016; Pieja, Rostkowski, et al., 2011; Pieja, Sundstrom, et al., 2011). Addition of methanol to methane-grown cultures can enhance PHB production (Song et al., 2011; Y. Zhang et al., 2008). Zhang et al. observed improved bacterial growth and PHB production in *Methylosinus trichosporium* IMV3011 when methanol was added to cultures grown with methane, compared to methane alone (Y. Zhang et al., 2008). *M. trichosporium* OB3b also showed increased PHB production when cultured in a methane and methanol combination, suggesting that methanol could be utilized as a potential stress inducer for PHB accumulation (Lazic et al., 2021; Zaldívar Carrillo et al., 2018).

2.6.2. Nitrogen sources

Most methanotrophs can utilize either nitrate or ammonium as a nitrogen source (Whittenbury et al., 1970) while some also have capacity to fix N_2 (Bowman, 2006; Murrell & Dalton, 1983; Stein, 2018). Generally, alphaproteobacterial methanotrophs prefer ammonium for growth, whereas gammaproteobacterial methanotrophs mainly prefer nitrate as a N-source (Nyerges et al., 2010; Tays et al., 2018). In some alphaproteobacterial methanotrophs, ammonium reduces the uptake of methane, most likely due to structural similarity between ammonium and methane leading to inhibition of methane monooxygenase enzymes and production of cytotoxic products such as hydroxylamine and nitrite (Nyerges & Stein, 2009; Nyerges et al., 2010). While methane and ammonia oxidation are physiologically similar, the ability to utilize electrons from

hydroxylamine oxidation differs. Unlike ammonia-oxidizing bacteria (AOB) which use cytochromes c552 and c554 to transfer electrons from hydroxylamine oxidation to the ubiquinone pool, known as the hydroxylamine ubiquinone redox module (HURM), for energy generation, methanotrophs cannot use ammonia as an energy source because they do not possess these cytochromes (Klotz & Stein, 2008). While some methanotrophs can utilize nitrate as a terminal electron acceptor and survive in low-oxygen conditions or can detoxify nitrite using reductase enzymes (Kits et al., 2015), *Methylocystis* sp. Rockwell is particularly sensitive to nitrate/nitrite toxicity, resulting growth inhibition (Klotz & Stein, 2008).

Typically, nitrogen limitation triggers a stress response towards PHB accumulation. However, preference to N-source was also reported in PHB accumulation among the alphaproteobacterial methanotrophs. *M. trichosporium* OB3b accumulated PHB up to 50% of the cell dry weight when grown in 20 mM nitrate as the N-source (Shah et al., 1996). Similarly, another study on *M. trichosporium* OB3b produced 13% PHB when grown in 10 mM ammonium as the N-source; however, when the N-source was changed to nitrogen gas, the PHB accumulation was increased to 45% (Rostkowski et al., 2013). In another recent study, similar lower yield of PHB was reported when *M. trichosporium* OB3b was grown in ammonium compared to nitrate (Zhang et al., 2017). *M. parvus* OBBP grown on ammonium accumulated 60% PHB when it was subsequently transferred to nitrogen limited conditions (Rostkowski et al., 2013), while, on the contrary, only 36% PHB was accumulated when this strain was grown in nitrate (Pieja, Rostkowski, et al., 2011). Similarly, *Methylocystis hirsuta* grown on ammonium accumulated 51% PHB once transferred to a nitrogen deficient conditions (Rahnama et al., 2012). When comparing the PHB yield, *M. parvus* OBBP, yielded more PHB than *M. trichosporium* OB3b when grown in ammonium (Rostkowski et al., 2013). Moreover, a total of 30% PHB was achieved when *M. trichosporium* OB3b was grown in a combination of both nitrate (10 mM) and ammonium (8 mM)

(Doronina et al., 2008). These studies indicate that methanotrophs are fairly specific in selecting their preferred N-source for growth and PHB production; however, factors such as the optimization of nitrogen concentrations (more specifically the N:C ratio) play a critical role in PHB production. For example, a study on *M. trichosporium* OB3b showed a higher yield of PHB at a low N:C ratio of 0.017, highlighting the role of nitrogen limitation in PHB accumulation (Zaldívar Carrillo et al., 2018).

2.6.3. Oxygen

Proteobacterial methanotrophs are aerobes, requiring oxygen to catabolize methane and as their primary terminal electron acceptor. Even though some methanotrophs can achieve methane conversion at imperceptible oxygen concentrations (Kalyuzhnaya et al., 2013), the rate of methanol to formaldehyde conversion is vastly increased at high oxygen levels (approximately 35% headspace) (Costa et al., 2001). The partial pressure of oxygen thus affects biomass production and PHB accumulation in methanotrophs. Rostkowski et al. observed a maximum biomass production from *M. trichosporium* OB3b and *M. parvus* OBBP at an oxygen partial pressure of 0.4 atm, whereas higher PHB accumulation was observed at 0.2 atm for *M. trichosporium* OB3b (45% of cell dry weight) and at 0.3 atm for *M. parvus* OBBP (60% of cell dry weight) (Rostkowski et al., 2013). When comparing *M. trichosporium* OB3b cultures in oxygen-rich conditions (O:C = 9.7:1) and oxygen-limiting conditions (O:C = 2.2:1), a similar level of bacterial growth was observed but a 10-fold increase in PHB production was detected under oxygen-rich conditions, suggesting a critical role of oxygen in PHB accumulation (Zaldívar Carrillo et al., 2018). Both methane and oxygen are essential during PHB biosynthesis in alphaproteobacterial methanotrophs; an oxygen-to-methane stoichiometric ratio of 1.5:1 is generally required for efficient PHB production

(Rostkowski et al., 2013), indicating that oxygen limitation is unfavorable for PHB accumulation (Zaldívar Carrillo et al., 2018; Rostkowski et al., 2013).

2.6.4. Other nutrients

Microbial tolerance to inhibitors has great industrial significance. Several factors including the microbial strain used, gas composition, and concentrations and proportions of nutrients, can influence growth and polymer synthesis in microbes. Generally, polymer synthesis is induced by an imbalanced supply of essential macronutrients such as nitrogen or phosphorus and microelements like magnesium, sodium, calcium, potassium, iron, manganese, sulfur, or copper (Sundstrom & Criddle, 2015; Kim & Lenz, 2001). A study on *Methylobacterium* sp. showed an inhibition of cell growth due to a high concentration of sodium, potassium and magnesium. On the other hand, deficiency in magnesium and potassium in *M. parvus* OBBP cultures resulted in increased PHB accumulation. Similar decreases in calcium concentration increased PHB accumulation on *M. parvus* OBBP (Sundstrom & Criddle, 2015). Phosphorus also influences growth and PHB accumulation in alphaproteobacterial methanotrophs with a concentration of 2-25 mM necessary to maintain normal sMMO activity. Phosphorus concentration above 40 mM inhibited the growth of *M. trichosporium* OB3b (Park et al., 1991) by suppressing the activity of methanol dehydrogenase (Mehta et al. 1987). This, in turn, results in inadequate production of formaldehyde for the assimilation of carbon in the cells or for the generation of NADH through dissimilation (Bowman & Sayler, 1994). However, phosphorous deficiency resulted in an increased PHB accumulation in *M. parvus* OBBP (Sundstrom & Criddle, 2015). Similar increases in PHB accumulation (from 16% to 26.5% cell dry weight) were observed in *M. trichosporium* IMV3011, when the phosphorus concentration was decreased from 7.3 mM to 5.7 mM (Y. Zhang et al., 2008). Furthermore, cultivation in the presence of citric acid under nutrient deficiency also

favored high PHB accumulation in *M. trichosporium* IMV3011 (Y. Zhang et al., 2008). Finally, low calcium concentrations under nitrogen-limited conditions also enhanced the PHB production in *M. parvus* OBBP (Sundstrom & Criddle, 2015).

2.6.5. Mass transfer limitation of gaseous substrates

In bioreactors and cultures, the mass transfer of gaseous substrates from the gas phase (bubbles or headspace) to the liquid medium and eventually into the cell is a complex process driven by thermodynamic parameters and differences in chemical potential. In methanotroph-based biotechnology, methane and oxygen uptake remains a major constraint, mainly due to the poor solubility and gas-liquid mass transfer limitations of these gases leading to slow substrate consumption and low cell density and product yield (Gęsicka et al., 2021). Although several efforts have been made to improve product titer and productivity – such as increasing partial pressure, changing reactor configuration and adding mass transfer vector (Kraakman et al., 2011) – a poor mass transfer rate and low solubility of gaseous substrates are major hurdles in designing and operating large scale bioprocesses (Yasin et al., 2015; Gęsicka et al., 2021). Most methanotroph bioconversion studies have been limited to laboratory or small-scale bottle cultures. However, significant progress has been made towards PHB production using various types of bioreactors – such as continuously stirred tank reactors (CSTR) (Chidambarampadmavathy et al., 2015), bubble-column bioreactors (BCB) (García-Pérez et al., 2018; Rodríguez et al., 2020; Sundstrom & Criddle, 2015; Ghoddosi et al., 2019), continuous-flow reactors (CFR) (Sundstrom & Criddle, 2015) and vertical loop bioreactors (Rahnama et al., 2012). Some fundamental techno-economic bottlenecks that need to be addressed include the design and adoption of optimal reactor configurations, and the selection of substrate and feeding strategy to ensure a higher substrate availability and cell density so as to achieve better process performance.

2.7. *Methylocystis* sp. Rockwell

In this work, *Methylocystis* sp. Rockwell was used as an experimental microorganism. It is an aerobic, Gram-negative, alphaproteobacterial methanotroph, isolated from an aquifer in Canoga Park California, United States (Stein et al., 2011). The complete genome of *Methylocystis* sp. Rockwell (ATCC 49242) was sequenced and annotated by the Joint Genome Institute (JGI) by the Organization for Methanotroph Genome Analysis (OMeGA). Its genome size is 4.6 Mbp with a GC content of 63% and 4,637 protein-encoding genes identified to date. This strain lacks sMMO but has a single *pmoCAB* operon encoding particulate methane monooxygenase (pMMO) for methane oxidation (Stein et al., 2011). *Methylocystis* sp. Rockwell showed an exceptional level of tolerance to high ammonium concentration (Tays et al., 2018) and grow well with ammonium over nitrate as the N-source (Nyerges et al., 2010; Tays et al., 2018), while maintaining a sensitivity to nitrite (Nyerges et al., 2010). Although *Methylocystis* sp. Rockwell can use ammonium, nitrate or fix N_2 as a source of nitrogen, its preference is ammonium for optimal growth (Tays et al., 2018; Lazic et al., 2021) and the presence of hydroxylamine detoxification enzymes (hydroxylamine dehydrogenase; HAO) (Stein & Klotz, 2011) indicate its potential application for remediation of ammonia-containing wastewater. For instance, a potential application of *Methylocystis* sp. Rockwell would be to use ammonium rich wastewater as the N-source in combination with waste methane and methanol as the carbon source for production of PHB. While *Methylocystis* sp. Rockwell is an aerobic methanotroph requiring oxygen to carry out its metabolic processes, it has also been found to adapt in low-oxygen conditions and respond to diverse environmental conditions (Dam et al., 2013) especially in utilizing different nitrogen sources by changing its growth rate and activity (Nyerges et al., 2010; Nyerges & Stein, 2009; Sharma et al., 2022; Lazic et al., 2021). This ability to adapt to varying nutrients makes *Methylocystis* sp. Rockwell a valuable research tool for studying the mechanisms underlying methanotrophy and its environmental and industrial

significance (section 2.11). Its potential use in bioplastics and biofuel production and in methane mitigation emphasizes the importance of studying and harnessing the capabilities of this remarkable bacterium. For these reasons, *Methylocystis* sp. Rockwell has been the subject of several studies investigating its physiology, metabolism, value-added products and ecology in the Stein and Sauvageau laboratories. Additionally, our lab groups have examined the metabolic pathways of *Methylocystis* sp. Rockwell involved in carbon and nitrogen assimilation (Lazic et al., 2021), its ability to grow on methane or methanol (Tays et al., 2018) and its production of PHB under stress conditions (Lazic et al., 2021; Sharma et al., 2022). However, a suitable combination of inoculum, nutrients, and feeding strategy for balancing high biomass and PHB production by *Methylocystis* sp. Rockwell is the rationale behind the work in this thesis.

2.8. Microbial cultivation systems

Microbial cultivation is a technique for reproducing microorganisms with defined media under controlled environmental conditions. Based on the strains and target products selected, the physicochemical parameters of cultivation changes. Cultivation can be executed in shake flasks or bottles, cell culture plates or dishes, and bioreactors or fermentors. Shake flask or bottle cultivation is the most commonly employed submerged culture technique in laboratory research, developed about 85 years ago, to increase oxygen supply by surface aeration. However, shake flask cultures are sensitive to operating conditions as the shape, size and surface properties of the flask, shaking speed, and liquid volume and properties are quite variable (Takahashi & Aoyagi, 2018). Plate cultures in single- or multi-well formats can be used to grow and propagate cells at a small scale. Cultures grown on solid agar or in liquid medium can be used to analyze metabolic activities or perform biochemical testing. Cultivation in bioreactors or fermenters is a technological advance to facilitate microbial growth, biochemical reactions, and production of value-added end products and

metabolites. Although fermentation techniques have been known for thousands of years, the first scientific study was conducted by Louis Pasteur in the 1850s. Since then, many advancements have been adopted to offer suitable environments to optimize growth and metabolic activity.

2.9. Bioprocessing and mode of operation

2.9.1. Batch culture

Batch cultivation is a closed system where there is no subsequent feeding into the system until the end of the process (Fig. 2.6a). Thus, the working volume 'V' of a culture remains constant. Here, medium is prepared, sterilized and inoculated at the beginning of the process, which allows cells to grow until the limiting nutrient is completely consumed. Suitable environmental conditions are established to produce a desired product. The culture broth or biomass is harvested and processed once the product formation reaches its optimal level.

During batch cultivation, cells exhibit four growth phases: lag phase, exponential phase, stationary phase and death phase (usually avoided in cultivation). Lag phase is an initial period of growth phase where cells grow in size, start adapting to their environment, and prepare for cell division by synthesizing cofactors, amino acids, enzymes, etc., for active metabolism. While lag phase is a non-productive phase for industries, efforts have been made to minimize lag time by inoculating adequate concentrations of exponentially growing cells. Fresh inoculum sizes of 5 - 10% (volume per liquid reactor volume) are generally used in microbial fermentation. Cells replicate during exponential growth phase to achieve maximum biomass. As the cell population grows exponentially, there comes a point where the availability of nutrients and space for growth, as well as the accumulation of harmful by-products, can no longer support the same rate of cell division. This leads to a decrease in the growth rate and eventually a plateau in the number of cells, marking the onset of the stationary phase. Balanced growth is observed when the nutrient supply

is adequate and no or few toxic by-products are generated. However, as the nutrient level starts to drop when cells enter stationary phase, conditions become more favourable for production of secondary metabolites. The exponential growth equation (Eq. 1) is utilized to determine the population size as it grows over time, as well as to anticipate the number of cells at any given moment during the exponential growth phase, given that the doubling time (Eq. 2) stays unchanged and there are no limiting factors like the exhaustion of nutrients or the accumulation of harmful waste products (Maier & Pepper, 2015).

General equation during exponential phase is written as $\frac{dX}{dt} = \mu X$. ---------- (Eq. 1)

The doubling time can be calculated using $t_d = \frac{\ln 2}{\mu}$ ---------- (Eq. 2)

Where μ = specific growth rate, X = cell dry weight and t = time.

2.9.2. Fed-batch culture

Fed batch cultivation is an open system where nutrients are fed either continuously or discontinuously without removal during operation. A fed-batch process typically begins with an initial batch cultivation followed by subsequent supply of fresh nutrients usually through addition of a concentrated medium (Fig. 2.6b). Such addition of substrate promotes further growth and product formation over time. As the culture broth is retained into the system, addition of fresh medium increases the working volume "V" over time. Typically, in the course of operation, the growth rate of an organism is controlled by controlling the amount of substrate fed to the system. Depending on the substrate, the organism, and experimental design, the addition of substrate can be controlled by adopting various feeding strategies such as constant feeding, exponentially increasing feeding, step-wise feeding, pulse feeding, continuous feeding, and feedback controlled feeding, among others.

2.9.3. Continuous culture

Continuous culture is an open system, where fresh nutrient medium is continuously supplied into the fermenter and the culture broth is removed simultaneously, maintaining a constant working volume "V" throughout operation (Fig. 2.6c). As the consumed nutrients are replaced and toxic metabolites are removed from the fermenter, this exchange of medium is performed at the same rate to maintain a constant internal environment (i.e. steady state) and continue production of a desired end product. In general, continuous cultures can achieve a steady state after going through four or five residence times. Once the steady state is achieved, the Monod equation (Eq. 3) developed by Jacques Monod in the 1940s is applied to describe the bacterial growth in response to substrate consumption (Maier & Pepper, 2015). Here the Monod equation at steady state would be:

$$\mu = D = \mu max \frac{S}{(Ks+S)} \qquad \text{---------- (Eq. 3)}$$

Where, μ is specific growth rate, D is the dilution rate, μmax is maximum specific growth rate, *S* is substrate concentration and *Ks* is the half saturation constant.

The dilution rate plays a critical role in adjusting the specific growth rate (μ) and achieving the steady state condition. The dilution rate describes the relationship between medium inflow rate (F) and working volume (V) of the bioreactor (expressed as D = F/V). Cell growth can be calculated using the biomass equation dX/dt = μX – DX. During steady state, dX/dt = 0 resulting in μ = D; meaning that the bacterial growth rate equals the dilution rate. The cell density will increase when the dilution rate is lower than the growth rate, whereas density will decrease and eventually the cells wash out from the bioreactor if the dilution rate is higher than the growth rate. This implies that depending upon the needs of bioprocess, the cell density can be controlled and maximized to saturation by manipulating the dilution rate.

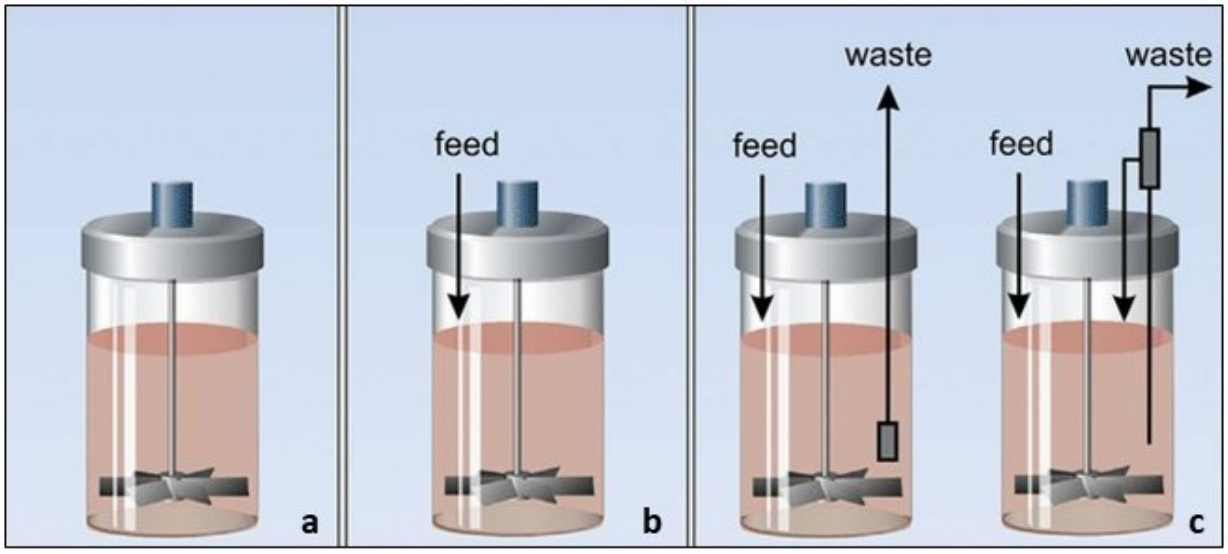

Fig. 2.6. Schematic representation of different types of bioprocessing. Batch culture (a), fed-batch culture (b), continuous culture (c). Adopted with permission from (Kropp et al., 2017). Copyright 2023, Elsevier.

Table 2.2. Comparative study on batch, fed-batch and continuous cultures (Abdel-Rahman et al., 2013; Chotteau, 2015)

Categories	Batch culture	Fed-batch culture	Continuous culture
Operating system	Closed system	Open system	Open system
Type of process	Not a continuous process	Semi-continuous process	Continuous process
Nutrient feeding	Nutrients are provided at the beginning of cultivation.	Nutrients are systematically added over the cultivation time.	Nutrients are continuously added to the system with simultaneous removal of culture broth (cells + media).
Fresh nutrients	No addition of fresh nutrients	Addition of fresh nutrients	Addition of fresh nutrients
Working volume	Constant	Increase	Constant
Growth phases	Lag phase, log phase, stationary phase and decline phase	Lag phase, log phase, stationary phase and decline phase	Lag phase at the beginning and then continue log phase
Cell density	Low cell density	Moderate to high cell density	Cell density remain same
Harvesting time	Product harvest at the end of process	Product harvest at the end of process	Continuously harvest the product for longer period
Cell growth and product yield	Less control over microbial growth and desired product.	Control over microbial growth and desired product.	Control over microbial growth and desired product.
Productivity	Low	Moderate	High
Internal environment	Change in internal environment	Change in internal environment with feeding	Constant internal environment
Fermentation setup	Fermentation setup is not altered during the process	Fermentation setup can be changed if needed.	Fermentation setup can be changed as per requirement.
Contamination and mutation	Lower chance of contamination and mutation	Higher chance of contamination and mutation.	Higher chance of contamination and mutation.
Advantages	• Simple and easy setup • Low cost of labor and equipment • Complete substrate utilization is possible • High product concentration • Useful in production of secondary metabolites such as antibiotics.	• Overcome substrate inhibition or catabolic repression problem • High product concentration • Useful in production of primary metabolites like, alcohol and proteins.	• Minimal labour cost • High productivity • Control growth rates • Fewer shut down process • Useful in production of enzymes, primary metabolites (amino acids, organic acids)
Disadvantages	• Product yield is low • Risk of substrate and/or end product inhibition	• Culture setup is complex, time-consuming, difficult to implement and maintain. • Chances of end product inhibition • Labor demand is high. • Involve high investment.	• Incomplete utilization of carbon • Culture setup is complex, time-consuming, difficult to implement and maintain. • Sometime cells clumping & foaming may block the inlet • Labour demand and investment are high. • Not suitable for production of secondary metabolites

2.10. Scale up of PHA production: challenges and perspectives

The global increase in production and use of petroleum-based plastic materials, their persistence in nature, and their environmental impacts have opened up an avenue towards innovation and adoption of biodegradable and renewable biopolymers which can offer similar functionalities of conventional plastics. PHA is one such category of biopolymers synthesized by a wide array of microorganisms via bioconversion of carbon-rich resources such as carbohydrates, glycerol, or fatty acids (Koller & Mukherjee, 2022). For example, alphaproteobacterial methanotrophs can convert C1-substrates (methane and methanol), Cyanobacteria utilize carbon dioxide, and Rhodospirilli utilize syngas for PHA production (Koller et al., 2017). However, the production of only a few of PHAs including poly(3-hydroxybutyrate), poly(4-hydroxybutyrate), poly(3-hydroxybutyrate-co-3-hydroxy-valerate), poly(3-hydroxybutyrate-co-4-hydroxybutyrate), and poly(3-hydroxybutyrate -co-3-hydroxyhexanoate), have been explored in detail (Koller & Mukherjee, 2022). There is no doubt that data generated from small laboratory scale studies are important in designing appropriate scale up strategies from bench to pilot or production scale. However, one must understand how changes in bioreactor size and types can lead to dissimilar process performance. Generally, in the course of scaling up bioprocesses, diligent attention is required to provide sufficient mixing, mass transfer, dissolved oxygen, and appropriate pH, temperature, substrate concentration etc. to improve or conserve process performance. Otherwise, heterogeneity in cultivation environments may result into various physiological responses in the cells, which affect their growth and overall productivity. Moreover, maintaining sterile conditions and the energy requirements for heating, agitation and aeration during upstream processing to centrifugation, filtration, heating, drying and pumping during downstream processing, all demand higher investment to establish efficient large-scale production (Patel et al.,

2006). Furthermore, many conventional PHA production facilities rely on two-stage production processes, in which bacterial strains are grown (using various feedstocks) in a first stage followed by nutrient limiting conditions for PHA production in a second stage. However, such two-stage bioprocesses may not be suitable for some applications, as it can be time-consuming, inefficient, and are tied to high feedstock costs, high investment and large energy requirements for scale-up (Johnson et al., 2009).

Over 25 start-up companies and 30+ brand owner chemical companies have started to produce and utilize PHA materials, representing 230 MT/year bioplastics production using various carbon-rich renewable resources for industrial and consumer applications (Koller & Mukherjee, 2022). Such pilot or larger-scale PHA producing companies include Newlight Inc, Biomer, Mango Materials, Imperial Chemical Industry Biological (ICI), Genecis Bioindustries Inc., TerraVerdae Bioworks among others. Biomer is a Schwalbach-based industrial producer of P(3HB) using *Azohydromonas australica* from sucrose as the carbon feedstock. Mango Materials is a San Francisco-based start-up company that uses crude biogas as a feedstock for PHB production from robust methanotrophic communities. ICI is a London-based company that produces P(3HB) using *Cupriavidus necator* from glucose as a feedstock. Genecis is an Ontario-based start-up company for PHA production. It uses discarded organic food waste as a raw material to grow acidogenic bacteria for production of fatty acids, which are in turn converted into P(3HB-co-3HV) by PHA-producing microbes. TerraVerdae Bioworks is a Canadian cleantech company, with product development facilities in Edmonton, AB and Charlottetown, PE, that uses municipal and forestry waste or agricultural residues as feedstocks for PHA production. For more details on PHA-producing companies and their stories, refer to this review article written by Koller & Mukherjee, (2022).

While the costs of PHA production have limited scale-up and production, the use of methane or biogas (a mixture of methane and carbon dioxide) generated from landfills or wastewater treatment plants as a source of feedstock is a promising strategy to minimize investment. Use of these common municipal waste facilities offers substantial opportunities to reduce greenhouse gas emissions, reduce depletion of fossil resources, and minimize plastic pollution and related environmental impact. Although several small-scale studies in serum bottles have been conducted to analyse the effect of various parameters on growth and PHA accumulation in methanotrophs (Zaldívar Carrillo et al., 2018; Rostkowski et al., 2013; T. Zhang et al., 2017; Sundstrom & Criddle, 2015; Rahnama et al., 2012), studies on larger-scale bioreactors, including sequencing membrane bioreactors, bubble-column bioreactors, continuous-flow reactors, vertical loop bioreactors and a continuous multistage dispersed growth configuration, have gained considerable attention due to their industrial context (see above, section 2.6.5). However, one must overcome several obstacles to achieve high cell density and productivity in scaled up production. Gas-liquid mass transfer, low solubility of gases, bioreactor design and feeding strategies are some of the key factors influencing large-scale cultivation of methanotrophs. The mass transfer limitation of methane and oxygen can be improved by designing efficient bubble column or U-loop bioreactors (Kantarci et al., 2005; Petersen et al., 2017), supplying gases under pressure, or by using transfer vectors such as silicon oil (Quijano et al., 2009). Methanotrophic bacteria can also be immobilized in hydrogel to increase mass transfer of methane in a bioreactor for higher PHB productivity (Ruelas, 2018).

While emphasis is generally given to bioreactor design to achieve better process performance, adoption of high cell density cultivation techniques, such as continue gas flow bioreactors and perfusion bioreactors using ultrafiltration membranes, could be efficient

approaches suitable for scale up PHA production by methanotrophs. In this thesis, I focused on designing a methanol fed-batch operation using a continuous gas flow bioreactor to improve growth and PHB yield from *Methylocystis* sp. Rockwell (see Chapter 5). This study recommends optimal culture conditions in addition to an efficient process design for improved production of PHB from *Methylocystis* sp. Rockwell that is applicable towards commercializing its biopolymer production. Once the optimum parameters have been identified in terms of overall process productivity, culture parameters including inoculum health and size, growth kinetics, aspect ratio of the bioreactor, media volume to headspace ratio, diameter of impeller to bioreactor ratio, Reynolds number, productivity etc. can be analysed to design the appropriate scale-up strategy for industry (Lim & Shin, 2013; Mahdinia et al., 2019).

2.11. Application of methanotroph biotechnology

2.11.1. Carbon sequestration

Because of their versatile biotransformation abilities, methanotrophs are viewed as promising candidates for sequestering harmful greenhouse gases like methane and carbon dioxide. Methanotrophs are known for their ability to control the global carbon cycle by lowering the methane concentration prior to its emission as atmospheric methane. Out of ca. 558 Mt of methane emission annually, nearly 515 Mt is removed from atmospheric reaction with hydroxyl radicals (Saunois et al., 2016). However, both aerobic and anaerobic methanotrophs play a major role in ameliorating atmospheric methane by consuming nearly 80-90% of methane at the site of production prior to its release (Cai et al., 2016; Frenzel et al., 1992). While few evidence on endophytic methanotrophs is known so far (Stępniewska et al., 2017; Raghoebarsing et al., 2005; Parmentier et. al., 2011), they get benefit from submersed vegetation in wetlands as cooperation

between endophytic methanotrophs and plants have a mutual relationship wherein carbon dioxide produced by methanotrophs feeds the vegetation, and in return the vegetation provides oxygen to the methanotrophs. Moreover, methanotrophs have shown their unique ability to provide methane-derived carbon to promote both plant and microbial growth in ecosystems (Kip et al., 2010). Such cooperation and carbon recycling between plants and partly endophytic methanotrophic bacteria act as an *in-situ* methane biofilter by lowering methane emissions and sequestering methane and carbon dioxide (Kip et al., 2010; Stępniewska & Kuåniar, 2013).

2.11.2. Bioremediation and environmental protection

Use of microorganisms and/or plants to remove contaminants, pollutants, and toxins for environmental restoration is called bioremediation. Various microorganisms including methanotrophic bacteria have a beneficial role for in-situ bioconversion and bioremediation due to their ubiquitous nature and diverse metabolic activities. Consumption of methane and production of biopolymers using methanotrophs biotechnology have shown an appealing solution to greenhouse gas emissions and plastic pollution. As methane is a low-cost carbon source, it is considered as an economical and environmentally friendly bioremediation technology. However, the effectiveness of methanotrophy in bioremediation is complicated by the toxicity of pollutants, the requirement for reducing equivalents, and the availability of methane and oxygen together. As adequate methane is necessary for growth and survival of methanotrophs, pollutant degradation by MMOs is a co-metabolic process that occurs over an extended period only in presence of methane (Semrau, 2011). Besides reducing methane emission, methanotrophs can also remove nitrogen from wastewater and degrade the hazardous organic materials. In fact, due to the broad substrate range of MMOs, methanotrophs have shown their abilities to degrade heavy metals, aliphatic compounds, alkanes, alkenes and aromatic compounds present in soils, sediment, and

groundwater (P. J. Strong et al., 2015). Moreover, methanotrophs can perform methane-dependent denitrification in nitrogen-contaminated wastewater in conjunction with other microorganisms (P. J. Strong et al., 2015). Depending on oxygen availability, such mixed culture denitrification can occur under both aerobic and anaerobic conditions (Deutzmann et al., 2014; Islas-Lima et al., 2004; Sun et al., 2013). It can be divided into two categories based on oxygen availability: aerobic methane oxidation coupled to denitrification (AME-D) or anaerobic methane oxidation coupled to denitrification (ANME-D). For example: *Methylomonas denitrificans* FJG1can directly reduced nitrate to produce substantial amounts of nitrous oxide under hypoxic conditions where methane donate electron to nitrate. Another anaerobic type of methane oxidation is completed by bacteria *Methylomirabilis oxyfera* of NC10 phyla. It reduces the nitrite to nitric oxide, which is then converted to dinitrogen and dioxygen using nitric oxide dismutase. The internally produced oxygen is then utilized by MMO for methane oxidation. Biogas production during industrial processes or anaerobic treatment of organic wastes can be minimized by methanotrophic bioconversions offering a new perspective in methane mitigation while recovering value added products (Cantera et al., 2019; Hwang et al., 2018).

2.11.3. Production of value-added products and industrial platform chemicals

The cellular metabolism of methanotrophs involves a plethora of enzymes that contribute to efficient methane conversion. The catalytic bioconversion of methane produces various soluble metabolites and intermediate compounds such as methanol, formaldehyde, organic acids, amino acids, lipids, biopolymers, etc. (P. J. Strong et al., 2015; Fei & Pienkos, 2018). These metabolites have promising applications in nutrition, healthcare, bioremediation, and agriculture (Singh et al., 2017). Besides these metabolites, production of cellular biomass itself from methanotrophs has

garnered industrial interest because of its lower environmental footprint to serve as an alternative source of protein for animals (Fei & Pienkos, 2018; Matassa et al., 2016). Methanotrophic strains like *Methylococcus capsulatus, Methylomonas* sp., and *Methylocystis* sp. can be considered as a potential protein substitute (Valverde-Pérez et al., 2020). As the protein content of *Methylococcus capsulatus* Bath comprises 45% of its dry biomass (Rasouli et al., 2018), it can be a reliable source of protein for animals such as pigs, chickens, salmon, and trout (Øverland et al., 2010). Additionally, ectoine is a natural amino acid derivative and is secreted by several species of halophilic bacteria to fight against extreme osmotic stress. It protects biomolecules from environmental stresses such as salinity, freezing, drying and high temperatures (Schröter et al., 2017). Currently, ectoine has been used in cosmetics as a moisturising cream (Graf et al., 2008), nasal sprays and eye drops for the treatment of allergic rhinitis (Werkhäuser et al., 2014). Studies have shown that the moderately halophilic methanotrophs *Methylobacter alcaliphilus* 20Z and *Methylobacter modestohalophilus* 10S consume methane to accumulate ectoine to up to 20% of their dry biomass (Khmelenina et al., 1999). Bacterial lipids can be used for production of medical supplements and biofuels (Fei et al., 2014). Methanotrophs such as *Methylomicrobium buryatense* and *Methylococcus capsulatus* can accumulate lipid molecules up to 10% of their total biomass and thus have potential for biofuel production (T. Dong et al., 2017). Moreover, the PHA biopolymers produced from alphaproteobacterial methanotrophs have huge potential to replace conventional petroleum-derived polymers (P. Strong et al., 2016).

2.12. References

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CHAPTER 3. Optimization of methane feed and N:C ratio for biomass and polyhydroxybutyrate production by the alphaproteobacterial methanotroph *Methylocystis* sp. Rockwell

3.1. Abstract

The consumption of methane and production of biodegradable polymers by alphaproteobacterial methanotrophs offers a promising strategy to mitigate greenhouse gas emissions and reduce non-biodegradable plastic pollution. This study identified an ideal amount of added methane and N:C ratio in 100 mL batch cultures of the alphaproteobacterial methanotroph *Methylocystis* sp. Rockwell growing in 1 L sealed bottles using Response Surface Methodology (RSM) to achieve both high biomass and high polyhydroxybutyrate (PHB) production. RSM analysis showed achievement of optimal biomass at 474.7 ± 10.1 mg/L in nitrate mineral salts (NMS) medium and 480.0 ± 65.5 mg/L biomass in ammonium mineral salts (AMS) medium with 8 mmol of methane and an N:C ratio of 0.022. However, optimal PHB concentration was achieved with 6 mmol methane at N:C ratios of 0.012 in NMS medium (149.7 ± 16.1 mg/L) and 0.022 in AMS medium (200.3 ± 5.1 mg/L). A multi-objective RSM analysis projected maxima in PHB production and %PHB cell content (based on dry weight) when using 4.88 mmol methane and N:C ratio of 0.016 in NMS cultures, and 6.28 mmol methane and the 0.016 N:C ratio in AMS cultures. Cultures grown under these projected conditions produced 173.7 mg PHB/L with 46.8% PHB cell content in NMS, and 196.9 mg/L with 53.1% PHB cell content in AMS. Taken together, these analyses predicted the optimal conditions for growth and PHB production in batch cultures of *Methylocystis* sp. Rockwell and confirmed a preference for ammonium as the N-source for PHB production. This information is valuable for media formulation in industrial scale-up of *Methylocystis* sp. Rockwell in PHB production.

3.2. Introduction

The continued rise in atmospheric methane concentration and human reliance on petroleum-based plastics are of grave concern as both have negative environmental impacts and lead to escalating health issues. Thus, there is a need for development and adoption of mitigating technologies. Bioconversion of methane into biodegradable plastics using methanotrophic bacteria is an appealing premise and a rapidly growing industry (Cantera et al., 2018). Alphaproteobacterial methanotrophs, including species of *Methylocystis* and *Methylosinus*, produce the biodegradable polymer polyhydroxybutyrate (PHB) during nutrient deprivation, most commonly triggered by nitrogen starvation (Pfluger et al., 2011; Pieja, Rostkowski, et al., 2011; Rostkowski et al., 2013). These bacteria oxidize methane, assimilate formaldehyde via the serine pathway and, under nitrogen limitation, gate acetyl-CoA towards PHB biosynthesis to store excess carbon into intracellular granules.

Alphaproteobacterial methanotrophs can use nitrate, ammonium or nitrogen fixation to support growth and PHB production, although each isolate has varying preference for its N-source (Stein, 2018). Moreover, the efficiency of growth and PHB production by methanotrophic bacteria varies with different combinations of carbon (methane/methanol) and nitrogen (ammonium/nitrate/N_2 fixation) sources (Lazic et al., 2021; Rostkowski et al., 2013; Tays et al., 2018; T. Zhang et al., 2017). Because nitrogen limitation is a common trigger for PHB production, optimization for industrial scale-up of this process can be achieved by determining an appropriate N:C ratio that is sufficient to support growth but limiting enough to support PHB accumulation, and thus avoid a two-stage production process (Zaldívar Carrillo et al., 2018). Generally, nitrate is preferred over ammonium as the N-source for growing methanotrophs due to competitive inhibition of methane monooxygenase enzymes and the production of toxic intermediates -

hydroxylamine, nitric oxide, and nitrite; when methanotrophs oxidize ammonia as a co-substrate (Nyerges et al., 2010; Nyerges & Stein, 2009; Stein, 2018). *Methylocystis* sp. Rockwell has exceptional tolerance to high ammonium concentrations, is sensitive to nitrite, and shows better growth rates and biomass production with ammonium rather than nitrate as the N-source (Nyerges et al., 2010; Tays et al., 2018). Moreover, *Methylocystis* sp. Rockwell can produce PHB during log-phase growth and produces a higher %PHB content (based on cell dry weight) when grown with ammonium than with nitrate (Lazic et al., 2022).

A study using Response Surface Methodology (RSM) to pinpoint the optimal N:C ratio for growth and PHB production in *M. trichosporium* OB3b showed that the highest biomass was achieved at N:C of 0.025 - 0.028, whereas highest PHB production was achieved at N:C of 0.017 (Zaldívar Carrillo et al., 2018). This difference in N:C is logical as higher N-availability supports cell growth rather than PHB biosynthesis. Despite a considerable progress has been made in methanotrophs biotechnology, due to the variation on nutritional requirement for the growth and metabolites productions among the methanotrophs, it would be imprudent to give a generalization viewpoint of optimal cultivation parameters of one specific study to a much broader species range, which necessitates a species-specific experimental approach among methanotrophs (Tays et al., 2018; Stein, 2018). Since no earlier work has been conducted in balancing higher biomass and PHB production from *Methylocystis* sp. Rockwell, this optimization study is novel to the methanotrophs biotechnology which could have potential industrial application. In the present study, we used a multi-objective optimization approach (Deb & Deb, 2014) to find the optimal methane amount per culture volume and N:C ratio that simultaneously support growth and PHB biosynthesis in *Methylocystis* sp. Rockwell. This goal is achievable as *Methylocystis* sp. Rockwell produces PHB during exponential growth, even in the absence of nitrogen limitation (Lazic et al.,

2022). Using methane as a carbon source and ammonium or nitrate as the nitrogen source, N:C was optimized using one-variable-at-a-time (OVAT) followed by RSM with full factorial design. Our results confirmed that ammonium is the preferred N-source, and the optimal methane amount and N:C ratio supporting both robust growth and PHB production in batch cultures of *Methylocystis* sp. Rockwell were identified.

3.3. Materials and methods

3.3.1. Media preparation and bacterial cultivation

Methylocystis sp. Rockwell is a methanotrophic isolate from an aquifer in Canoga Park California, USA (Stein et al., 2011). Cultures (100 mL) were grown and maintained in nitrate mineral salts (NMS) or ammonium mineral salts (AMS) medium (Whittenbury et al., 1970) in 1 L gas-tight bottles. 10X stock media containing 99 mM of KNO_3 or NH_4Cl and 10X nutrient media were diluted with deionized water to achieve the desired concentrations of N-source and other essential nutrients for each set of experiments. 1 mL sterilized phosphate buffer (26 g of KH_2PO_4, 33 g of Na_2HPO_4 and milli-Q water up to 1000 mL; pH 6.8) was added to 100 mL sterilized and cooled culture medium followed by addition of 2 mL fresh inoculum (2% v/v) into 1 L Kimble bottles. The bottles were kept gas tight with screw top lids lined with butyl rubber septa. Methane was injected through a 0.22-µm Millex-GS syringe filter unit (Millipore, Sigma) and atmospheric pressure was maintained by previously removing the same amount of air as the volume of methane injected.

Cultures were incubated at 30 °C with shaking at 150 rpm (G10 Gyrotory shaker, New Brunswick Scientific) until reaching stationary phase (Supplementary Fig. A-1a). Bacterial growth was analysed by harvesting 500 µL of culture into each well of a 48-well plate and measuring

optical density at 540 nm (OD; Multiskan Spectrum, Thermo Fisher Scientific). Cell dry weights were determined by centrifuging 25 mL of culture at 10,000 × g (Sorvall Evolution RC, SS-34 rotor, Thermo Fisher Scientific) at 4 °C for 20 min. The cell pellets were resuspended in 10 mL ultrapure milli-Q water, transferred to a tared weigh dish, and dried at 60 °C to a constant mass (Zaldívar Carrillo et al., 2018).

3.3.2. Composition of headspace gases

Methane, carbon dioxide and oxygen were measured in the gas headspace of each culture by extracting 100 µL gas with a 250-µL gas-tight syringe (SGE Analytical Science) and injecting it into a gas-chromatograph with thermal conductivity detector (GC-TCD) (GC 8A, Shimadzu). The GC-TCD was fitted with packed columns: a molecular sieve 5A column (80/100 6ft, 2mm ID SS, Restek) to detect oxygen and a Hayesep Q 8A column (80/100 6ft, 2mm ID SS, Restek) to detect methane and carbon dioxide. The sample injection ports and detector temperatures were kept at 120 °C for both columns; the molecular sieve column temperature was 90 °C with TCD current of 90 mA and the Hayesep Q column temperature was 20 °C with TCD current of 120 mA. Ultrahigh purity helium (Praxair) was the carrier gas at 160 kPa. Analyte sandard curves were generated using pure methane, oxygen and carbon dioxide gases (Praxair).

3.3.3. Experimental design and statistical analysis

Optimization of methane amount and N:C ratio was accomplished using a one-variable-at-a-time (OVAT) approach and a response surface methodology (RSM) based on a full factorial design (Akar Sen, 2016; Myers et al., 2016). The conventional OVAT approach was used to determine the range of N:C ratios for RSM experiments, whereas the RSM approach was used to

determine a model-based multi-objective optimal conditions (MOOC). This procedure was conducted independently for NMS and AMS media. For wider coverage of effects from variable interactions, the RSM experiments were conducted using 4^k full factorial experimental design (4^2 = 16 runs) consisting of two independent variables: methane amount and N:C ratio. The responses of interest included biomass yield, PHB concentration, and %PHB cell content on a dry weight basis. The data were visualized in 3D surface plots using square root transformation in quadratic models (Freeman & Tukey, 1950; Iwueze & Johnson, 2011; Myers et al., 2016). Each surface response was analysed by ANOVA where $p < 0.05$ showed a significant contribution to the fit of the model. Further, post hoc Tukey's test was used to determine significant differences between treatment conditions. The experimental data were used to develop mathematical models on ANOVA using Design Expert software (Stat-Ease Inc., USA) (Akar Sen, 2016). The model was then used to predict expected optimal conditions.

3.3.4. PHB quantification

PHB production was quantified via depolymerization/derivatization and gas chromatography, as described previously (Zaldívar Carrillo et al., 2018) but with slight modifications. Briefly, a 10-mL culture sample harvested after 6 days of incubation was placed into a 12-mL screw-capped glass tube. The tube was centrifuged at 4,000 × g (Sorvall Evolution RC, SA-600 rotor, Thermo Fisher Scientific) at room temperature for 30 min. Cell pellets were resuspended in 2 mL chloroform. Further digestion of the sample was achieved by adding 1 mL methanol and 1 mL acidified methanol solution (1 mL benzoic acid standard solution mixed in 24 mL methanol and 1.5 mL concentrated sulfuric acid). The reaction mixture was mixed and submerged in a boiling water bath for 5 h. The digestion process depolymerizes PHB to 3-

hydroxybutyric acid, which is derivatized by methylation into the volatile methyl 3-hydroxybutyrate. The methyl benzoate formed from methylation of benzoic acid is used as an internal standard. After 5 h of reaction, the sample was allowed to cool, and 1 mL of deionized water was added, after which the sample was vortexed for 20 sec. The sample phases were allowed to separate and the organic phase (bottom layer) was collected into a glass vial capped with a butyl rubber septum cap. 3 µl of sample was injected into a GC-FID (Hewlett Packard, HP-5890A, Agilent Technologies) fitted with a DB-5ms column (30 m × 250 µm × 0.25 µm; Agilent Technologies). The split ratio was maintained at 1:10 and helium was used as the carrier gas (flow rate of 1.5 mL/min). The injector and detector temperatures were held at 250 °C and 300 °C, respectively. The temperature was programmed at an initial oven temperature of 80 °C, held for 1 min, raised to 120 °C at a rate of 10 °C/min, then to 270 °C at 30 °C/min, and finally held for 3 min. The area ratio of the methyl 3-hydroxybutyrate to methyl benzoate peaks was converted to PHB concentration using a standard curve of crystalline PHB (Sigma-Aldrich) depolymerized and derivatized using the same method.

3.4. Results

3.4.1. One-variable-at-a-time (OVAT) analysis of biomass and PHB production

Our study indicated that *Methylocystis* sp. Rockwell had a faster doubling time in batch cultures when grown in ammonium mineral salts (AMS; 8.5 h) versus nitrate mineral salts (NMS; 9.8 h) media (Supplementary Fig. A-1). Methane was supplied to 100 mL batch cultures at amounts ranging from 2 to 10 mmol. While methane provided at 6 mmol resulted in the highest biomass in both NMS and AMS media, PHB production was optimal when methane was supplied at 8 mmol for NMS and 4 mmol for AMS media (Table 3.1). We selected 6 mmol methane to pinpoint the

optimal N-concentration for PHB production as this amount led to the highest biomass for both nitrogen sources. The Pearson correlation coefficient values between the final optical density (OD) and dry weight biomass were found to be 0.985 for Table 3.1 and 0.995 for Table 3.2, showing a strong positive correlation between OD and biomass. Optimal biomass was then achieved with 8 mM nitrate or ammonium, whereas optimal PHB production occurred with 0.5 mM nitrate or 1 mM ammonium (Table 3.2). Moreover, maximum PHB production was significantly lower (p-value = 0.024) for cultures grown with nitrate compared to with ammonium.

Table 3.1. Effect of methane amount (mmol) on growth (optical density (OD) = 540 nm)), biomass (dry weight (DW) in mg/L)) and PHB production (mg/L culture and PHB% of cell dry weight (DW)) in batch cultures of *Methylocystis* sp. Rockwell grown with either nitrate or ammonium (10 mM) as the N-source (*n=3*). Values in bold are the highest levels achieved for each set of measurements.

KNO_3 (mM)	**NH_4Cl (mM)**	**CH_4 (mmol)**	**Final OD_{540}**	**Dry wt. (mg/L)**	**PHB (mg/L)**	**PHB cell content (%DW)**
10	-	0	0.009 ± 0.001	5.33 ± 2.31	0.00	0.00
10	-	2	0.266 ± 0.028	157.33 ± 8.33	0.00	0.00
10	-	4	0.417 ± 0.051	270.67 ± 22.03	9.16 ± 3.33	3.47 ± 1.54
10	-	6	**0.596 ± 0.012**	**378.67 ± 43.88**	41.70 ± 16.51	11.20 ± 4.89
10	-	8	0.558 ± 0.098	340 ± 24	**56.75 ± 6.81**	**16.81 ± 2.98**
10	-	10	0.550 ± 0.045	334.67 ± 32.33	35.28 ± 10.21	10.45 ± 2.49
-	10	0	0.014 ± 0.001	5.33 ± 2.31	0.00	0.00
-	10	2	0.393 ± 0.015	232 ± 10.58	19.83 ± 1.08	8.55 ± 0.16
-	10	4	0.596 ± 0.003	320 ± 20.78	**50.69 ± 13.72**	**15.90 ± 4.33**
-	10	6	**0.679 ± 0.004**	**461.33 ± 26.63**	12.85 ± 1.31	2.86 ± 0.41
-	10	8	0.626 ± 0.004	397.33 ± 8.33	12.09 ± 2.34	3.04 ± 0.54
-	10	10	0.601 ± 0.013	325.33 ± 33.31	11.95 ± 0.92	3.72 ± 0.70

Table 3.2. Effect of nitrogen concentration (mM) on growth (optical density (OD) = 540 nm)), biomass (dry weight (DW) in mg/L)) and PHB production (mg/L culture and PHB% of cell dry weight (DW)) in batch cultures of *Methylocystis* sp. Rockwell initially containing 6 mmol methane (*n=3*). Values in bold are the highest level for each set of measurements.

CH_4 (mmol)	NH_4Cl (mM)	KNO_3 (mM)	Final OD_{540}	Dry wt. (mg/L)	PHB (mg/L)	PHB cell content (%DW)
6	-	0	0.153 ± 0.016	81.33 ± 14.05	24.19 ± 6.60	30 ± 7.94
6	-	0.5	0.470 ± 0.013	274 ± 8.49	**96.69 ± 2.67**	**35.32 ± 2.07**
6	-	1	0.612 ± 0.025	408 ± 6.93	65.06 ± 29.82	16.03 ± 7.60
6	-	2	0.635 ± 0.015	416 ± 33.94	18.33 ± 2.82	4.39 ± 0.32
6	-	4	0.685 ± 0.045	466 ± 59.40	7.34 ± 3.24	1.63 ± 0.90
6	-	8	**0.731 ± 0.012**	**469.33 ± 8.33**	10.83 ± 1.17	2.31 ± 0.29
6	0	-	0.153 ± 0.016	81.33 ± 14.05	24.19 ± 6.60	30 ± 7.94
6	0.5	-	0.424 ± 0.015	276 ±10.58	113.31 ± 9.78	41.15 ± 4.63
6	1	-	0.621 ± 0.050	404 ± 31.24	**196.12 ± 29.75**	**48.54 ± 6.55**
6	2	-	0.668 ± 0.015	457.33 ± 25.40	133.62 ± 26.24	29.20 ± 5.24
6	4	-	0.720 ± 0.031	462.67 ± 25.72	97.15 ± 12.67	21 ± 2.37
6	8	-	**0.742 ± 0.003**	**465.33 ± 9.24**	93.33 ± 5.83	20.05 ± 1.20

3.4.2. Analysis of response surface methodology (RSM) for biomass and PHB production

To explore the combined influence of methane amount and N-source, we created a full factorial design of experiments for RSM analysis. Growth, biomass, and PHB amounts for cultures initiated with methane ranging from 2 to 8 mmol and N:C ranging from 0.002 to 0.032, with either nitrate or ammonium as N-source, were compared using this method. In both NMS and AMS media, methane at 8 mmol and an N:C ratio between 0.022 to 0.032 led to highest biomass levels (Fig. 3.1). However, different methane amounts and a lower range of N:C ratio (0.012 to 0.022) supported the highest PHB production levels in both NMS and AMS media (Fig. 3.2). Although

the optimal %PHB cell content was similar, the total amount of PHB was significantly lower ($p < 0.05$) for cultures grown in NMS than in AMS media (Fig. 3.2).

Analysis of the measurements, including final cell dry weight of biomass, PHB concentration, and %PHB cell content, was performed for each medium (AMS or NMS) with one-way analysis of variance (ANOVA). The ANOVA of the RSM plots showed that fit of the quadratic model with square-root transformation was statistically significant ($p < 0.05$). The final model equations for PHB concentration and %PHB content for AMS medium are found in Supplementary Fig. A-2 and Tables A-1 to A-4, and for NMS medium in Supplementary Fig. A-3 and Tables A-5 to A-8. Moreover, one-way ANOVA was performed to determine the contributing factors to net PHB concentration using Minitab-16. When *Methylocystis* sp. Rockwell was grown in NMS, the average PHB concentration ranged from 25.7 ± 7.9 to 149.7 ± 16.1 mg/L and one-way ANOVA showed significant variation among the samples ($p < 0.05$). Further, post hoc Tukey's analyses revealed that PHB concentration of cultures grown in NMS with 6 mmol of methane and N:C ratio of 0.012 was significantly greater ($p < 0.001$) than all other conditions tested with NMS medium (Supplementary Fig. A-4a). Similarly, bacteria grown in AMS medium showed an average PHB concentration ranging from 20.9 ± 2.3 to 200.3 ± 5.1 mg/L and one-way ANOVA showed a significant difference among the samples ($p < 0.05$). Post hoc Tukey's analyses revealed that PHB concentration for cultures grown in AMS at an N:C ratio of 0.022 and 6 mmol of methane was significantly higher ($p < 0.001$) than all other conditions tested in AMS medium (Supplementary Fig. A-4b). Together, these results indicate that the optimal methane amount and N:C ratio depended on whether *Methylocystis* sp. Rockwell was grown in NMS or AMS; but overall growth in AMS resulted in both greater biomass and PHB content.

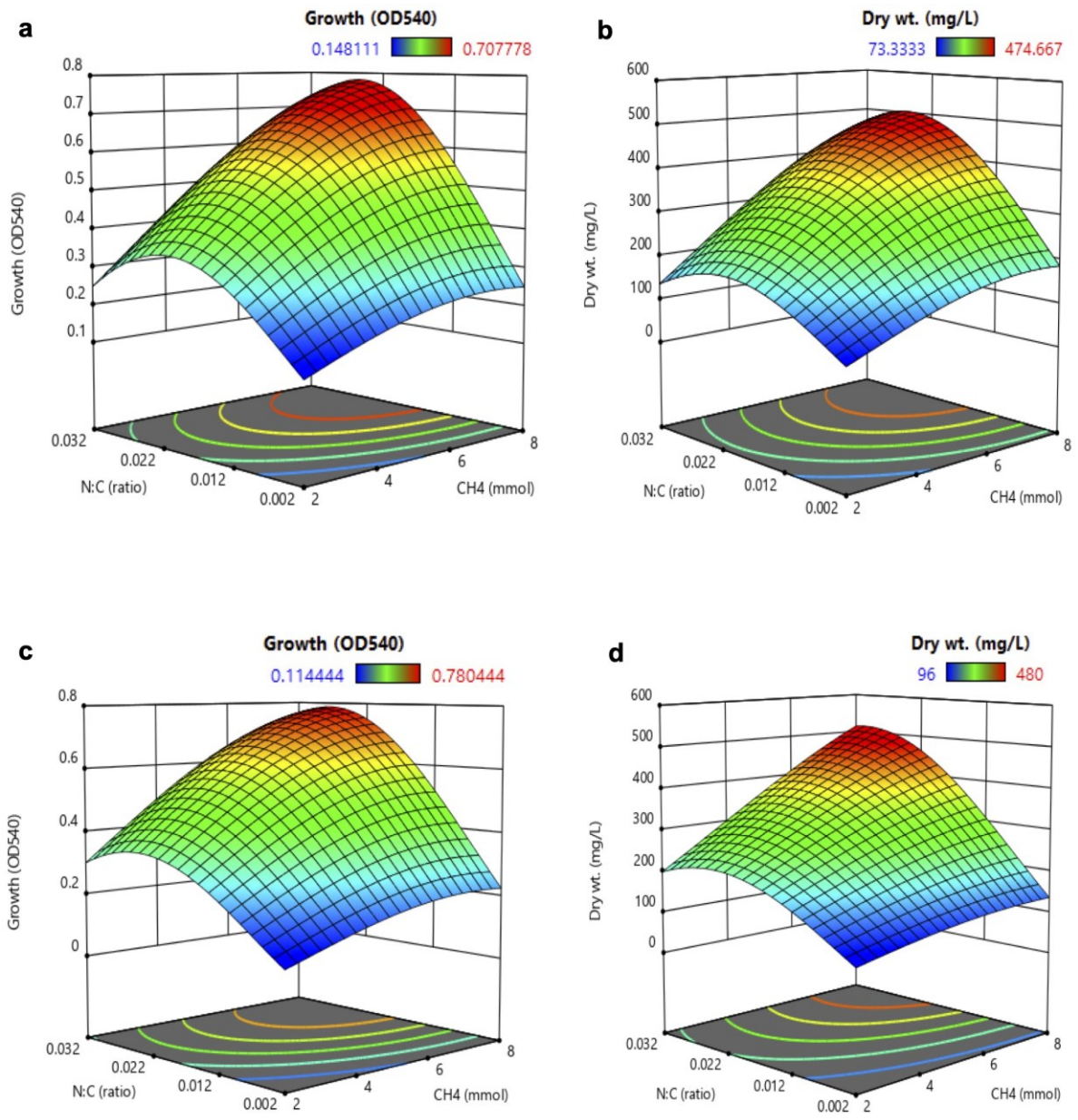

Fig. 3.1. RSM results for growth (OD = 540 nm) and biomass (Dry wt. in mg/L) in stationary phase batch cultures of *Methylocystis* sp. Rockwell grown with varying amounts of methane against varying N:C ratios. Results are shown for cultures grown in NMS (a, b) and AMS (c, d) media (n = 3).

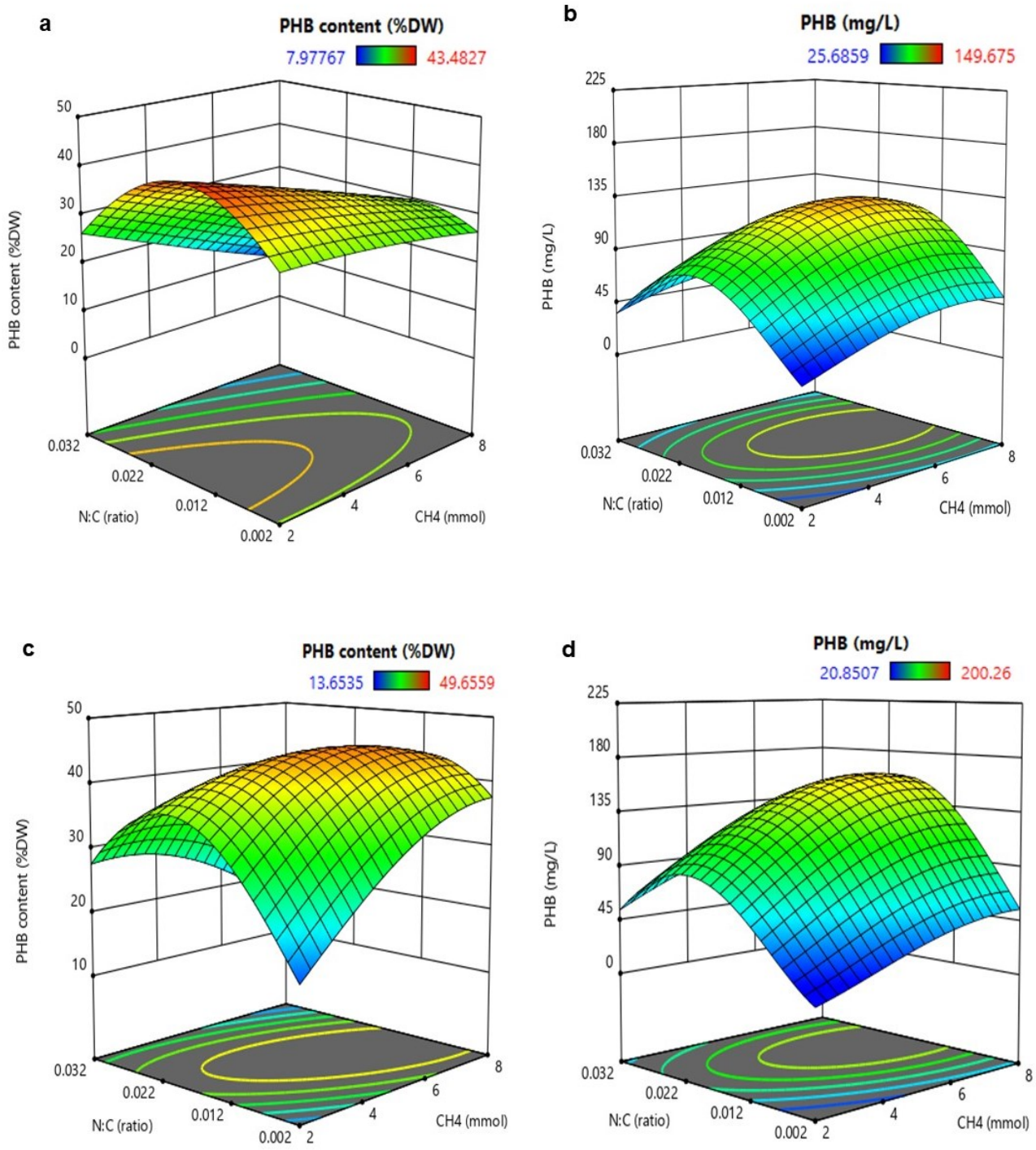

Fig. 3.2. RSM results for PHB production (PHB% cell content as % dry weight and PHB amount in mg/L) in stationary phase batch cultures of *Methylocystis* sp. Rockwell grown with varying amounts of methane against varying N:C ratios. Results are shown for cells grown in NMS (a, b) and AMS (c, d) media (n = 3).

3.4.3. Validation of multi-objective optimal conditions (MOOC)

Both the OVAT and RSM experiments revealed that, as expected, nitrogen limitation favored PHB production over biomass yield, albeit with differences in PHB yield between nitrate and ammonium as N-source. While PHB concentration was higher at low N:C ratio (0.012 to 0.022) (Supplementary Fig. A-4) and bacterial biomass was higher at high N:C ratio (0.022 to 0.032) (Fig. 3.1), achieving substantial biomass with a high %PHB cell content is crucial for industrial production. Thus, two multi-objectives optimal conditions (MOOC) analyses were performed with the RSM data to equally balance two parameters - PHB yield and %PHB cell content; or three parameters - biomass yield, PHB yield and %PHB cell content (Supplementary Fig. A-5, Table 3.3). The analysis aimed at achieving optimal PHB yield and %PHB cell content predicted methane at 4.88 and 6.28 mmol for NMS and AMS media, respectively, with an N:C ratio of 0.016. Validation experiments for these conditions showed strong agreement between the predicted and experimental biomass, but significantly higher ($p < 0.05$) experimental PHB concentration than predicted (Table 3.3). When aiming for high biomass in addition to PHB yield and %PHB cell content, predicted optimal conditions were 6.07 and 6.88 mmol methane for NMS and AMS, respectively, and an N:C ratio of 0.017. However, experimental validation for these conditions showed slightly higher biomass than predicted for AMS, but lower PHB yield for NMS (Table 3.3). Thus, optimizing for two outcomes (PHB yield, %PHB cell content) led to the best parameterization. The highest biomass (372 ± 38.15 mg/L) with the highest %PHB cell content (53.1% on a cell DW basis) produced by *Methylocystis* sp. Rockwell was similar to values previously found in *Methylosinus trichosporium* OB3b (52.5% and 55.5% PHB cell content) (Zaldívar Carrillo et al., 2018; T. Zhang et al., 2019).

Table 3.3. Prediction and experimental validation of multi-objective optimal conditions. Based on the complete set of RSM experimental responses, the multi-objective optimal condition was predicted for NMS and AMS by Design Expert® version-11 software. The optimization #1 was intended to yield maximum %PHB cell content (on the basis of cell dry weight) and PHB concentration, whereas Optimization set #2 was intended to yield maximum biomass, %PHB cell content, and PHB concentration. The projected amount of methane (mmol) and N:C ratios were then tested experimentally for comparison. Standard deviations for n=3 replicates are reported.

Optimization	N-source	N:C ratio	Methane (mmol)	Biomass yield (mg/L)		PHB yield (mg/L)		PHB content (% cell DW)	
				Projection	Experimental	Projection	Experimental	Projection	Experimental
1- %PHB cell content, PHB concentration	NMS	0.016	4.88	352.46	372 ± 20	124.44	173.65 ± 13.10	36.08	46.79 ± 4.74
	AMS	0.016	6.28	364.84	372 ± 38.15	158	196.93 ± 12.80	44.50	53.11 ± 3.03
2- Biomass, %PHB cell content, PHB concentration	NMS	0.017	6.07	414.42	431.67 ± 34.03	130.56	102.04 ± 2.88	32.58	23.73 ± 1.83
	AMS	0.019	6.88	408.66	488.33 ± 7.64	162.88	164.44 ± 3.21	41.72	33.68 ± 0.79

3.5. Discussion

The conversion of methane into PHB varies among the alphaproteobacterial methanotrophs depending on optimal combinations of C- and N-sources (Khosravi-Darani et al., 2013; Zaldívar Carrillo et al., 2018), oxygen availability (Zaldívar Carrillo et al., 2018), and trace nutrients like copper (T. Zhang et al., 2018), among others. It is generally assumed that high N:C leads to high biomass production, while low N:C leads to high PHB accumulation (Zaldívar Carrillo et al., 2018). *Methylocystis* sp. Rockwell differs from most characterized alphaproteobacterial methanotrophs as it prefers ammonium over nitrate as its N-source for growth and PHB production (Lazic et al., 2021, 2022; Nyerges et al., 2010; Tays et al., 2018). It also has the ability to produce PHB during log phase of growth (Lazic et al., 2022) at 36 and 72 h rather than only in stationary phase at 108 h (Supplementary Fig. A-1c).

OVAT and RSM experiments were conducted using a range of methane amounts (added to 100 mL cultures in 1 L bottles) and N:C ratios to evaluate the effects of nutrient sufficiency versus limitation on cell biomass and PHB production with the aim of finding the optimal combination. The initial RSM analysis showed that, with 6 mmol methane, an N:C of 0.022-0.032 was suitable for improving biomass production, whereas an N:C of 0.012-0.022 was more suitable for PHB accumulation. Growth and PHB production were inversely related as biomass was compromised while PHB production was stimulated under nitrogen limitation, a process regarded as a nutrient stress response (Bordel et al., 2019; Juengert et al., 2017; Pieja et al., 2012).

A full factorial experimental design combined with RSM analysis projected that a multi-optimization based on PHB concentration and %PHB cell content, but not for biomass, produced the best results for PHB production. While an N:C of 0.016 was found to be optimal for both NMS and AMS media, the optimal amount of methane differed depending on whether nitrate (4.88 mmol methane) or ammonium (6.28 mmol methane) was the N-source. Statistically equivalent ($p < 0.05$) amounts of biomass were produced under these two conditions, but more PHB was produced when ammonium was the N-source.

It should be noted that all experiments in this study were conducted in 1 L bottles to prevent cultures from experiencing oxygen limitation. The high oxygen requirement to achieve optimal biomass and PHB production was similar to that found for *Methylosinus trichosporium* OB3b (Zaldívar Carrillo et al., 2018). In smaller bottles (250 mL), 100 mL cultures of *Methylocystis* sp. Rockwell generally reached an OD_{540} ~0.3 due to oxygen limitation, whereas cultivation of 100 mL culture in 1 L bottles did not experience oxygen limitation and achieved OD_{540} ~0.7 (Tables 3.1 & 3.2). The molar oxygen:methane consumption ratios were consistent at 1.35 ± 0.05 for all experiments, which is slightly lower than the oxygen:methane consumption ratio of 1.5 observed

for *Methylocystis parvus* cultures (Pieja, Sundstrom, et al., 2011) and 1.46 ± 0.05 for *Methylocystis hirsuta* cultures (Bordel et al., 2019).

Several studies have been conducted to improve PHB production in a variety of alphaproteobacterial methanotrophic strains. In *Methylosinus trichosporium* IMV 3011, improved bacterial growth and PHB production (0.6 g/L) was reported when methanol was added to a methane-grown culture (Y. Zhang et al., 2008). High PHB concentration (3.43 g/L) was also observed in *M. parvus* OBBP when grown in a continuous-flow reactor (Sundstrom & Criddle, 2015). In the present study, under optimal conditions, *Methylocystis* sp. Rockwell at high biomass produced PHB at a similar % cell content (53.1%) as that measured from batch cultures of *M. trichosporium* OB3b (52.5%) under similarly oxygen replete conditions (Zaldívar Carrillo et al., 2018). The projected optimal N:C ratio of 0.016 for *Methylocystis* sp. Rockwell was also in agreement with the optimum N:C ratio of 0.017 observed for *M. trichosporium* OB3b (Zaldívar Carrillo et al., 2018); both of which were experimentally confirmed. These results confirm that *Methylocystis* sp. Rockwell can balance growth and PHB production, even in small-scale batch cultures, perhaps due to its ability to produce PHB during exponential growth rather than only in stationary phase (Supplementary Fig. A-1c).

3.6. Conclusion

Bioconversion of methane for production of bioplastics by alphaproteobacterial methanotrophs is an attractive alternative to sugar-based feedstocks as methane is an inexpensive, readily available, non-food-based feedstock; let alone a potent greenhouse gas that must be mitigated. As methanotrophs show distinct preferences for nutrient combinations to support both growth and PHB production, a species-specific MOOC approach can be used to optimize the strain

and process towards industrialization. This study highlights *Methylocystis* sp. Rockwell as a promising candidate for industrial PHB production if a prolonged exponential growth phase is maintained by adopting appropriate feeding strategy, particularly when using ammonium as an N-source. We further intend to achieve a high cell density with improved PHB production by fine-tuning of optimal conditions using continuous gas flow bioreactor, a suitable approach for technical scale up.

3.7. References

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CHAPTER 4: Impact of culture history on growth and fate of polyhydroxybutyrate in *Methylocystis* sp. Rockwell.

4.1. Abstract

Polyhydroxybutyrate (PHB) synthesis and degradation is a cyclic process among PHB producing microbes. An assessment of biomass production, PHB synthesis and degradation together with the physiological role of PHB under nutrient stress is essential for establishing a bioprocess for production and recovery of PHB from methanotrophic biomass. This study tested the responses of *Methylocystis* sp. Rockwell in terms of its growth and fate of PHB in batch cultures under nutrient stress using two metabolic states of inocula, well-fed and starved cells. The rates of synthesis and degradation of PHB changed with nutrient availability in the culture media and the metabolic state of the inoculum. Unlike starved inocula, well-fed inocula could grow under nitrogen-depleted conditions and accumulate PHB while growing. However, the culture with recurrent nitrogen limitation (i.e. starved inocula in the absence of N-source) were not able to grow or produce PHB. Regardless of the duration of nutrient deprivation (30, 60, or 120 days), *Methylocystis* sp. Rockwell cultures sustain themselves by utilizing carbon derived from PHB degradation during prolonged starvation and they recover effectively when methane and ammonium are reintroduced, indicating that duration of starvation had no cumulative effect on bacterial survival. Furthermore, this study emphasized the superiority of a single-step process compared to the two-step process for PHB production. It also provided valuable insights on inoculum preparation, medium formulation, and process design, which are essential for developing effective bioprocess strategies to produce and recover PHB from *Methylocystis* sp. Rockwell.

4.2. Introduction

Bacterial growth and cellular functions depend, to some degree, on the proportion of carbon and nitrogen available in their environment. As microbes exhibit remarkable adaptability to thrive in environments characterized by fluctuating resource availability, their ability to survive and flourish under feast-or-famine conditions provides valuable insights into the maintenance of diverse and complex microbial communities over extended periods. Nutrient fluctuation due to microscale spatial heterogeneity is a characteristic feature of many natural habitats. Bacterial growth under nutrient-rich environments is typically leads to metabolically active cells containing abundant reducing equivalents, whereas growth under nutrient-deficient conditions is characterised by starved cells with a paucity of reducing equivalents. These well-fed and starved conditions represent two metabolic states in which the carbon and energy balances play vital roles in bacterial survival. Nitrogen limitation, on the other hand, triggers the nitrogen regulation (Ntr) mediated stress response in many bacteria, leading to accumulation of the polyhydroxybutyrate (PHB). In these cases, PHB not only functions as a carbon and energy storage molecule but also as an electron sink (Batista et al., 2018; Koch et al., 2020). However, PHB accumulation and intracellular degradation is a cyclic process catalyzed respectively by PHB synthase and PHB depolymerase enzymes. These processes connect with overall metabolism, ensuring PHB turnover to sustain metabolic equilibrium in the cell (Arias et al., 2013; Velázquez-Sánchez et al., 2020; Ren et al., 2009). Despite considerable progress in elucidating the roles of different enzymes involved in PHB accumulation and degradation in methanotrophic bacteria, it is unclear how the metabolic state of cells affects in their subsequent growth and fate of PHB when the cells experience nutrient stress such as- carbon, nitrogen, or oxygen deprivation. These nutrient stresses are common in the natural environment and can significantly impact the survival and growth of

methanotrophic bacteria. In fact, some aerobic methanotrophs can thrive at the oxic/anoxic interface in the environment, where nutrient fluctuation is discernable (Guerrero-Cruz et al., 2021; Hakobyan & Liesack, 2020). In such conditions, it is plausible that intracellular PHB accumulation or degradation could be a beneficial survival strategy. Indeed, some methanotrophs are known to produce fermentation products, suggesting their use of anaerobic metabolism as a survival strategy (Tays et al., 2018; Vecherskaya et al., 2001; Vecherskaya et al., 2009).

Understanding how *Methylocystis* sp. Rockwell responds to different nutrient states is crucial to comprehend the cyclic process of PHB synthesis and degradation, which has direct implication in bioprocess design for higher PHB yield. Moreover, it is equally important to know the biology of how the starved bacteria recover from a state of deprivation and whether the recurrent N-limitation is suitable for higher PHB accumulation, particularly from an industrial perspective, holds equal significance. Therefore, this chapter aimed to investigate how the growth and fate of PHB in *Methylocystis* sp. Rockwell are influenced by well-fed or starved inocula when subjected to nutrient deprivation and replenishment. Well-fed inocula exhibited lower PHB content, while starved inocula displayed higher PHB content. After incubating the cultures in 16 different combinations of nutrient (carbon, nitrogen, oxygen) deprivation, microbial growth and PHB fate were assessed. Study identified the specific nutrient combinations and importance of time points to maximize growth and PHB yield/recovery in industrial bioprocesses. Furthermore, the resilience of *Methylocystis* sp. Rockwell has demonstrated its ability to adapt to limited resources over extended periods by undergoing metabolic shifts and utilizing survival strategies.

4.3. Materials and methods

4.3.1. Inocula preparation

Methylocystis sp. Rockwell cells were grown in 100 ml of culture medium containing ammonium as N-source in 1-L Kimble bottles tightly sealed with caps inlayed with butyl rubber septa incubated at 30 °C, 150 RPM for five days. Starved inocula with high PHB content were prepared using 6.28 mmol methane and 0.1 mM of ammonium (maintaining an N:C ratio of 0.016, See chapter 3)(Sharma et al., 2022), while well-fed inocula were prepared with 5 mmol methane and 10 mM ammonium (maintaining an N:C ratio of 0.20) in mineral medium. The inocula representing starved and well-fed cells were prepared after the stationary phase is achieved, followed by appropriate dilution to maintain a similar optical density (OD) for all subsequent cultures that received 20 ml of inoculum.

4.3.2. Design of experiments (DOE)

As demonstrated schematically (Supplementary Fig. B-1), first well-fed and starved inocula were prepared as discuss above, then they were re-cultured under specified nutrient combinations (Table 4.1). To investigate microbial growth and fate of PHB (i.e. PHB accumulation or degradation) as a consequence of nutrient limitation, 9 replicate cultures for each condition were grown in 100 ml of AMS medium supplemented with 2 ml of phosphate buffer (pH 6.8) in 250 ml bottles (for low oxygen) or 1 L bottles (for high oxygen). Culture conditions included three variables: presence and absence of methane, presence and absence of ammonium, and low and high oxygen levels (Table 4.1).

Table 4.1. DOE: Nutrient combinations used to analyse bacterial growth and fate of PHB in subsequent cultivation of well-fed (W) or starved (S) inucula as consequences of nutrient deprivation. The letter C, N, O represent carbon (methane), nitrogen (ammonium) and oxygen respectively.

Combinations of carbon and nitrogen		Oxygen		Inocula
		O_0	O_1	
$CH_4 + NH_4^+$	(C_1N_1)	$C_1N_1O_0W$	$C_1N_1O_1W$	**W**
		$C_1N_1O_0S$	$C_1N_1O_1S$	**S**
CH_4 only	(C_1N_0)	$C_1N_0O_0W$	$C_1N_0O_1W$	**W**
		$C_1N_0O_0S$	$C_1N_0O_1S$	**S**
NH_4^+ only	(C_0N_1)	$C_0N_1O_0W$	$C_0N_1O_1W$	**W**
		$C_0N_1O_0S$	$C_0N_1O_1S$	**S**
No carbon No nitrogen	(C_0N_0)	$C_0N_0O_0W$	$C_0N_0O_1W$	**W**
		$C_0N_0O_0S$	$C_0N_0O_1S$	**S**

Culture conditions

C_1= 5 mmol of methane

C_0= No carbon

N_1= 10 mM of ammonium

N_0= No nitrogen

O_1= High O_2 (1000 ml bottles)

O_0= Low O_2 (250 ml bottles)

S= Starved inoculum (PHB rich cells)

W= Well-fed inoculum (low PHB cells)

4.3.3. Effect of formate on growth and fate of PHB

To determine whether PHB is degraded earlier to start the methane oxidation and to understand the effect of formate on growth and fate of PHB, a separate set of experiment was conducted using PHB rich cells (starved inocula) with and without formate. For this, first the PHB rich starved inucula were prepared as it was discussed above followed by subsequent cultures of 20 mL inocula in 1 L Kimble bottles containing 100 mL of AMS medium supplemented with 2 ml of phosphate buffer (pH 6.8) and tightly sealed with caps inlayed with butyl rubber septa. The sodium formate concentration of 2.5 mM was maintained in the culture with formate. Multiple replicate cultures of each condition were incubated in shaker (150 RPM) at 30 °C until sacrificed (n = 3) at different timepoints.

4.3.4. Analysis of bacterial growth, headspace composition and PHB content

The growth of *Methylocystis* sp. Rockwell was measured by optical density (OD) using a spectrophotometer (Multiskan Spectrum, Thermo Scientific) every 24 h up to 144 h. Dry weight biomass (mg/L) production was measured gravimetrically by vacuum filtering and drying 25 ml of culture. Gas headspace concentrations for methane and oxygen concentration were measured by GC-TCD, as described in Chapter 3. PHB content was analyzed using GC-FID (Zaldívar Carrillo et al., 2018), as described in Chapter 3. For biomass and PHB measurements, three replicate cultures for each nutrient condition were sacrificed at 48, 96, and 144 hours.

4.4. Results

4.4.1. Effects of well-fed or starved inoculum on bacterial growth under various nutrient deprivation conditions

i. Growth of cultures initiated with well-fed inocula. *Methylocystis* sp. Rockwell grew with methane with or without a nitrogen source (Fig. 4.1). No growth was observed in the absence of methane (C_0N_1) (Fig. 4.1). Final OD of 0.784 ± 0.01 (from C_1N_1) and 0.533 ± 0.003 (from C_1N_0) and their respective biomass production of 568.33 ± 23.09 mg/L and 443.33 ± 5.77 mg/L observed in oxygen rich environments (Fig. 4.1a-b) were significantly higher ($p < 0.05$) than those with low oxygen, that corresponded to 0.210 ± 0.005 (for C_1N_1) and 0.222 ± 0.009 (for C_1N_0) and their respective biomass of 201.67 ± 5.77 mg/L and 193.33 ± 2.89 mg/L (Fig. 4.1c-d). This highlights the importance of oxygen for methane oxidation and bacterial growth.

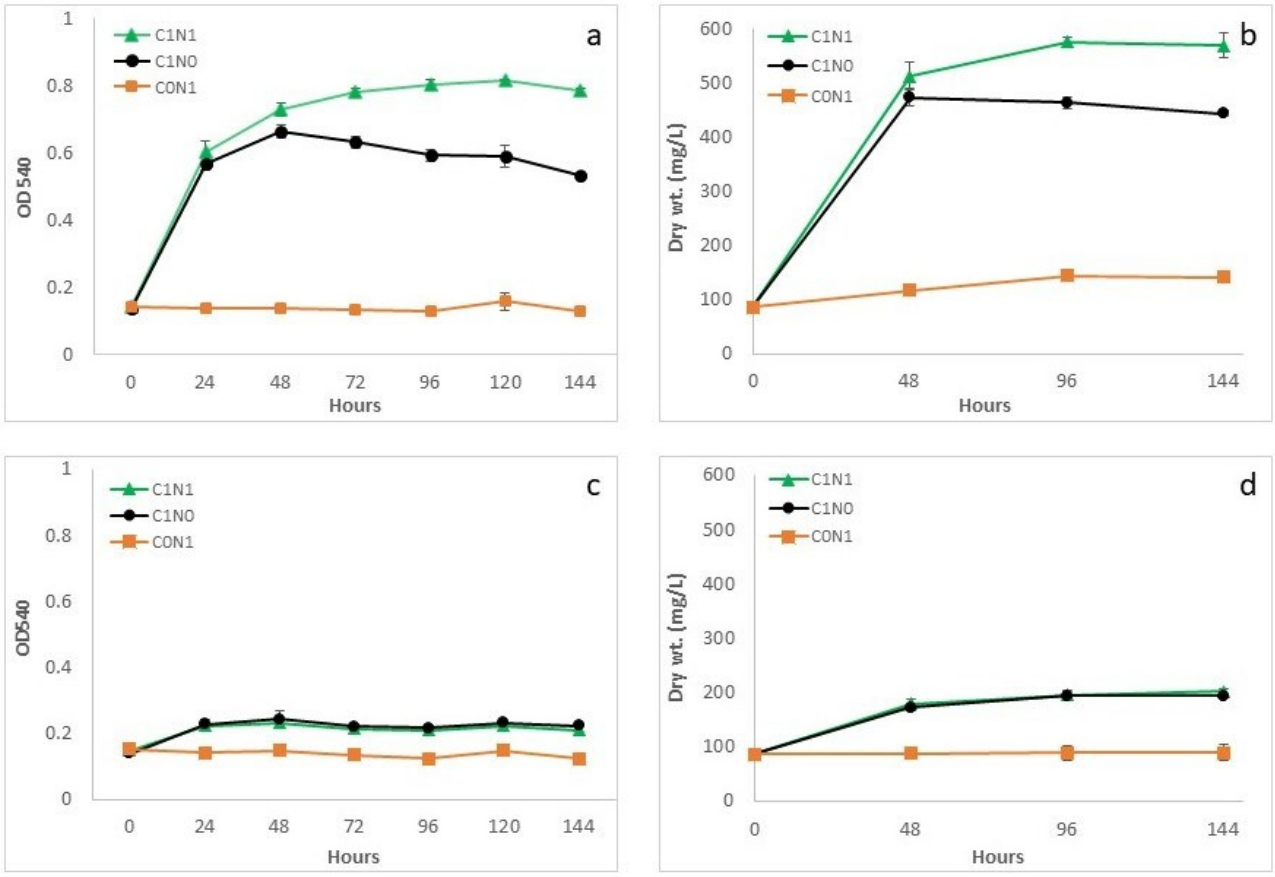

Fig. 4.1. Growth of *Methylocystis* sp. Rockwell (a, c) and dry weight (b, d) when well-fed inocula were used for cultures containing methane (C1) and nitrogen (N1), or no methane (C0) and no nitrogen (N0) under high oxygen (a, b) and low oxygen (c, d) conditions. Error bars represent the standard deviation (n =3).

ii. Growth of cultures initiated with starved inocula. For cultures initiated with starved inocula, final OD of 0.748 ± 0.010 (from C_1N_1) and biomass production of 454.67 ± 16.65 mg/L with high oxygen (Fig. 4.2a-b) were significantly higher ($p < 0.05$) than with low oxygen, which corresponded to OD of 0.162 ± 0.003 (for C_1N_1) and biomass production of 118.67 ± 4.62 mg/L (Fig. 4.2c-d). Growth was not observed in cultures with either methane or nitrogen starvation (C_0N_1 or C_1N_0 condition) (Fig. 4.2).

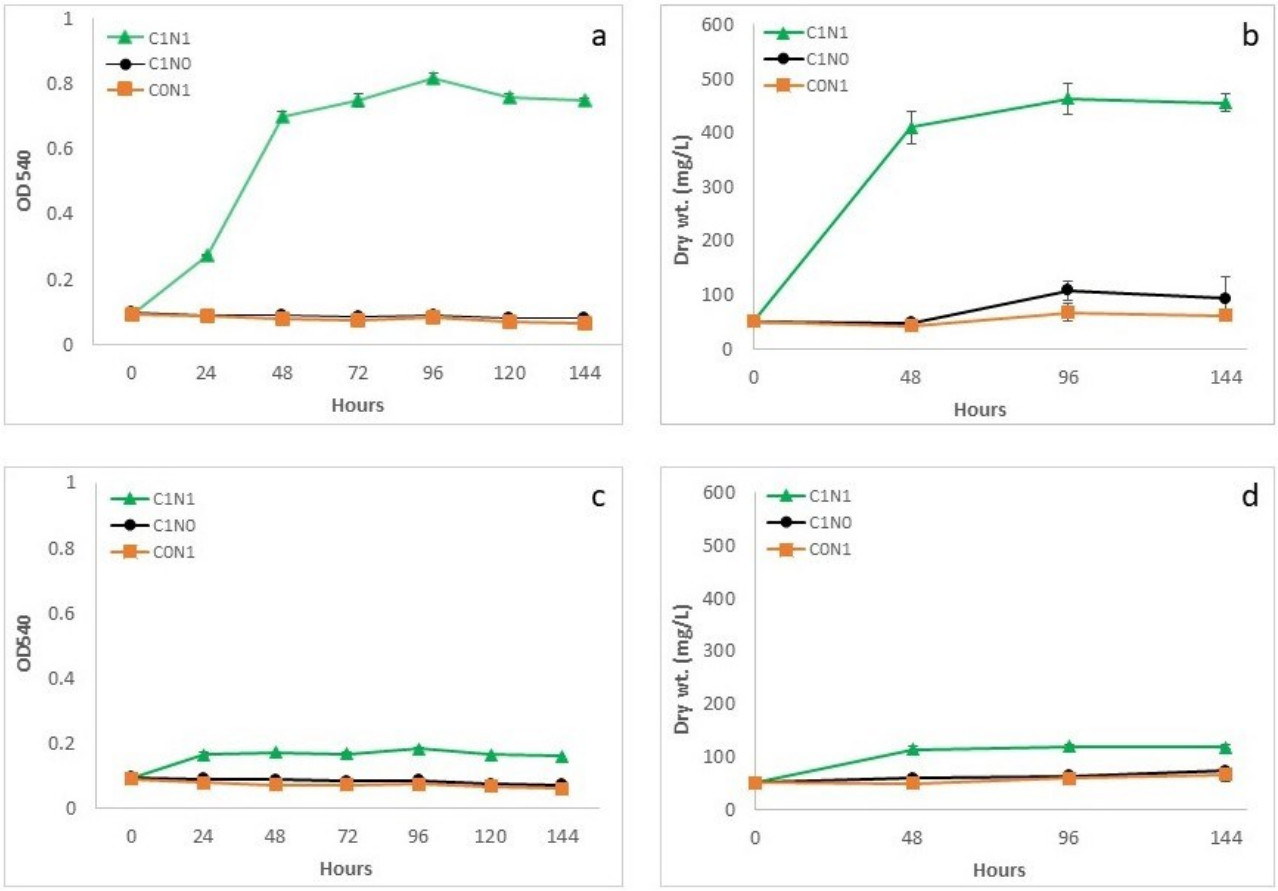

Fig. 4.2. Growth of *Methylocystis* sp. Rockwell (a, c) and biomass production (b, d) when starved inocula were used in presence of methane (C1), presence of nitrogen (N1), absence of methane (C0) and absence of nitrogen (N0) under high oxygen (a, b) and low oxygen (c, d) conditions. Error bars represent the standard deviation (n =3).

4.4.2. Effects of well-fed or starved inoculum on fate of PHB for cultures exposed to nutrient deprivation

i. Fate of PHB for cultures initiated with well-fed inocula. Cultures of *Methylocystis* sp. Rockwell initiated with well-fed inocula growing in high levels of oxygen accumulated PHB when growing in the presence of methane, regardless of the presence or absence of ammonium (C_1N_1 and C_1N_0) (Fig. 4.3a-b). At the 48-hour time point, PHB production was significantly higher in the

absence of nitrogen (C_1N_0) compared to in the presence of both methane and nitrogen (C_1N_1), supporting that nitrogen limitation favors PHB production. However, accumulated PHB was degraded over time under both conditions and maintained a constant level, indicating stabilization between PHB synthesis and degradation (Fig. 4.3). The maximum level of PHB production was significantly higher ($p < 0.05$) in cultures grown with high-oxygen levels rather than with low-oxygen levels, suggesting an essential role of oxygen in PHB biosynthesis (Fig. 4.3).

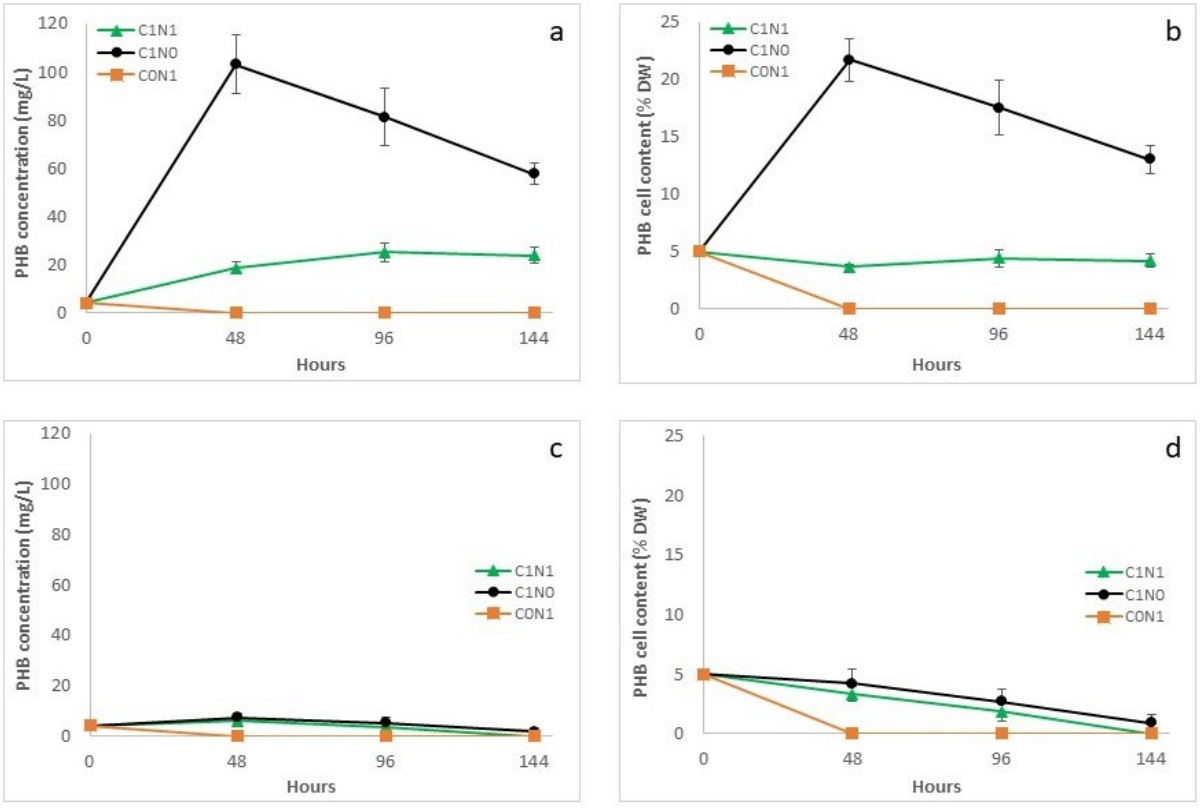

Fig. 4.3. Fate of PHB when well-fed inocula were used to cultivate *Methylocystis* sp. Rockwell with methane (C1), ammonium (N1), no methane (C0) and no ammonium (N0) under high oxygen (a, b) and low oxygen (c, d) conditions. Error bars represent the standard deviation (n =3).

ii. Fate of PHB for cultures initiated with starved inocula. For cultures initiated with starved inocula, *Methylocystis* sp. Rockwell produced PHB when growing in the presence of methane and ammonium (C_1N_1) and at high oxygen levels; but unlike cultures initiated with well-

fed inoculum, these cultures did not produce PHB in the absence of ammonium (C_1N_0). PHB production was observed in C_1N_1 when the cells were actively dividing (up until the 96-hour time point), but then a significant reduction in PHB was recorded for cultures grown on methane and ammonium (C_1N_1). The lack of PHB production in the absence of ammonium (C_1N_0) suggests that PHB production is dependent on cells that have ample internal nitrogen supply, or the ability to kick-start nitrogen fixation (Pieja et al., 2011). After 96 hours, PHB was degraded to a similar level as other nutrient combinations (Fig. 4.4) indicating metabolic balance in the cell. There was no PHB production in cultures grown under low-oxygen conditions.

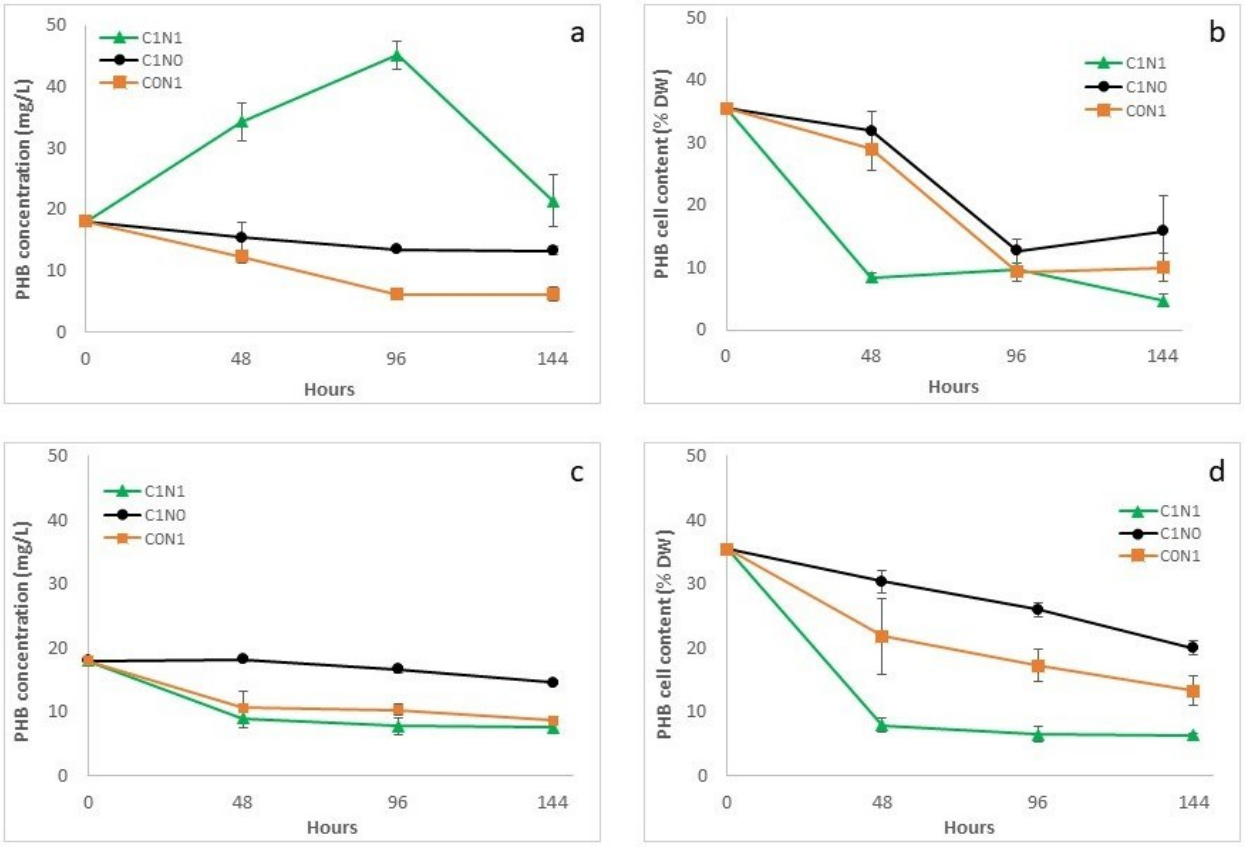

Fig. 4.4. Fate of PHB when starved inocula were used to grow cultures in the presence of methane (C_1), presence of ammonium (N_1), absence of methane (C_0) and absence of ammonium (N_0) under high oxygen (a, b) and low oxygen (c, d) conditions. Error bars represent the standard deviation (n =3).

4.4.3. Survival of *Methylocystis* sp. Rockwell in the absence of nutrients for 120 days

Since we observed PHB degradation from starved inocula when cultured in absence of carbon or nitrogen, we sought to investigate if *Methylocystis* sp. Rockwell could continue to grow and sustain themselves by utilizing the carbon released from PHB degradation during an extended period of nutrient deprivation. Experiment involved well-fed and starved inocula to initiate cultures- one with oxygen and the other without oxygen, but no methane or ammonium. The cultures were monitored and incubated for 120 days under the specified conditions. *Methylocystis* sp. Rockwell cultures initiated with well-fed inocula completely degraded their PHB within 30 days, whereas cultures initiated with starved inocula degraded PHB over the full 120 days, both with and without oxygen in the headspace (Fig. 4.5b-c). However, instead of growing, the OD values decreased in all treatment conditions (Fig. 4.5a). This suggests that, while PHB was degraded during 120 days of nutrient starvation, it was not used as a carbon source for bacterial growth (Pieja et al., 2011). To determine whether the cells were losing viability over time, three replicate cultures were sacrificed from each experimental set at 30, 60, and 120 days and were used to inoculate new cultures (2% inoculum) grown with 5 mmol of methane, 10 mM of ammonium, in presence of air. All of these new cultures recovered their growth, regardless of the presence of remnant PHB, albeit with different lag phase (Fig. 4.5d-f). Cultures that were originally initiated with the starved inoculum (high PHB content) exhibited a longer lag phase than those originally initiated with the well-fed inoculum (low PHB content).

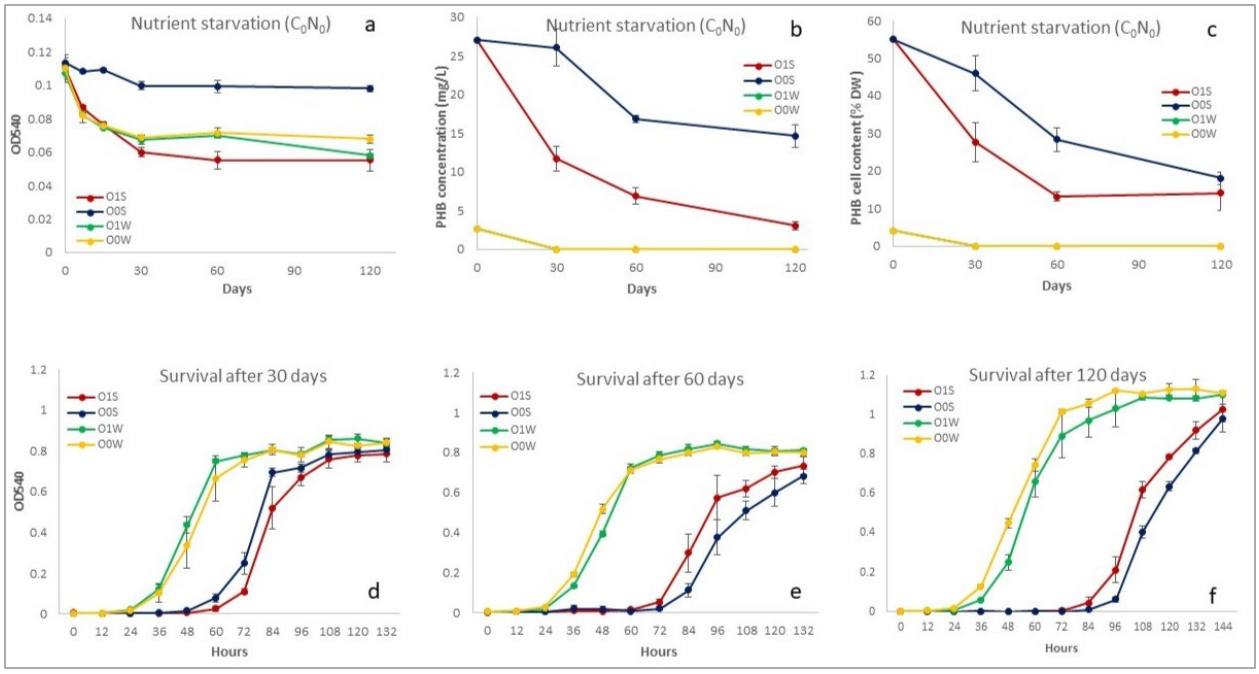

Fig. 4.5. Bacterial response to nutrient starvation and recovery after starvation. Well-fed inocula (W) and starved inocula (S) were cultivated up to 120 days in the absence of carbon (C_0) and nitrogen (N_0) in oxic (O_1) or anoxic (O_0) conditions (a). Change in PHB concentration (b) and change in PHB cell content (c) observed over the duration of starvation. Survival/recovery of starved cells observed in presence of methane and ammonium after 30 days of starvation (d), after 60 days of starvation (e) and after 120 days of starvation (f). Error bars represent standard deviation (n = 3).

4.4.4. Effect of formate on growth and PHB degradation in *Methylocystis* sp. Rockwell

As we observed decreased in PHB content when starved inocula were cultivated in absence of carbon or nitrogen, we further examined if PHB undergoes degradation to fulfill the reductant demand for the initiation of methane oxidation (Hanson & Hanson, 1996; Lipscomb, 1994; Fergala et al., 2021). This experiment utilized starved inoculum containing cells abundant in PHB, both with and without the presence of formate. Addition of 2.5 mM of sodium formate when culturing PHB-rich starved inocula (F_1P_1) had an impact on growth rate. Higher growth rate (0.011 h^{-1}) was observed in the culture without formate (F_0P_1) than presence of formate (0.008 h^{-1}) during its

exponential growth phase, but no significant effect was observed on final OD at 96 h (OD of 0.638 ± 0.006 with formate and 0.717 ± 0.021 without formate, Fig. 4.6a). PHB degradation was delayed during early (8 – 24 h) growth phase in the culture containing formate compared to without (Fig. 4.6c-d). However, PHB maintained at a similar level after 48 h in each condition.

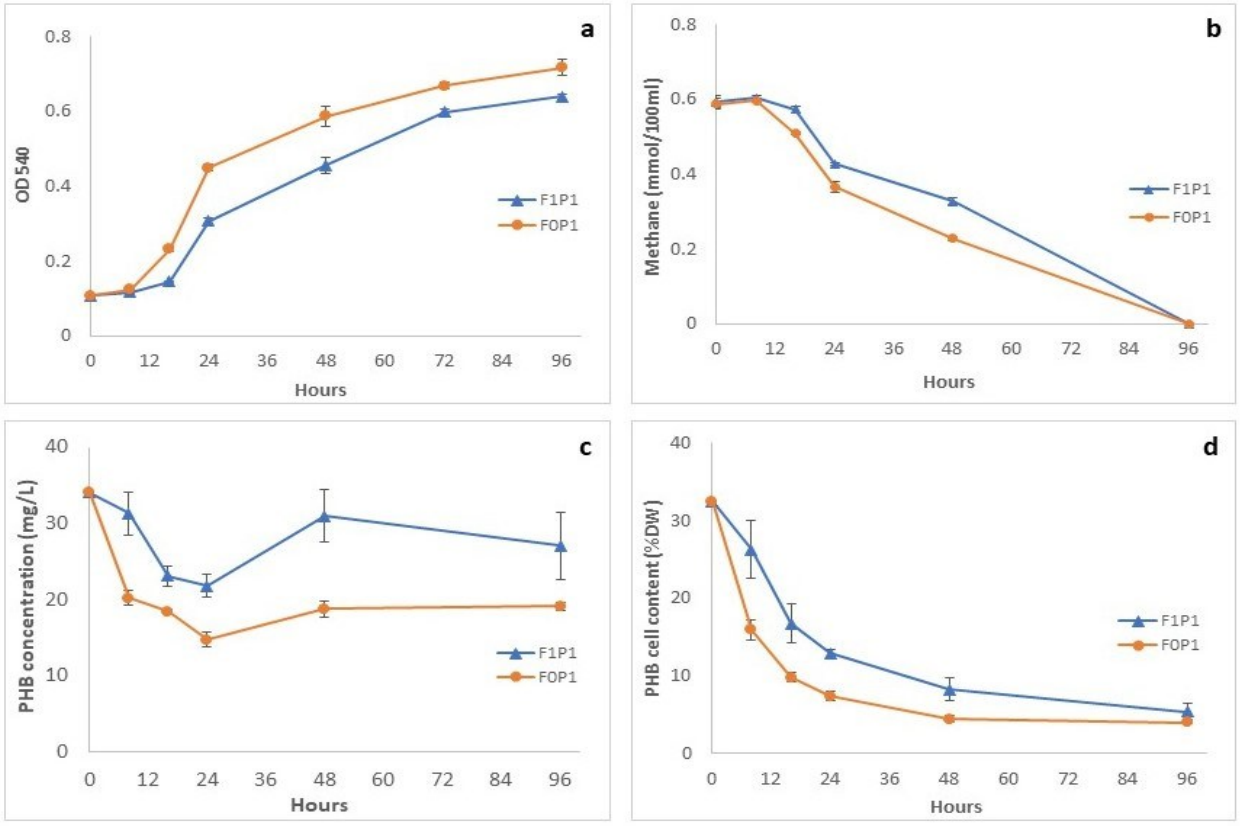

Fig. 4.6. Effect of formate on growth and PHB degradation. Growth of *Methylocystis* sp. Rockwell in cultures initiated with starved (high PHB) inocula in presence (F1) or absence (F0) of formate (a), change in headspace methane (b), change in PHB concentration (c), change in PHB cell content (d). Error bars represent standard deviation (n = 3).

4.5. Discussion

PHB production in *Methylocystis* sp. Rockwell is mainly stimulated by nutrient limitation. While increased PHB production is desirable for commercial applications, a lack of understanding on how the metabolic state of culture inocula affects growth and fate of PHB under nutrient

starvation conditions hinders the process design. A well-fed inoculum is represented by cells having less PHB content but sufficient reducing equivalents to initiate immediate methane oxidation, facilitating growth in the presence of methane, nitrogen, and oxygen. However, growth of this inoculum can also occur in the absence of nitrogen, likely due to internal N-storage (Pieja et al., 2011). Although *Methylocystis* sp. Rockwell showed PHB production during logarithmic growth, significantly higher PHB accumulation occurred at 48 h in subsequent growth of well-fed inocula under nitrogen starved conditions (Fig. 4.3a-b) indicating an importance of nitrogen limitation in PHB accumulation (Muhammadi et al., 2015). On the other hand, PHB degradation after 48 h may be a consequence of low oxygen levels, further limiting PHB production and stimulating PHB degradation (Williams, 1988) to maintain metabolic balance (Pieja et al., 2011). This highlights an important cyclic process- PHB production and degradation occurred in *Methylocystis* sp. Rockwell. moreover, the factors such as nitrogen limitation, elevated oxygen levels, and selecting an appropriate time point play a crucial role to avoid PHB loss over time and achieve a maximal PHB recovery in *Methylocystis* sp. Rockwell. The bacterial growth at 48 h (OD = 0.66 ± 0.24) from this experiment (Fig. 4.1a) is at similar level with the final OD of 0.65 ± 0.13 from optimal N:C ratio (Chapter 3), but the PHB production differ between these two sets of experiments. Although current data suggest the potential of two-phase strategy of cultivating *Methylocystis* sp. Rockwell in replete nutrient conditions followed by nitrogen limitation for higher PHB accumulation, PHB production was significantly higher ($p < 0.05$) in cells grown at an initial optimal N:C ratio of 0.016 (Chapter 3) (Sharma et al., 2022) than two-phase process indicating our strategy of PHB production in one-phase seems reasonable in terms of higher PHB production, time efficiency and economic viewpoint for the industries. Therefore, it can be argued that if the

bioreactor operation utilizes appropriate combinations of nutrient and feeding strategies, further improvements in PHB production could be seen in a continuous cultivation approach.

The decrease in PHB in cultures initiated from starved inoculum (initially have high PHB content) in the absence of methane indicated that PHB was actively degraded (Fig. 4.4), perhaps as a nutrient stress response (Pieja et al., 2011; Sadykov et al., 2017; Müller-Santos et al., 2020). However, *Methylocystis* sp. Rockwell did not use carbon from PHB degradation to support growth (Fig. 4.2). Similarly, a decrease in PHB (Fig. 4.4) and growth (Fig. 4.2) for cultures initiated with starved inocula in absence of nitrogen suggests that these cells have already undergone nutrient stress (N-limitation) during inocula preparation and are not further capable of oxidizing methane or commencing growth in subsequent culture. As a result, further growth of these cultures was only observed when they were introduced into media containing both methane and ammonium, where they could degrade PHB to kick-start methane oxidation (Pieja et al., 2011; Cai et al., 2016). To determine whether PHB is degraded before methane oxidation can commence, an experiment was conducted using starved inoculum (PHB rich cells) with and without formate. Given that both formate oxidation and PHB degradation yield NADH, there is a possibility that bacteria conserve stored PHB when external formate is supplied, as it allows them to preserve carbon and energy resources (Sipkema et al., 2000; Fergala et al., 2021). As expected, during the initial 8 hours of growth, there was a significant delay ($p < 0.05$) in the utilization of PHB when formate was present (F_1P_1) compared to when it was absent (F_0P_1). However, it is important to note that no methane consumption was observed under either condition (Fig. 4.6b). Such delay in PHB utilization when formate was present is linked to the availability of NADH due to initial formate oxidation (Hanson & Hanson, 1996). Ishikawa et al., (2017) also observed generation of NADH due to exogenous addition of sodium formate in *Methylococcus capsulatus* Bath culture. Whereas faster utilization

of PHB in absence of formate can be due to intracellular degradation of PHB fulfilling the NADH demand (Sipkema et al., 2000; Fergala et al., 2021). Additionally, the depletion of PHB in both conditions (F_1P_1 and F_0P_1) between 8 and 24 hours suggests a simultaneous use of both substrates (Fig. 4.6c-d), which is supported by the work of Pieja et al. (2011). This change in PHB utilization under different conditions suggests an energy conservation strategy by the bacteria. Additional investigation into the cellular energy status and formate assay under various treatment conditions (including the presence or absence of formate utilization in cells with and without PHB) will provide further understanding of the underlying mechanism, and connection between formate oxidation and PHB degradation to fulfil NADH demand for methane oxidation in *Methylocystis* sp. Rockwell.

To investigate the role of PHB on the growth and survival of *Methylocystis* sp. Rockwell, a study was conducted on the effect of starved and well-fed inocula by incubating them under further starvation conditions for up to 120 days. Unlike non-methanotrophic bacteria which can degrade PHB to enable their survival and growth under carbon starvation (Handrick et al., 2000; Ratcliff et al., 2008), *Methylocystis* sp. Rockwell didn't grow during the period of nutrient starvation (Fig. 4.5a) but was able to degrade PHB (Fig. 4.5b-c) and remained viable, indicating that methanotrophs do not replicate using stored PHB (Pieja et al., 2011). As they fully recovered once methane, ammonium, and oxygen became available (Fig. 4.5d-f), the physiological role of PHB granules in methanotrophs can be described as stored carbon, where PHB degradation likely provides carbon and energy to maintain cellular integrity during nutrient starvation (Pieja et al., 2011). This further support earlier findings that methanotrophs typically use PHB degradation to enable survival, but not growth, during nutrient starvation (Pieja et al., 2011; Cai et al., 2016; Henrysson & McCarty, 1993; James et al., 1999). However, a much longer lag phase was observed

in high PHB content cells (starved inocula) versus low PHB content cells (well-fed inocula) during recovery from starvation indicating the mechanisms and timelines for recovery can vary depending on the nature and duration of starvation, and environmental factors. The possible reason for this difference could be the effect of nutrient availability on starting cellular metabolism (Zhang et al., 2019), with higher levels of reducing equivalents present in well-fed inocula (Pieja et al., 2011) and the possible formation of resting bodies in starved inocula (Whittenbury et al., 1970; Stevenson & Socolofsky, 1973) affecting the response. Methanotrophs have developed various mechanisms to endure nutrient scarcity. They can form exospores or cysts to survive under nutrient deprivation (Whittenbury et al., 1970). While some methanotrophs have been observed to endure up to 70 days without methane (Roslev & King, 1994), *Methylocystis* sp. Rockwell exhibited an impressive survival period of 120 days without methane, ammonium, or oxygen. Here, degradation of PHB in *Methylocystis* sp. Rockwell might be linked to a long-term survival strategy, involving the formation of resting bodies. Research has indicated a positive correlation between the amount of PHB accumulation and the degree of cellular encystment (Stevenson & Socolofsky, 1973), where PHB degradation plays a role in supplying the required intracellular carbon and energy during the process of cyst formation (Sadykov et al., 2017). Further analysis, such as microscopy, energy level assessment, and gene expression analysis of starved vs. well-fed inocula, could provide insights into the fundamental mechanism underlying starvation response and long-term survival mechanisms of *Methylocystis* sp. Rockwell which has implications in various fields, including microbiology, ecology, and biotechnology.

The research in this chapter demonstrated the importance of inoculum history in maximizing growth potential and maintaining PHB yield under varied nutrient combinations. These findings offer valuable insights into the key factors that impact a cyclic process of PHB

production and degradation, and inform strategies for inoculum preparation, media formulation, and process design to optimize PHB production and recovery in methanotrophic bioprocesses.

4.6. Conclusion

Studying and comprehending the cycling processes of PHB production and degradation helps researchers to optimize nutrient parameters and culture conditions that can be used to develop more efficient and economically viable methods for large-scale PHB production, necessary to meet industrial demands. For this reason, a clear understanding of bacterial response in terms of biomass and PHB production is crucial before implementing nutrient stress conditions in industrial setting. The results suggest that two-step process (growth followed by nitrogen limitation) is likely a less effective strategy than one-step process that utilizes an optimal N:C ratio to achieve higher growth and PHB yield in *Methylocystis* sp. Rockwell (Chapter 3). Furthermore, the well-fed inocula of *Methylocystis* sp. Rockwell can accumulate PHB while growing under nitrogen limitation conditions. Thus, another possible approach to enhance biomass production and PHB accumulation is to employ a single-step strategy that involves pulsing low ammonium concentration while ensuring a continuous supply of methane and air to the culture. Intermittent supply of reduced ammonium concentration curbs excessive cell growth, while a constant supply of methane ensures a consistent carbon source that increase PHB accumulation in *Methylocystis* sp. Rockwell cultures. This understanding could help developing an improved bioreactor design, mode of cultivation, and feeding strategies to enhance the overall performance of the bioprocess.

4.7. References

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CHAPTER 5: Improving growth and polyhydroxybutyrate production of *Methylocystis* sp. Rockwell using methanol fed-batch bioreactors.

5.1. Abstract

Methane emissions from various industries and methanol waste from pulp and paper are desirable single carbon feedstocks for microbial bioconversion into valuable products. In addition, disposal of petroleum-based plastic materials is a major global environmental challenge that causes pollution and threatens the survival of organisms, including humans. *Methylocystis* sp. Rockwell can consume both methane and methanol and accumulate polyhydroxybutyrate (PHB), a molecule used to produce biodegradable plastics, offering a promising mitigation measure for both single carbon wastes and petroleum-based plastic pollution. Therefore, it is essential to improve bioprocessing strategies to increase productivity in order to make this methanotrophic bacterium a viable option for single carbon waste reduction and PHB production at industrial scale. In this study, *Methylocystis* sp. Rockwell was cultivated in bioreactors using ammonium as a nitrogen source and methane and/or methanol as a carbon source. Batch and fed-batch cultivation methods were compared for biomass and PHB production, and fed-batch cultivation was found to be the most effective. A pulse feeding strategy with a low initial concentration of methanol was implemented to reduce toxicity effects. The results indicated that 22.5 mM methanol exceeded the toxicity threshold, and a sustained nitrogen concentration of 0.1 to 0.2 mM was ideal for high PHB accumulation. Compared to growth on methane alone, biomass and PHB production were significantly higher with methanol feeding. The fed-batch process with pulsed methanol feeding (15 mM to 17.5 mM every 24 h) was found to be the most productive for generating a high yield of both PHB and biomass from *Methylocystis* sp. Rockwell. PHB productivity reached 45.05 ± 5.77 mg/L/d, making the fed-batch approach suitable for a methanol-based bioprocess.

5.2. Introduction

Pollution due to accumulation of methane, methanol (both C1 compounds) and plastic waste is a global challenge and results from many different types of industrial operations. For example, the pulping industry produces methanol as a major by-product during de-methylation of lignin and xylan in the Kraft process (Zhu et al., 2000). Methanotrophic bacteria, however, can use C1 compounds as their sole source of carbon and energy for production of biomass and various metabolites (Benstead et al., 1998; Tays et al., 2018). In fact, alphaproteobacterial methanotrophs are considered as potential cell-factories for production of biopolymer called polyhydroxybutyrate (PHB). However, the production and commercialization of biopolymers require an effective microbial feeding strategy for industrial scale-up. While the demand for biopolymers is increasing due to their biodegradable, biocompatible, renewable and eco-friendly nature (Muhammadi et al., 2015; Liu et al., 2020), the scale-up of PHB production via high cell density cultivation using a readily available single carbon feedstock can lower the carbon footprint and offer a promising techno-economic strategy in commercialising biopolymer production.

Different feeding strategies and operational conditions have been attempted by using methane, and methanol as feedstocks to achieve a high cell density and improve PHB production from different methanotrophic strains (e.g. Sundstrom & Criddle, 2015; Pieja et al., 2012; García-Pérez et al., 2018; Song et al., 2011; Dong, 2012). These strategies were species-specific, involved myriad optimization parameters in the processes, and varied in compatibility with different nutrient sources. Moreover, the solubility and mass transfer limitation of methane, the toxicity of methanol, the slow growth rate of most methanotrophic bacteria, and the challenges of achieving high cell density at commercial scale are major hurdles to industrial scale methanotroph-based bioprocesses.

Therefore, it is obvious that methanotroph growth must be improved to achieve commercial feasibility.

Methanol is water miscible, readily available to the culture and requires only moderate levels of oxygen to be catabolized (Khosravi-Darani et al., 2013). Although methanol can be a source of carbon and energy for methanotrophs, a high concentration of methanol is toxic to methanotrophic bacteria (Tays et al., 2018). However, they can be preadapted to grow on methanol as a sole carbon source (Hou et al., 1979), and addition of methanol following growth on methane can amplify PHB production (Song et al., 2011; Zhang et al., 2008) compared to bacteria grown on methane as a sole carbon source (Zaldívar Carrillo et al., 2018; Zhang et al., 2008). Such improved growth and PHB production were reported for *M. trichosporium* IMV 3011 when methanol was added to a methane-grown culture (Zhang et al., 2008). Similar improvements in PHB production were observed for *M. trichosporium* OB3b grown with a combination of methane and methanol (Zaldívar Carrillo et al., 2018), indicating a potential application of methanol as a stress inducer for PHB accumulation (Lazic et al., 2021; Zaldívar Carrillo et al., 2018).

PHB production from *Methylocystis* sp. Rockwell revealed that this strain can balance a higher biomass and PHB production in small-scale batch cultures (Sharma et al., 2022) than several other alphaproteobacterial methanotrophs cultivated under different growth and operating conditions (Pieja, Rostkowski, et al., 2011; Pieja, Sundstrom, et al., 2011; Sundstrom & Criddle, 2015; García-Pérez et al., 2018; Dong, 2012; Xin et al., 2007). Interestingly, *Methylocystis* sp. Rockwell can start accumulating PHB during the exponential growth phase and attain the highest PHB yield during stationary phase (Lazic et al., 2022; Sharma et al., 2022). Moreover, nitrogen limitation at a N:C ratio of 0.016 was found to be equally important for PHB production (Sharma

et al., 2022). Thus, a proper carbon and nitrogen feeding strategy could achieve a prolonged exponential growth phase resulting in higher cell density with improved PHB accumulation.

This thesis chapter aimed to enhance both biomass and PHB productivity of *Methylocystis* sp. Rockwell in continuous air flow bioreactors using methane and/or methanol as carbon sources and ammonium as a nitrogen source. We implemented fed-batch operation through an adapted pulse feeding of methanol with or without continuous methane supply, supplemented with controlled nitrogen feeding to maximize the PHB yield from *Methylocystis* sp. Rockwell in a benchtop bioreactor. The performance of batch and fed-batch operations was evaluated to deduce which process conditions were most effective to achieve the highest biomass and PHB productivity. This study showed that methanol as the carbon source provided the best outcome for achieving both biomass and PHB productivity in *Methylocystis* sp. Rockwell.

5.3. Materials and methods

5.3.1. Inoculum preparation

Methylocystis sp. Rockwell (ATCC 49242) was cultivated in 100 ml ammonium mineral salt (AMS) medium supplemented with 1.5 mL of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) at pH 6.8 in 1-L Kimble bottles sealed with butyl-rubber septa caps. To maintain atmospheric pressure, 5 mmol of air was removed from the bottle headspace and replaced with 5 mmol of methane (Praxair) using a 0.22-µm Millex-GS syringe filter unit (Millipore, Sigma). The cultures were grown in an incubator-shaker (G10 Gyrotory shaker, New Brunswick Scientific) maintained at 30°C and 150 rpm (Tays et al., 2018). For experiments involving methanol feeding, after three days of incubation, 20 µl methanol (HPLC grade, Sigma-Aldrich) was added to the culture to adapt them to growth on methanol.

5.3.2. Bioreactor experiments

Bioreactor experiments were conducted using 3-L benchtop units (New Brunswick™ BioFlo®/CelliGen®115). The mode of cultivation and feeding strategy are shown in Table 5.1, and the experimental setup is shown in Supplementary Fig. C-1. The vessel contained 1.93 L pre-autoclaved AMS medium maintained at 30 °C using a thermosensor, heating blanket, and condenser while being stirred at 300 RPM. To create the culture medium, 40 mL (2% v/v) of exponentially growing inoculum and 30 mL of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) at pH 6.8 were added, bringing the final working volume to 2 L. The CO_2 sensor (Vernier Science) was used to monitor metabolic activity, with a decrease in CO_2 indicating a reduction in nutrient utilization, with outlet gas exiting through a filter and vented into a chemical fume hood. Additionally, a dissolved oxygen (DO) sensor (P0720-6282, Inpro 6000, Mettler Toledo) and pH probe (P0720-5584, Ingold, Mettler Toledo) were calibrated and used for culture monitoring. Methanol was fed to the culture medium as a pulse every 24 hours using a 500-µL air-tight syringe until a decline in growth or maintenance of stationary phase was observed. Methane and air were introduced to the bioreactor at a constant rate of 0.077 standard liter per minute (slpm) and 0.177 slpm, respectively, using mass flow controllers (SmartTrak 50, Sierra) and were dispersed by two Rushton impellers placed 5.5 cm apart. To ensure that the gas addition was aseptic, the inlet and exhaust were connected with 0.3-µm Whatman Hepa-Vent™ filters.

Table 5.1. Bioreactor experiments showing mode of cultivation and nutrients feeding strategy.

Expt. run	Cultivation mode	Nutrients feeding		
		Carbon	Ammonium	Air flow
1a	Batch	Methanol (15 mM)	0.1 mM	0.177 slpm
1b	Batch	Methanol (15 mM)	1.0 mM	0.177 slpm
2a	Fed-batch	Methanol (1 – 22.5 mM)/d	0.1 mM/d	0.177 slpm
2b	Fed-batch	Methanol (1 – 22.5 mM)/d	1.0 mM/d	0.177 slpm
3a	Fed-batch	Methane (0.077 slpm)	0.1 mM	0.177 slpm
3b	Fed-batch	Methane (0.077 slpm)	1.0 mM	0.177 slpm
4	Fed-batch	Methane (0.077 slpm)	0.1 - 0.2 mM/d	0.177 slpm
5	Fed-batch	Methanol (1 – 17.5 mM)/d	0.1 - 0.2 mM/d	0.177 slpm
6	Fed-batch	Methane (0.077 slpm) and Methanol (1 – 17.5 mM)/d	0.1 - 0.2 mM/d	0.177 slpm

5.3.3. Batch cultivation of *Methylocystis* sp. Rockwell growing on methanol in bioreactors

Methanol (15 mM) was added to the inoculated bioreactor containing either 0.1 mM or 1.0 mM ammonium at the beginning of the batch experiments. Experiments were conducted in two separate runs (Table 5.1, expt. run- 1a and 1b), and bacterial growth, biomass, PHB, and remaining ammonium were regularly monitored by harvesting 20 mL of culture every 24 hours up to stationary phase.

5.3.4. Fed-batch cultivation of *Methylocystis* sp. Rockwell growing on methanol in bioreactors

The cultivation of *Methylocystis* sp. Rockwell on methanol in fed-batch mode was carried out in four separate runs (Table 5.1, expt. run 2a, 2b, 5 and 6), either with methanol only (Table 5.1, expt. run 2a, 2b, and 5) or methane + methanol combination (Table 5.1, expt. runs 6) where each subsequent run incorporated modifications to the culture conditions based on the results of the earlier experimental runs to enhance biomass and PHB production. First the fed-batch experimental run 2a and 2b were conducted to determine the methanol toxicity threshold for growth followed by subsequent fed-batch run 5 and 6. The flow rate of methane remained constant

(0.177 slpm) in methane + methanol combination as of methane-only fed-batch. Growth, biomass, PHB, and remaining ammonium were analyzed by harvesting 20 mL of culture every 24 hours throughout the experiments. To capture the range of ammonium and carbon feed necessary to maximize production, various concentrations of nitrogen (0.1 mM, 0.2 mM or 1.0 mM of ammonium) were tested as well as pulse-feeding with methanol once every 24 hours for a duration of 10 to 17 days. Following the 20 mL culture harvest, an equal volume of concentrated AMS (10 mM, 20 mM or 100 mM) enriched with essential nutrients was introduced to sustain the 2-L culture volume while achieving a final concentration of 0.1 mM, 0.2 mM, or 1.0 mM ammonium in the corresponding reactors.

5.3.5. Fed-batch cultivation of *Methylocystis* sp. Rockwell growing on methane in bioreactors

Fed-batch cultivation of *Methylocystis* sp. Rockwell using methane as a carbon source was carried out in three separate runs (Table 5.1, expt. run 3a, 3b and 4) where various concentrations of nitrogen (0.1 mM, 0.2 mM or 1.0 mM of ammonium) were tested with continuous gas flow in bioreactors. Methane was continuously supplied at a rate of 0.077 slpm and air at rate of 0.177 slpm to the inoculated bioreactor. Bacterial growth, biomass, PHB, and remaining ammonium were regularly monitored by harvesting 20 mL of culture every 24 hours up to stationary phase.

5.3.6. Analysis of growth and biomass production

Samples (20 mL) were extracted from the reactor vessel via a sterile filter-fitted syringe and collected into a sterile glass sampler tube every 24 hours. Bacterial growth was measured by absorbance at 540 nm using a spectrophotometer (Multiskan Spectrum, Thermo Scientific) and biomass content (mg/L) was measured gravimetrically after vacuum filtration (10 mL of extracted

sample) through a pre-weighed hydrophilic 0.22-µm membrane filter (MCE, MF-Millipore) dried for 48 hours.

5.3.7. Determination of remaining ammonium

To ensure complete nitrogen consumption, ammonium assays were conducted on the samples every 24 hours. The supernatant collected from samples for PHB measurement were used to measure ammonium concentration. A spectrophotometer (Multiskan Spectrum, Thermo Scientific) was used to quantify the total remaining ammonium via colorimetric assay, described previously (Kandeler & Gerber, 1988) with slight modifications. Briefly, 0.25 mL of sample supernatant was added to a 48-well plate and 125 µL of a solution containing sodium hydroxide (12 g/L) plus sodium nitroprusside solution (85 g/L sodium salicylate, 0.6 g/L sodium nitroprusside) at a 2:1 ratio was added. Subsequently, 50 µL of sodium dichloroisocyanurate solution was added to each sample, and the plate was gently tapped and incubated in the dark for 30 minutes at room temperature. Ammonium concentration was determined by measuring the absorbance at 660 nm, using MilliQ water as a reference blank, and against a calibration curve using a range (10 to 200 µM) of NH_4Cl_2 concentrations.

5.3.8. Analysis of PHB production

10 mL of culture harvested from the bioreactors was transferred to a 12 mL screw-capped glass tube and centrifuged at 5,000 rpm (Sorvall Evolution RC, SA-600 rotor, Thermo Fisher Scientific) at room temperature for 30 minutes. Supernatant was collected for the ammonium assay and the cell pellet was used for PHB measurement by GC-FID as described previously (Sharma et al., 2022; Zaldívar Carrillo et al., 2018; Chapter 3, Section 3.3.4 of this thesis).

5.4. Results

5.4.1. Growth and PHB yield of *Methylocystis* sp. Rockwell in batch operation with methanol

The growth of *Methylocystis* sp. Rockwell was monitored in methanol batch mode of cultivation (expt. runs 1, Table 5.1) in the bioreactor containing either 0.1 mM ammonium (expt. runs 1a) or 1 mM ammonium (expt. runs 1b) with continuous air sparging. Stationary phase was achieved within 48 hours, and the final OD was 0.08 on day 4 in bioreactor containing 0.1 mM ammonium. Bioreactor containing 1.0 mM ammonium exhibited slower growth and achieved a final OD of 0.102 on day 4 (Fig. 5.1a). Final biomass on day 4 was measured at 80 mg/L when grown in 0.1 mM ammonium, and 90 mg/L in 1 mM ammonium.

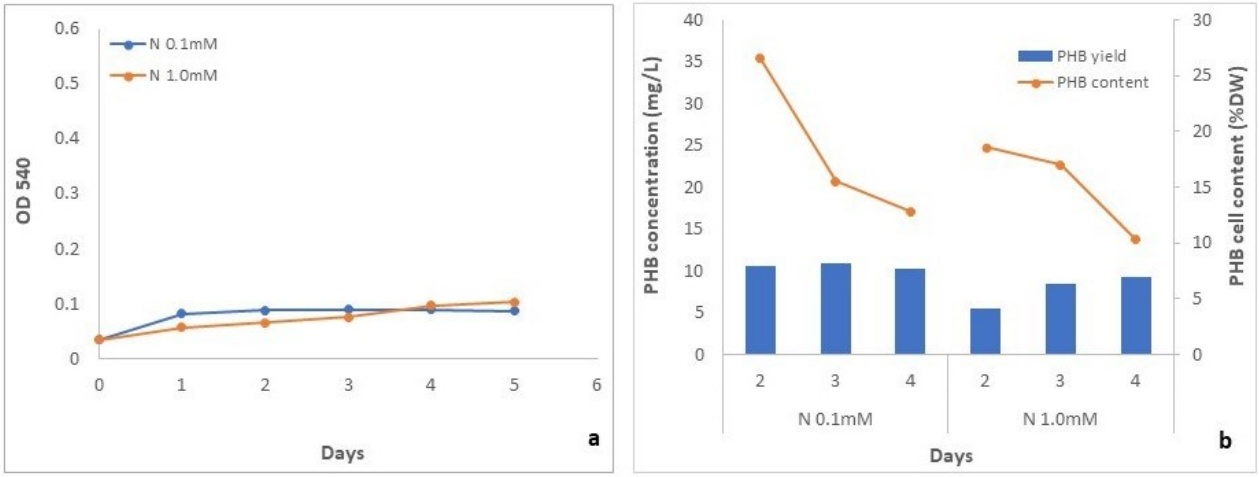

Fig. 5.1. Growth and PHB yield of *Methylocystis* sp. Rockwell in batch cultivation using 15 mM methanol and 0.1 mM or 1.0 mM of ammonium (Table 5.1, expt. run 1a and 1b). Bacterial growth in batch culture (a), PHB production in batch culture (b).

In experimental runs 1a (0.1 mM ammonium), PHB concentration remained consistent (about 10 mg/L) between days 2-4 (Fig. 5.1b). However, in runs 1b (1 mM ammonium), the PHB concentration increased from day 2 (5.57 mg/L) and eventually reached 9 mg/L (Fig. 5.1b), a level similar to that observed in the culture with 0.1 mM ammonium on day 4. Furthermore, the PHB

cell content was highest on the second day of cultivation (26.49% for 0.1 mM ammonium; 18.58% for 1.0 mM ammonium), but gradually decreased over time in both conditions (Fig. 5.1b). The PHB concentration and cell content were dependent on the ammonium concentration, with higher ammonium concentration (1.0 mM) resulting in lower PHB production compared to the culture with lower ammonium concentration (0.1 mM).

5.4.2. Methanol toxicity threshold of *Methylocystis* sp. Rockwell in fed-batch cultivation

Since higher concentration of methanol is toxic to *Methylocystis* sp. Rockwell (Tays et al., 2018), fed-batch cultivation was employed (expt. run 2a and 2b) to detect the methanol toxicity threshold of *Methylocystis* sp. Rockwell. A continuous adapted pulse feeding of methanol was used with an initial pulse to 1 mM with gradual increase up to 22.5 mM per day with simultaneous pulse feeding of either 0.1 mM (runs 2a) or 1 mM of ammonium (runs 2b). Feeding with 1 mM ammonium resulted in more robust bacterial growth than with 0.1 mM ammonium. The highest OD values were observed on day 9 (20 mM methanol pulse addition) regardless of nitrogen concentration (0.897 for 0.1 mM ammonium and 1.069 for 1 mM ammonium). A sharp decline in OD was observed on day 10 with the addition of 22.5 mM methanol on day 9 in either AMS concentration (Fig. 5.2a), indicating the threshold of methanol toxicity (Tays et al., 2018).

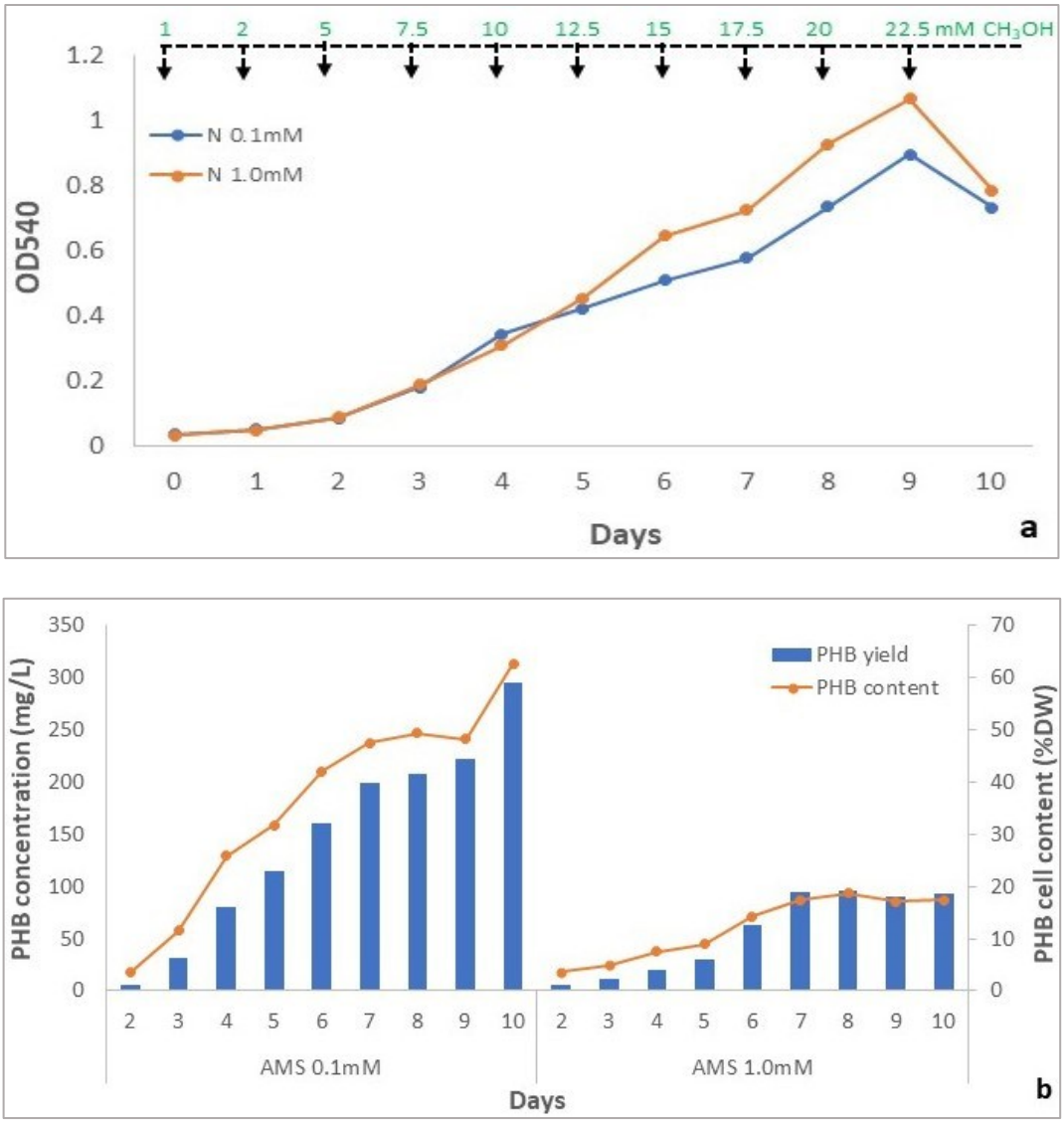

Fig. 5.2. Determination of methanol toxicity threshold showing growth of *Methylocystis* sp. Rockwell (a) and PHB production (b) in fed-batch process (Table 5.1, expt. run 2a and 2b). The numbers and arrows pointing from the top indicate the concentration of methanol pulse feeding.

The methanol fed-batch process with 0.1 mM or 1 mM of ammonium for toxicity threshold detection showed a steady increase in PHB production and became stable at approximately 200 mg/L with PHB cell content of 48% for 0.1 mM ammonium and at approximately 95 mg/L with 18% PHB cell content for 1.0 mM ammonium after day 7. Predictably, PHB production was higher

when using ammonium at 0.1 mM compared to 1 mM (Fig. 5.2.b) as nitrogen limitation is known to stimulate PHB production in bacteria (Pieja et al., 2012; Bordel et al., 2019). Ammonium was completely consumed every 24 hours (Supplementary Fig. C-2b) in 0.1 mM ammonium (runs 2a), resulting in higher PHB production. However, ammonium was not completely consumed in the 1.0 mM ammonium (runs 2b), resulting in ammonium accumulation over time (Supplementary Fig. C-2c) and low PHB production. A sharp increase in PHB production was observed on day 10 in bioreactors maintained with 0.1 mM ammonium (294.94 mg/L with cell content of 62.75%) that requires further confirmation (Fig. 5.2b). These findings indicate that AMS feeding at 0.1 mM is suitable for enhancing PHB production in *Methylocystis* sp. Rockwell.

5.4.3. Growth and PHB yield of *Methylocystis* sp. Rockwell in fed-batch operation with methane

The fed-batch cultivation with initial ammonium concentration of 0.1 mM (expt. runs 3a) and 1.0 mM ammonium (expt. runs 3b) were conducted using continuous methane flow (0.077 slpm) and air sparging until the stationary phase was achieved. These fed-batch experiments achieved stationary phase at 48 h in bioreactor with 0.1 mM of ammonium whereas a higher ammonium concentration (1.0 mM) sustained the growth of the culture over 120 h. The final OD values were 0.251 and 0.583 in bioreactors containing 0.1 mM and 1.0 mM ammonium (Fig. 5.3a) with corresponding final biomass contents of 250 mg/L and 420 mg/L, respectively, measured on day 6. The observed difference in growth and biomass production could be attributed to the increased availability of nitrogen in the culture medium.

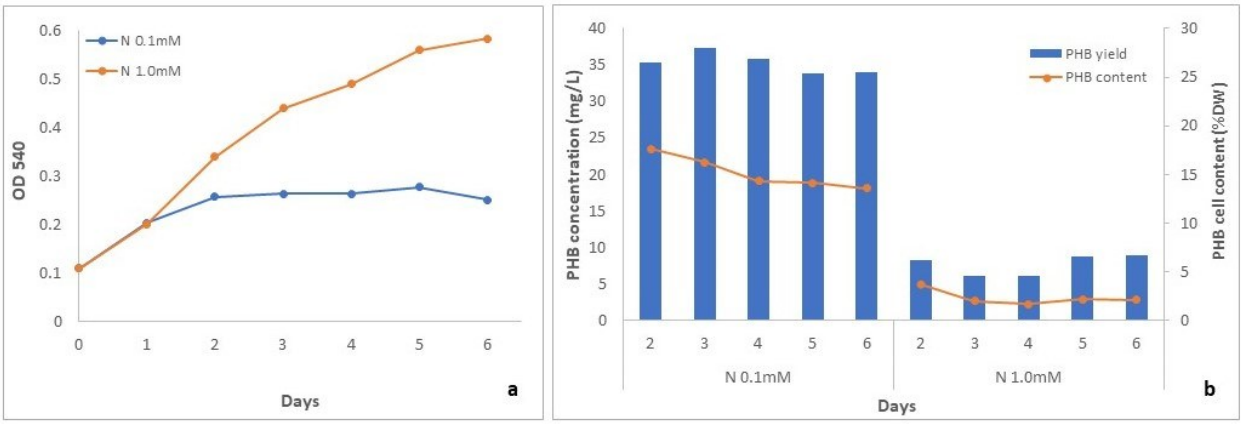

Fig. 5.3. Growth and PHB yield of *Methylocystis* sp. Rockwell in fed-batch cultivation using continuous methane flow and 0.1 mM or 1.0 mM of ammonium (Table 5.1, expt. run 3a and 3b). Bacterial growth in fed-batch culture (a), PHB production in fed-batch culture (b).

While cultivating *Methylocystis* sp. Rockwell on continuous methane flow fed-batch mode, the PHB concentration remained relatively consistent from day 2 onwards (about 35 mg/L) over experimental duration and was nearly 5 folds higher in 0.1 mM ammonium than 1.0 mM ammonium (Fig. 5.3). The highest PHB cell content of 17.64 % was measured on day 2, and gradually decreased from this point on to reached 13.59 % for 0.1 mM ammonium – a value still 6 folds higher than PHB content for 1.0 mM on day 6 (Fig. 5.3b). Based on these results, further methane flow fed-batch process was conducted with pulse feeding of low ammonium (0.1 – 0.2 mM) to improve the PHB production while sustaining the bacterial growth.

5.4.4. Growth and PHB yield of *Methylocystis* sp. Rockwell in fed-batch cultivation with different carbon sources and ammonium feedings

To determine the suitable carbon source for improving biomass and PHB production from *Methylocystis* sp. Rockwell in fed-batch cultivation, further expt. run 4, 5 and 6 were conducted with continuous feeding of methane, pulsed feeding of methanol, and a combination of continuous

methane and pulsed methanol feeding, respectively (Table 5.1). The fed-batch process involved multiple runs, each run was supplemented with an initial pulse of ammonium to 0.1 mM, increasing to 0.2 mM ammonium to maintain a lower N:C ratio for biomass and PHB production (Sharma et al., 2022).

In a separate experimental run with 0.1 mM of ammonium and 15 mM methanol pulse fed on day 6 resulted in peak PHB productivity on day 7 (Supplementary Fig. C-3). This level of methanol pulse was maintained between days 6 and 9, after which methanol pulse feed was increased to 17.5 mM until day 17, not exceeding the threshold limit of methanol toxicity. While the lag phase in methanol-only feeding was 48 h longer than with methane-only feeding, continuous growth was observed over the duration of the experiments in either methanol-only (expt. runs 5) or methane-grown cultures with methanol pulse feeding (expt. runs 6). There was a steady increase in growth and no signs of stationary phase or sharp decline in OD under methanol-only and methane + methanol feeding, with final ODs of 2.05 ± 0.01 and 1.897 ± 0.08, respectively, on day 17. On the other hand, faster cell growth was observed in methane-only feeding within 24 h; biomass increased to OD of 1.2 ± 0.10 to attain a stationary phase on day 13 and a final OD of 1.353 ± 0.14 achieved on day 17 (Fig. 5.4a).

Compared to the methanol batch mode with 0.1 mM ammonium (expt. runs 1a), the fed-batch mode with methanol-only (expt. runs 4) achieved 25-fold higher final OD, the methane + methanol combination (expt. runs 6) achieved 24-fold higher final OD, and methane-only (expt. runs 5) achieved 17-folds higher final OD. Although there was no significant difference in final biomass yield among methanol-only (1,140 ± 42.42 mg/L) and methane + methanol combination (1,135 ± 63.63 mg/L) on day 17, the biomass production was lower in methane-only conditions (870 ± 212 mg/L) (Fig. 5.4b). The final OD values for methanol fed-batch bioreactors (expt. run 5

and 6) were significantly ($p < 0.05$) higher than the fed-batch with methane-only feeding (expt. runs 4), indicating that methanol fed-batch is a suitable bioprocess for the growth of *Methylocystis* sp. Rockwell.

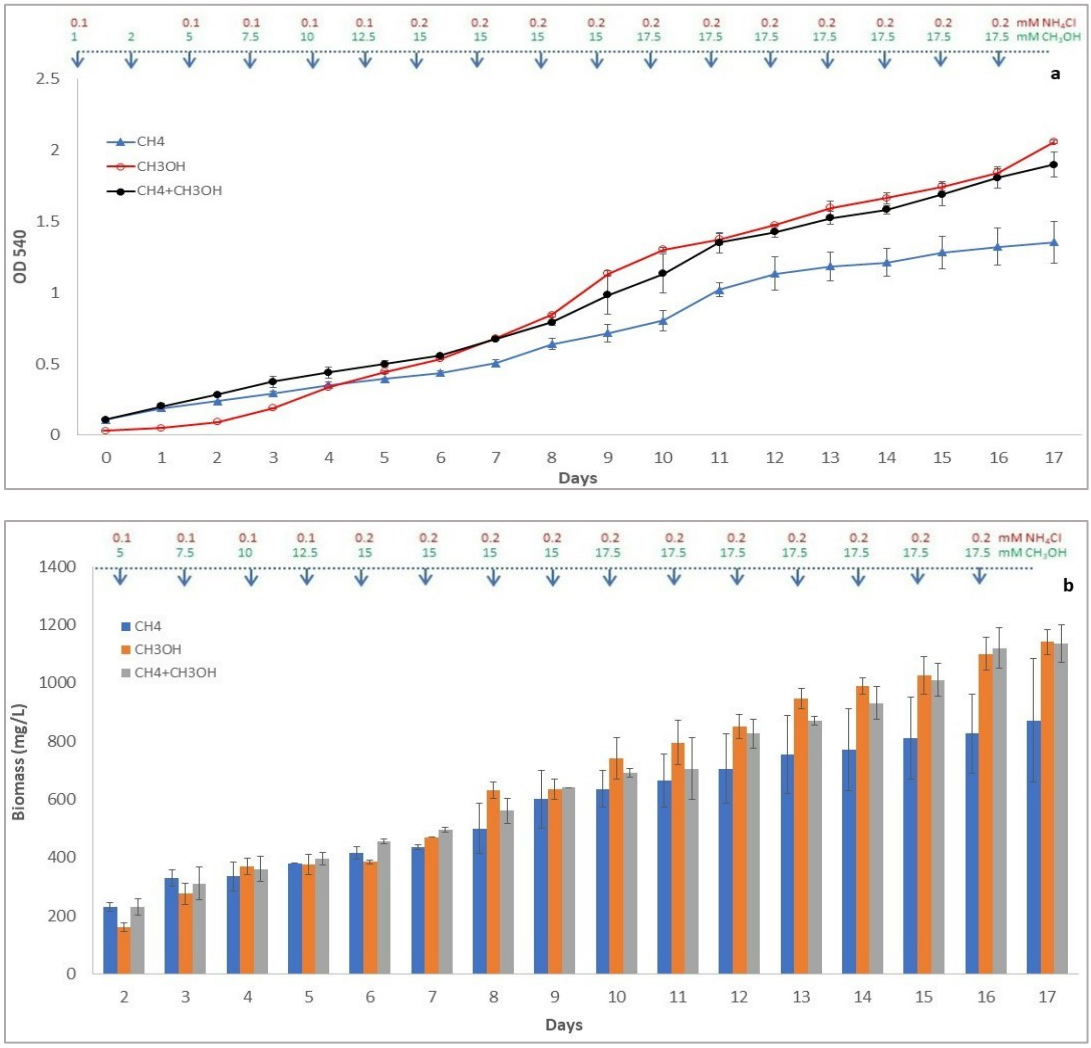

Fig. 5.4. Comparison of carbon sources and ammonium feeding for growth (a) and biomass production (b) of *Methylocystis* sp. Rockwell grown in fed-batch operation. Experiments were conducted using continuous flow of methane in methane-only fed-batch (expt. runs 4), and pulse feeding of methanol in methanol-based fed-batch (expt. runs 5), and a combination of continuous feeding of methane and pulsed methanol feeding (expt. runs 6) with 0.1-0.2 mM of ammonium feeding every day for all carbon combinations. The numbers and arrows pointing from the top indicate the concentration of AMS and methanol pulse feeding. Error bars represent standard deviation (n = 2).

The growth rate analysis of fed-batch cultivation with methane + methanol combination and methane-only experiments showed a decreasing trend in specific growth rate, whereas the methanol-only condition showed an initial increase in the specific growth rate over the first three days (Fig. 5.5). After six days, the specific growth rate remained consistent across all conditions, suggesting that nutrient availability remained consistent.

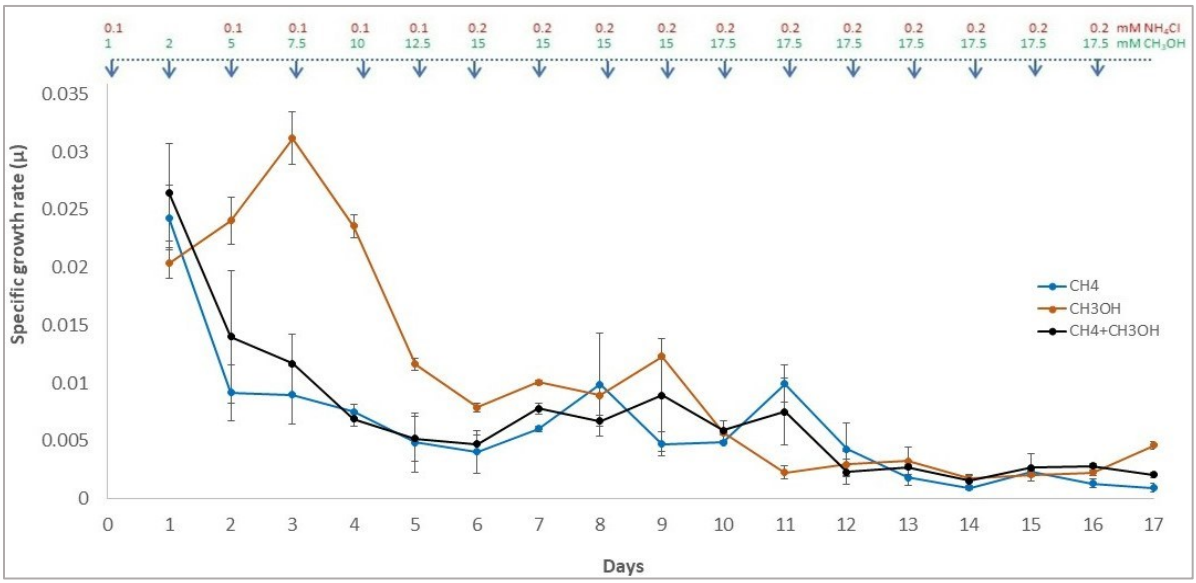

Fig. 5.5. Specific growth rate of *Methylocystis* sp. Rockwell in methane-only fed-batch and methanol-based fed-batch cultivation with 0.1-0.2 mM of ammonium feeding every day in all carbon combinations. Methane-only fed-batch (expt. runs 4) utilized continue flow of methane while methanol-based fed-batch (expt. run 5 and 6) used pulse feeding of methanol. The numbers and arrows pointing from the top indicate the concentration of AMS and methanol pulse feeding. Error bars represent standard deviation (n = 2).

Since higher PHB production was achieved at the lower ammonium concentration, further experiments were conducted using 0.1 and 0.2 mM of ammonium feeding. While the increase from 0.1 to 0.2 mM ammonium in the middle of experiment provided additional nitrogen to fulfill the demand from increasing cell density, complete consumption of ammonium was observed in all of

the fed-batch runs 4, 5 and 6 supplemented with 0.1 or 0.2 mM of ammonium feeding (Supplementary Fig. C-2b,d,e,f), resulting in higher PHB production.

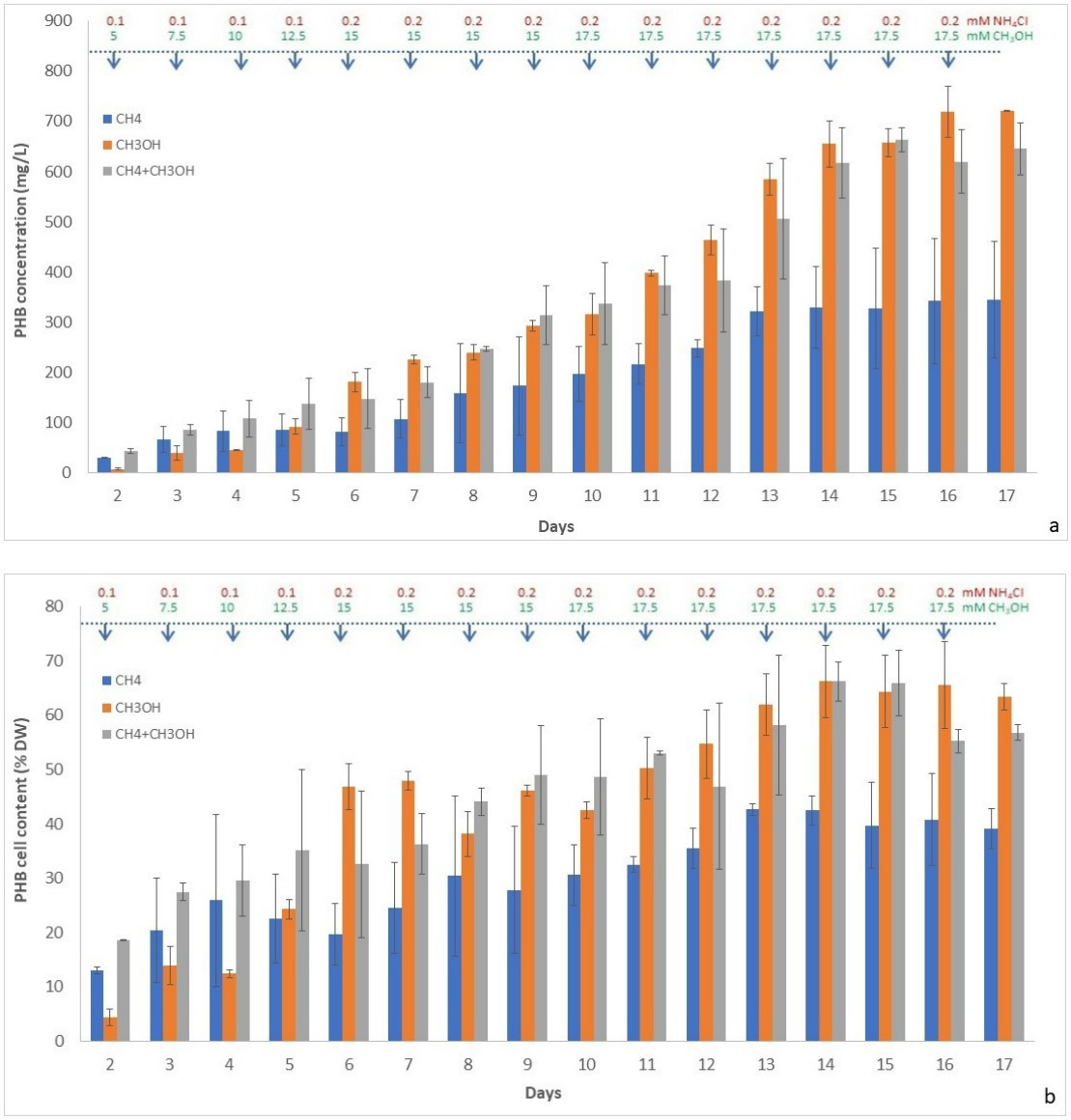

Fig. 5.6. PHB concentration (a) and PHB cell content (b) of *Methylocystis* sp. Rockwell in methane-only fed-batch and methanol-based fed-batch cultivation with 0.1-0.2 mM of ammonium feeding every day in all carbon combinations. Methane-only fed-batch (expt. runs 4) utilized continue flow of methane while methanol-based fed-batch (expt. run 5 and 6) used pulse feeding of methanol. The numbers and arrows pointing from the top indicate the concentration of AMS and methanol pulse feeding. Error bars represent standard deviation (n = 2).

All carbon-feeding conditions (methane-only, methanol-only and methane + methanol) showed a continued increase in PHB production. A maximum, stable PHB concentration was achieved on day 14 at 654.56 ± 47.04 mg/L for methanol-only and 622.05 ± 35.98 mg/L for methane + methanol. These PHB concentrations in methanol-fed bioreactors were significantly higher than bioreactors fed with methane-only (328.85 ± 80.93 mg/L) ($p < 0.05$) (Fig. 5.6a). Similarly, the PHB cell content of 66.21 ± 6.64% for methanol-only and 66.24 ± 3.58% seen on day 14 for methane + methanol fed-batches were significantly higher ($p < 0.05$) than methane-only (42 ± 2.71%) fed-batch mode (Fig. 5.6b). These findings suggest that the methanol fed-batch process enhances the production of PHB by *Methylocystis* sp. Rockwell under low nitrogen.

5.4.5. Overall productivity with different carbon sources and ammonium feedings

The overall biomass and PHB productivity (mg/L/day) were calculated for batch and fed-batch experiments. In the methanol batch operation (expt. runs 1a), the highest biomass productivity of 20 mg/L/d and PHB productivity of 5.3 mg/L/d was observed on day 2 with 0.1 mM of ammonium (Supplementary Fig. C-4). In the methanol-only (expt. runs 5) and methane + methanol (expt. runs 6) fed-batch processes, the productivity of biomass was higher at the beginning of the run, gradually decreased and remained stable at approximately 75 mg/L/d after day 5. However, the methane-only (expt. runs 4) condition achieved a lower productivity of approximately 60 mg/L/d after day 5. (Fig. 5.7a).

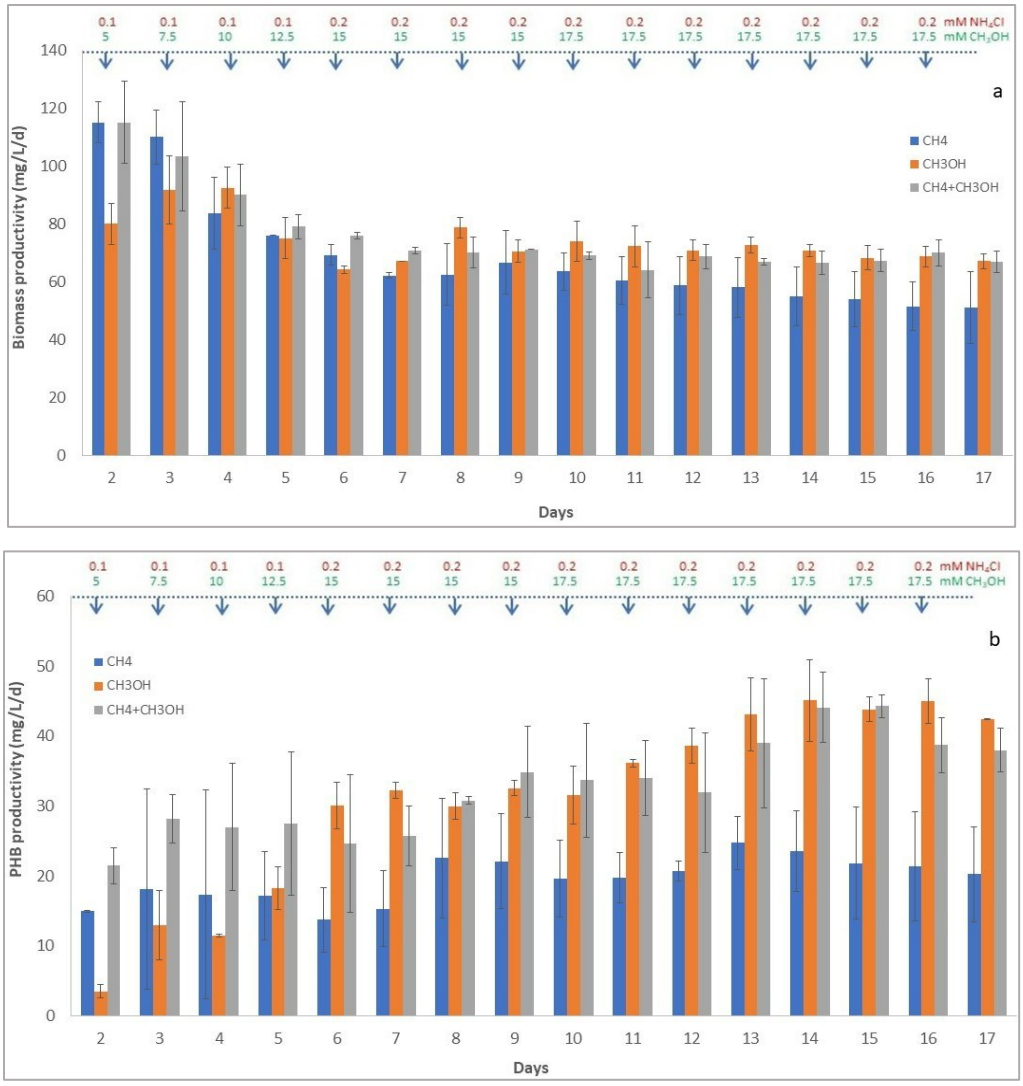

Fig. 5.7. Overall productivity of biomass (a), and productivity of PHB (b) using methane-only fed-batch and methanol-based fed-batch cultivation. Methane-only fed-batch (expt. runs 4) utilized continue flow of methane while methanol-based fed-batch (expt. run 5 and 6) used pulse feeding of methanol with 0.1-0.2 mM of ammonium feeding every day. The numbers and arrows pointing from the top indicate the concentration of AMS and methanol pulse feeding. Error bars represent standard deviation (n = 2).

PHB productivity increased up to 14 days and remained stable thereafter in all fed-batch processes (expt. run 4, 5 and 6) (Fig. 5.7b). The PHB productivity of 5.29 mg/L/d in methanol batch cultivation with 0.1 mM ammonium (expt. runs 1a) was nearly 9-fold lower than for the

methanol fed-batch cultivation (expt. runs 5). Moreover, the highest PHB productivity was reached at day 14 for methanol-only (45.05 ± 5.77 mg/L/d) and methane + methanol (44.07 ± 5.06 mg/L/d). Both values were significantly higher ($p < 0.05$) than for methane-only runs (23.49 ± 5.78 mg/L/d). The results imply that methanol supplemented conditions are better suited for PHB production in *Methylocystis* sp. Rockwell than the methane-only condition.

5.5. Discussion

Conversion of methane or methanol into biomass and PHB by *Methylocystis* sp. Rockwell is complex and depends on various factors including methanol toxicity, nitrogen and carbon availability, oxygen availability, and reactor configuration (Zaldívar Carrillo et al., 2018; Sharma et al., 2022; Tays et al., 2018). By understanding these factors, it is possible to optimize bioprocess design. Compared to methane as a feedstock, methanol is readily available to the culture and does not incur mass transfer limitations. However, methanol toxicity can be problematic for designing an appropriate bioprocess. Thus, we first conducted a fed-batch experiment by gradually increasing the daily methanol feed from 1 mM to 22.5 mM to determine the toxicity threshold level for methanol with pulse feeding (Fig. 5.2). As the poor solubility and mass transfer limitation of gaseous substrates (methane and oxygen) are major constraints for methanotrophic bioprocesses (Yasin et al., 2015; Gęsicka et al., 2021), we increased the gas flow into the system and adjusted the reactor configuration by using baffles and agitating at 300 RPM.

The proportion of available nitrogen and carbon is another component that directly impacts production. A high N:C ratio resulted in improved bacterial growth as reflected by the higher OD values with 1 mM ammonium compared to a low N:C ratio with 0.1 mM ammonium, but PHB production was compromised with higher ammonium concentrations, a situation also observed by

Lazic et al. (2021). Both PHB concentration and PHB cell content were higher for the 0.1 mM ammonium than the 1 mM ammonium condition in batch (Fig. 5.1b) and fed-batch cultivation (Fig. 5.2b). Unsurprisingly, complete consumption of ammonium with 0.1 mM ammonium and incomplete consumption with 1 mM ammonium was observed in methanol-grown batch cultures. Approximately 200 µM ammonium remained after the initial 24 hours and approximately 120 µM were maintained until harvesting on day 4, indicating incomplete use of the nitrogen source (Supplementary Fig. C-2a). For the methanol fed-batch experiment (runs 2b), daily addition of 1 mM ammonium resulted in accumulation of ammonium over time (Supplementary Fig. C-2c) with low PHB production (Fig. 5.2b), whereas addition of 0.1 mM ammonium (expt. runs 2a) showed complete consumption of ammonium (Supplementary Fig. C-2b) with improved PHB production (Fig. 5.2b). While a continuous air supply and agitation can improve the mass transfer and availability of oxygen, and reduce growth barriers (Tays et al., 2018; Zhong, 2010), incomplete use of ammonium was an indication of the high N:C ratio which was good for biomass but not PHB production (Tays et al., 2018; Zaldívar Carrillo et al., 2018; Sharma et al., 2022). In contrast, a low N:C ratio was maintained with 0.1 mM or 0.2 mM ammonium feeding, where complete consumption of ammonium favored higher PHB accumulation (Pieja et al., 2012; Bordel et al., 2019), regardless of operation mode (Supplementary Fig. C-2b,d,e,f).

Fed-batch experiments with increasing daily methanol concentration indicated a methanol toxicity threshold of 22.5 mM and a suitable nitrogen level to maintain a low N:C ratio. Thus, fed-batch experiments with daily feeding of 0.1 to 0.2 mM of ammonium with continuous air flow (0.177 slpm) were conducted to ensure there was low ammonium availability and reduce oxygen limitation to the system. As expected, daily addition of ammonium of 0.2 mM per day from day 6 to day 17 supplied sufficient nitrogen to improve biomass and PHB production. This adjustment

showed positive results as OD steadily increased without reaching stationary phase (Fig. 5.4a) with a simultaneous improvement in PHB production (Fig. 5.6). These experiments validated the hypotheses from Chapter 3 and 4 of this thesis that *Methylocystis* sp. Rockwell can produce PHB while growing under nitrogen limitation conditions. Relatively steady specific growth rate of *Methylocystis* sp. Rockwell (Fig. 5.5) observed after six days indicates a sufficient availability of substrates to sustain growth (Fink et al., 2023), whereas PHB accumulation is associated with nitrogen deprivation, which was reflected by higher PHB concentration and cell content with low ammonium (0.1 – 0.2 mM) availability in the medium (Fig. 5.6).

Previous studies demonstrated the PHB production from methane using different cultivation mode and feeding strategies. For example, a bubble column bioreactor (2.5 L) was used for batch cultivation of *Methylocystis hirsute*, with internal gas recycling and a methane feed of 4% (v/v) resulted in 34.6% of PHB production (García-Pérez et al., 2018), which was lower then PHB content (42 ± 2.71%) observed for *Methylocystis* sp. Rockwell in continuous methane fed bioreactors (Fig. 5.6b). In another study, a batch cultivation of *Methylocystis* sp. GB 25 DSMZ 7674 in pressure bioreactor using methane as a carbon source produced the PHB content of 51% in two step process of continuous growth followed by accumulation of PHB under nutrient deficient conditions (Wendlandt et al., 2001). However, *Methylocystis* sp. Rockwell in fed-batch cultivation with continuous methane flow produced a lower PHB content (42 ± 2.71%), which could be associated with low solubility of gaseous substrates and higher oxygen demand for methane oxidation (Steinle et al., 2017; Yasin et al., 2015; Gęsicka et al., 2021). Another high cell density cultivation of *Methylocystis hirsuta* DSMZ 18500 in a loop bioreactor containing 1:1 ratio of methanol:ethanol achieved 4.5 g/L of dry cell biomass with PHB content of 85% (w/w). Furthermore, an addition of phosphorus (1.05 g/L) and magnesium (5 g/L) in a bubble

column bioreactor supplemented with methane resulted in higher biomass yield (8 g/L) with PHB content of 73.4% (w/w) (Ghoddosi et al., 2019). These results suggest that growth and PHB synthesis are influenced by various factors besides the availability of carbon and nitrogen, such as effect of other elements or chemicals, strain selection and appropriate process design. While some of earlier research has reported greater biomass and PHB output using distinct strains and operational modes, this study stands out due to its selection of species, process design, and nutrient feeding strategy, which yielded higher levels of both biomass and PHB production from *Methylocystis* sp. Rockwell in methanol fed-batch bioreactors. The fed-batch process of methane + methanol combination resulted in significantly higher production of PHB (622.05 ± 35.98 mg/L) than methane-only (328.85 ± 80 mg/L) condition on day 14 ($p < 0.05$). Higher biomass and PHB production under fed-batch cultivation with methane + methanol combination was in agreement with higher PHB yield of *Methylosinus trichosporium* OB3B grown in combination of methane and methanol in batch culture (Zaldívar Carrillo et al., 2018). Zhang et al., (2008) also observed an improved PHB production (0.6 g/L) by *Methylosinus trichosporium* IMV3011 when methanol was added to a methane-grown culture. The fed-batch operation with methanol-only feeding also showed a similar level of biomass and PHB production of *Methylocystis* sp. Rockwell with no significant difference with the methane + methanol combination feeding. This finding indicates that cells grown on methanol can fix carbon more rapidly likely due to the absence of MMO as a consumer of reducing equivalents. Consequently, they reach the nitrogen limitation faster, which leads to a shift in their metabolism toward a stress-related pathway favouring PHB production, an observation consistent with the results seen by Lazic et al. (2021).

Although additional research is necessary to recommend a suitable scale-up strategy for industrialization, the results of this study and the overall performance observed suggest that the

methanol supplemented fed-batch process is a better feeding strategy than continuous methane feeding and methanol batch operations in terms of utilizing C1 substrates for higher biomass and PHB production by *Methylocystis* sp. Rockwell. Additionally, it is important to evaluate the system requirements, such as the liquid volume to headspace ratio, vessel dimensions, and the ratio of impeller diameter to bioreactor, among other factors, before developing a suitable feeding regimen and proposing any scaleup strategies for industrialization (See chapter 6) (Lim & Shin, 2013; Mahdinia et al., 2019).

5.6. Conclusion

This study was focused on biomass and PHB production by *Methylocystis* sp. Rockwell comparing the batch and fed-batch operations with ammonium as the nitrogen source and C1 compounds (methane and/or methanol) as the carbon substrate. This study pinpoints the methanol toxicity threshold limit for *Methylocystis* sp. Rockwell and highlights that methanol-based fed-batch under high aeration and low ammonium concentration can improve biomass and PHB production compared to continuous methane-only feeding. While further studies are required to propose a suitable scale-up strategy, the findings of this work provide an initial framework and direction for future work.

5.7. References

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CHAPTER 6: Conclusion and future directions

6.1. Summary of this study

Synthetic polymers have long benefited our lives, but with rapid industrialization and human activities have led to a dramatic surge in plastic pollution, increasing 20-folds in the past 50 years (Walker & Fequet, 2023). A massive production of plastic material and its improper disposal results in their accumulation in landfills and natural environments, thereby releasing toxic chemicals into the environment and posing a severe threat to wildlife and human well-being. Additionally, the plastic manufacturing process heavily relies on non-renewable resources, and the anthropogenic activities such as mining of fossil fuels, waste management, and agriculture practices further exacerbating climate change through greenhouse gas emissions (US EPA, 2023). Besides these, an extraction of oil and gas, waste from paper mill and wood industries contribute to the methanol production and release into the environment process (Zhu et al., 2000). Therefore, exploring alternative resources and adopting eco-friendly technologies are crucial steps in mitigating these environmental challenges. For this purpose, the methanotroph biotechnology has garnered considerable attention over the years as it has the potential to address greenhouse gas emissions, methanol waste and petroleum-based plastic pollution, while also producing valuable products (e.g. platform chemicals, biofuels, and biopolymers). Single-carbon (methane and methanol) bioconversion to biopolymer, polyhydroxybutyrate (PHB) production using alphaproteobacterial methanotrophs could be a promising solution, as it can offer a non-toxic, biocompatible, and biodegradable plastics, as an alternative to petroleum-based synthetic plastics. The carbon source for PHB production can be obtained from methane produced in landfills or wastewater treatment plants, as well as from methanol waste generated by chemical or wood industries. By adopting this approach, we can simultaneously address the issue of methane

emissions and methanol waste, thereby effectively reducing the carbon footprint. Additionally, this process establishes a closed-loop cycle, as the PHB can eventually decompose into methane at the end of its life cycle.

While the metabolic pathway of methane oxidation, carbon assimilation and PHB biosynthesis have been well understood, adopting methanotroph biotechnology for PHB production is facing huge challenges due to the mass transfer limitation, low solubility of gaseous substrates, lack of information on bioreactor design and species-specific nutrient preferences for growth and PHB production. This necessitates a strain-by-strain optimization of various physiochemical parameters to develop an appropriate feeding strategy for scale-up PHB production. Given these considerations, this thesis primarily focused on to understand the bacterial response to various environmental conditions and determine the optimal nutrient parameters for developing effective feeding strategies so as to enhance the biomass and PHB production from the alphaproteobacterial methanotroph *Methylocystis* sp. Rockwell. We explore various nutrient combinations and cultivation modes for bioconversion of methane and/or methanol using ammonium or nitrate mineral salt (AMS or NMS) medium, in order to identify conditions that promote both biomass and PHB production across different scales. By comprehending the scalability of this process, we can determine the feasibility of utilizing methanotrophs for PHB production as a viable alternative to synthetic plastics.

Important results from the third chapter of thesis were confirming that *Methylocystis* sp. Rockwell prefers ammonium rather than nitrate as its N-source (Tays et al., 2018), which contradicts the conventional belief that ammonium can impede growth of methanotrophs compared to nitrate (Nyerges et al., 2010; Nyerges & Stein, 2009; Whittenbury, Phillips, et al., 1970). A multi objective optimal N:C ratio of 0.016 was found to balance the higher biomass and

net PHB production when *Methylocystis* sp. Rockwell is cultivated in batch on methane and either nitrate or ammonium as the N-source. Nevertheless, due to the fact that both the growth and PHB production exhibited greater results when using AMS as compared to NMS, the subsequent investigation was narrowed down to solely examining ammonium as the nitrogen source. In the fourth chapter of this thesis, it was determined that the metabolic state of inocula and its subsequent culture environmental conditions can affect the cycle of PHB production and degradation over time. This study demonstrated that cultures initiated with a well-fed inoculum can continue to grow and accumulate PHB under nitrogen limitation in further cultivation, but that cultures initiated with N-starved inocula were unable to grow and produce PHB. Furthermore, this study showed that starved inocula containing high PHB amounts have a much longer lag phase when recovering from starvation, indicating that the starved state is not ideal for continued cultivation. This chapter provides fundamental insights indicating *Methylocystis* sp. Rockwell accumulate PHB while growing but initiates PHB degradation after reaching the stationary growth phase or experiencing nutrient starvation. Therefore, by utilizing well-fed inocula and employing a bioreactor setup that ensures continuous carbon supply with ample aeration, it becomes feasible to prolong the growth phase and achieve substantial PHB production in a single-step process when cultivating *Methylocystis* sp. Rockwell in an ammonium medium while maintaining a lower N:C ratio. In the fifth chapter of this thesis, we incorporate the findings from chapter 3 and chapter 4 to design the bioreactor experiments and tested different feeding strategies where we found *Methylocystis* sp. Rockwell accumulate PHB while growing where low ammonium feeding resulted in complete N-consumption thereby higher PHB production. Methanol-based fed-batch operation with high aeration and maintenance of a low ammonium concentration was found to provide the best strategy to achieve both high biomass and PHB over time. This result is contrary to a prior study (Tays et

al., 2018) indicating that *Methylocystis* sp. Rockwell is incapable of growth on methanol in the batch culture. Not only was this strain capable of robust growth on methanol, but higher biomass and PHB were achieved in methanol fed-batch operation than in methane fed-batch operation, even with N-limitation. This result expands the range of possible substrates and feeding strategies for PHB production bioprocesses using *Methylocystis* sp. Rockwell. Comparing methanol feeding with methane feeding for PHB production from methanotrophs, methanol feeding provides a more direct and efficient carbon source, as methanol is a readily available substrate which does not require reductant for oxidation. Methane feeding, on the other hand, requires oxygen and reducing equivalents for an additional step of methane oxidation by methanotrophs to convert it into methanol before PHB production can occur (Hanson & Hanson, 1996; Karthikeyan et al., 2015). However, methane is a more abundant and cost-effective carbon source, often readily available from sources like mining of oil and gas, landfills or wastewater treatment plants. Therefore, the choice between methanol feeding and methane feeding depends on factors such as cost, availability, and specific process requirements.

This thesis manuscript provides valuable insights into the potential advantages and obstacles involved in scaling up of methanotrophic bioprocess for the production of biodegradable plastics. The data gathered through this research will assist both academia and industry in devising an efficient and successful bioprocess development for harnessing the potential of methanotrophic bacteria in biodegradable plastics production. I firmly believe that my contributions have advanced our progress in tackling the global repercussions of overreliance on plastics and paving the way for a more sustainable and environmentally conscious approach towards mitigating plastic pollution.

However, numerous unanswered questions persist regarding the physiological aspects of *Methylocystis* sp. Rockwell, which can be further elucidated through investigations into PHB metabolism and the comparison of different cultivation modes, considering both methane and methanol feeding. Analyzing gene expression and metabolite profiles during PHB production under various environmental and cultural conditions can shed light on the activated pathways influenced by these factors, thereby playing a crucial role in microbial physiology research aimed at achieving high PHB yields. Implementing strategies such as modifying key regulatory proteins or genes, optimizing nutrient availability, or manipulating culture conditions can be employed to facilitate PHB accumulation. Furthermore, utilizing an integrated omics analysis to construct a genome-scale metabolic model (GEM) based on these findings can help identify metabolic and regulatory bottlenecks within PHB metabolism, providing a comprehensive approach to enhance PHB production through genetic engineering. Additionally, the GEM approach can offer a deeper understanding of how ammonium as a N-source regulates the metabolic processes and elucidate the fate of electrons produced during ammonium oxidation. Such integrated omics approach and data integration within a GEM framework will provide a comprehensive overview of *Methylocystis* sp. Rockwell physiology which is crucial in terms of establishing this strain as an industrial candidate. Some other future directions/recommendations include but not limited to.

6.2. Future directions

6.2.1. Bioreactor configuration and bacterial cultivation

i. As the mass transfer limitation of oxygen and methane from the gaseous to liquid phase and low gas solubility are major rate-limiting factors of methanotroph bioprocessing, some plausible solutions to these challenges include adjusting the configuration of the bioreactor,

increasing the partial pressure, or adding a mass transfer vector to the system (Sahoo et al., 2021). Using a high-affinity transfer vector such as 10% silicon oil can increase the mass transfer and solubility of gaseous substrates and maximize efficiency of the process. Quijano et al. showed that adding 10% silicon oil as a mass transfer vector resulted in the highest overall transfer of oxygen in both airlift and stirred tank reactors, leading to improved process performance (Quijano et al., 2009). Similar improvements in biomass and PHB production by *Methylocystis* sp. Rockwell could possibly be achieved by adopting similar strategies.

ii. Evaluation of downstream processing – such as extraction, degradation and purification methods – is equally important as optimization and development of scalable process for PHB production. Future research can focus on evaluating downstream processing methods to identify efficient and cost-effective ways for recovering PHB by methanotrophs.

6.2.2. Mode of cultivation and scale-up strategies

i. ***Cyclic fed-batch:*** Here, the microbial culture is allowed to grow in a closed bioreactor with a limited volume of medium. At the end of each growth cycle, a portion of the culture is removed, and the bioreactor is replenished with a fresh batch of nutrient medium. Cyclic fed-batch allows a greater control over the process and can result in higher product yields compared to traditional batch fermentation. While a higher PHB content was observed from *Methylocystis* sp. Rockwell grown in methanol-ammonium media indicating a positive effect of methanol-mediated stress on PHB accumulation (Lazic et al., 2021), a lower biomass production in batch cultivation showed growth inhibition due to methanol toxicity (Tays et al., 2018). Thus, a cyclic fed-batch mode of cultivation is a better strategy than batch operation

in terms of utilizing methanol as a carbon substrate by *Methylocystis* sp. Rockwell. This process can be repeated multiple runs, typically with increasing nutrient concentrations, to allow continued bacterial growth while minimizing the toxic waste accumulation and yielding higher PHB.

ii. ***Self cycling fermentation:*** The self cycling fermentation (SCF) method is a semi-continuous mode of fermentation where microorganisms are grown in a bioreactor with a set volume of nutrient medium. The process involves the periodic removal of exactly half of the working volume when the cells reach stationary phase and replacing it with a same amount of fresh medium. This process allows for the completion of exactly one generation of microbial cell replication during each cycle, and the continuous production of the desired product over multiple cycles. SCF can result in higher product yields compared to traditional batch fermentation methods and is commonly used in the production of biofuels and other high-value chemicals (Tan et al., 2022).

iii. ***Scale up strategies****:* In this thesis, overall productivity of bioprocesses was analysed in terms of biomass and PHB productivity. A scale up strategy can be proposed based on the system requirements and overall performance of the given bioprocess. Following geometric characteristics of the bioreactor vessel, operational conditions and biochemical properties (Table 6.1.) can be considered while proposing and conducting scale up bioprocess for higher PHB production from *Methylocystis* sp. Rockwell.

Table 6.1. Geometric parameters, operational conditions and biochemical properties for scale up bioprocess (Lim & Shin, 2013; Mahdinia et al., 2019).

Parameters consideration for scale up	Formulae	Values
Bioreactor aspect ratio: It is a ratio between height and diameter of the reactor vessel.	$\frac{Hr}{Dr}$	2.33
Headspace ratio: It is the ratio of headspace volume to the container volume	$\frac{(V - Vb)}{V}$	0.333
Media volume ratio: It is the media volume to headspace ratio	$\frac{Vb}{(V - Vb)}$	2
Impeller aspect ratio: It is the diameter of impeller to reactor ratio	$\frac{Di}{Dr}$	0.216
Horsepower: Power need/consumption to rotate two impellers at 300 RPM	$Di^5 \times RPM^3 \times (4.5 \times 10^{-13}) \times I$	79.18×10^{-5}
Reynolds number: It is used to describe the turbulence in the fluid by calculating ratio of inertial forces to viscous forces.	$\frac{\rho . Ni . Di^2}{\mu}$	16895.76
Specific growth rate: It is the number of divisions per cell per unit time.	$\frac{ln\left(\frac{x_2}{x_1}\right)}{(t_2 - t_1)}$	See chapter 5
Productivity: Calculation based on product titer per day.	Concentration per day (mg/L/d)	See chapter 5

D_r: Diameter of bioreactor, D_i: Diameter of impeller, H_r: Height of bioreactor, V: Volume of vessel, V_b: Working volume, RPM: Agitator speed (rotation per minute), I: Constant factor (1.8) for two impellers, ρ: Density of water, Ni: Rotational speed of impeller (in rotations per second, RPS or s^{-1}), μ: Dynamic viscosity of water, x_1: Initial optical density, x_2: Final optical density, t_1: Initial time (h), t_2: Final time (h)

6.2.3. Study of resting bodies

i. Certain microorganisms form the resting bodies as a survival mechanism against unfavorable conditions like nutrient deprivation, desiccation, or heat. Some methanotrophs have been observed to form resting bodies like lipid-cysts or exospores in response to harsh conditions (Titus et al., 1982; Whittenbury, Davies, et al., 1970; Kalyuzhnaya et al., 2019). The ability of *Methylocystis* sp. Rockwell to survive 120 days under nutrient deprivation suggests that it may

form a lipid-cyst under such circumstances (Whittenbury, Davies, et al., 1970). To understand the molecular mechanisms of cyst formation, gene expression profiles of actively growing cells can be compared to those forming cysts. Microscopy and biochemical assays could be used to study the morphology of cells during resting body formation and measure the production of PHB or other metabolites. Such studies could provide insights into the survival strategies of methanotrophs that have applications to biotechnology. For instance, understanding the molecular mechanisms of cyst formation could lead to the development of robust strains of methanotrophs, as well as providing insights into how these organisms survive in harsh environmental conditions.

ii. In the context of detecting *in situ* methanotroph activity in environmental samples, resting bodies can serve as a biomarker, another important application in environmental microbiology and microbial ecology. Their presence and quantification, along with the associated changes in cellular metabolism, provide valuable information about the dormant methanotroph population and their potential for methane oxidation. By studying resting bodies, scientists can gain insights into the dynamics and resilience of methanotroph communities in various ecosystems. Furthermore, the detection of lipid cysts can aid in identifying environments with high methanotrophic activity, which can have implications for bioremediation strategies.

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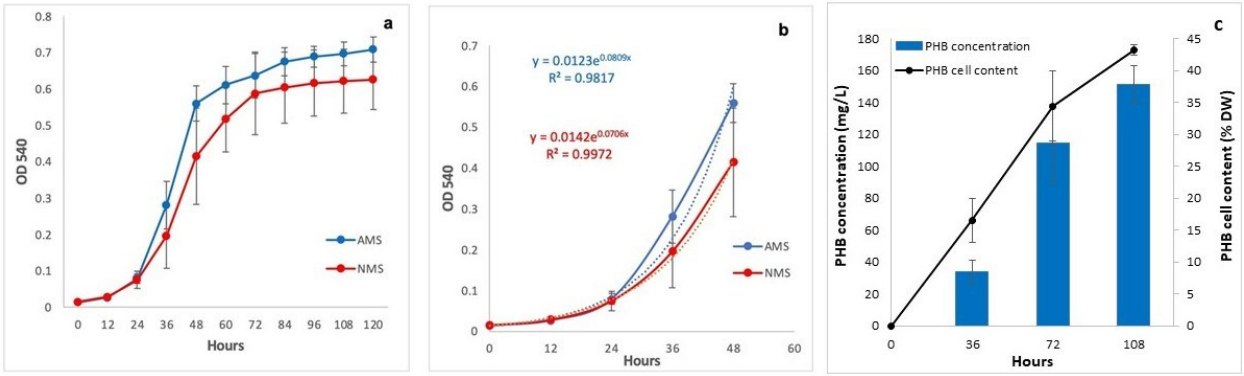
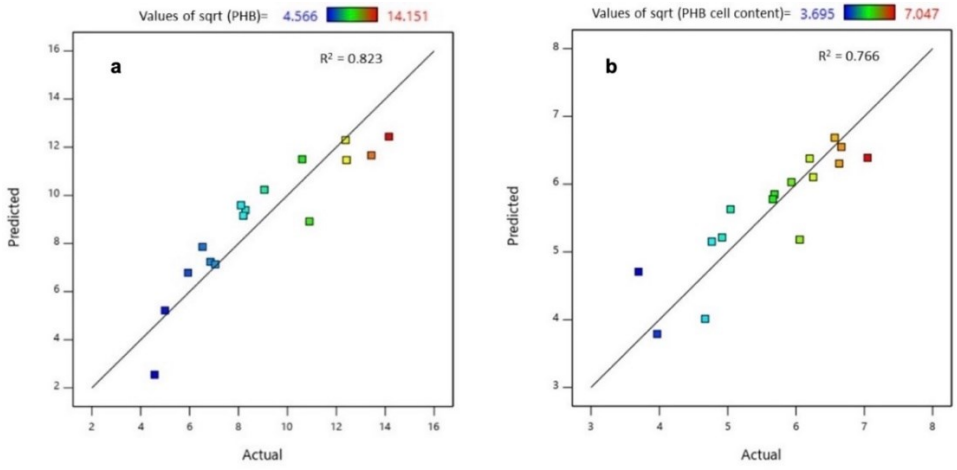
Appendices

A. Supplementary information for chapter 3

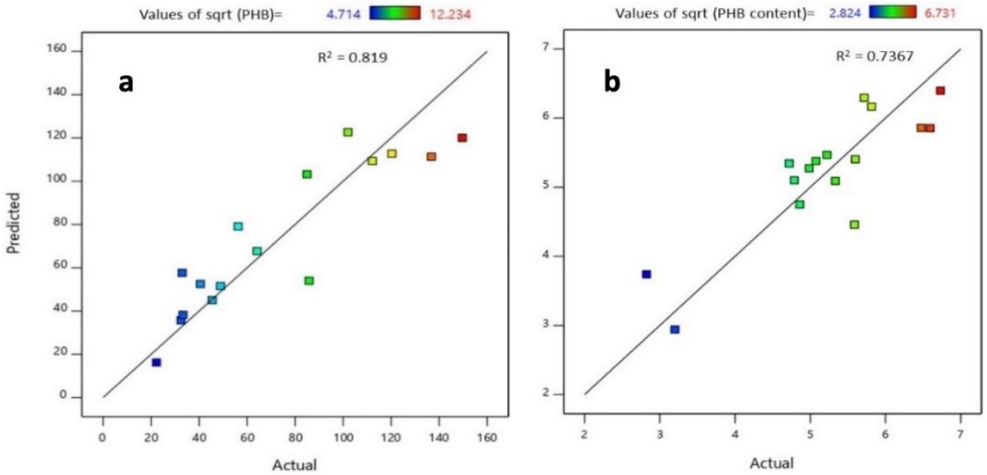
Supplementary Fig. A-1. Growth of *Methylocystis* sp. Rockwell in nitrate (NMS; red) and ammonium (AMS; blue) mineral salts medium provided with 6 mmol methane gas injected into the headspace of a 100-ml culture in a 1-L bottle (a). Dotted trendlines represent the exponential growth phase of the bacteria, and the doubling times were calculated using ln(2)/B, where value of B is obtained from exponential growth equation ($\mathbf{Y = ae^{Bx}}$) (b). Doubling times were calculated as 9.8 h in NMS and 8.5 h in AMS. Error bars represent SD (n = 4). PHB production from *Methylocystis* sp. Rockwell during growth phases (c). Culture was grown in AMS medium provided with 6 mmol CH_4 and an N:C ratio of 0.016; samples (n = 3) were harvested every 36 h for biomass and PHB analysis.



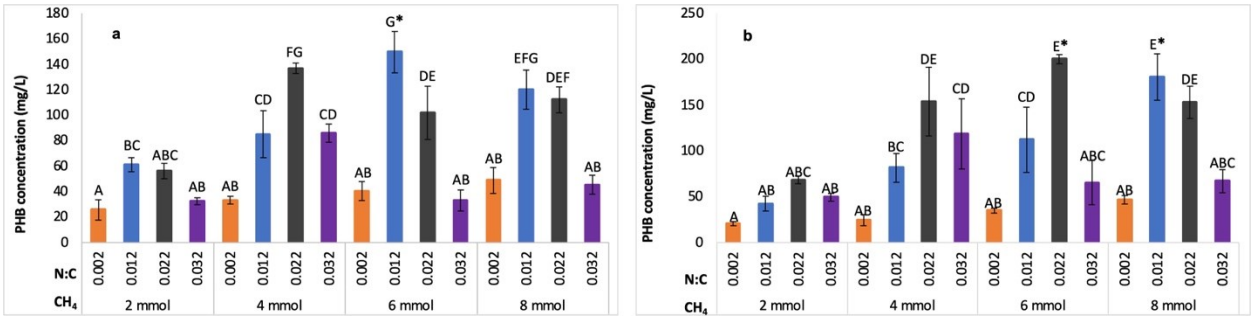
Supplementary Fig. A-2. Comparison of the regression model between predicted and actual values for PHB concentration (a) and %PHB cell content (b) when *Methylocystis* sp. Rockwell cultures were provided with various amounts of methane in AMS media. The colour points represent the square root values. The square root transformation was used to normalize the data, reduce heteroscedasticity and for clearer visualization. The R^2 values are indicators of the fit of the regression model.



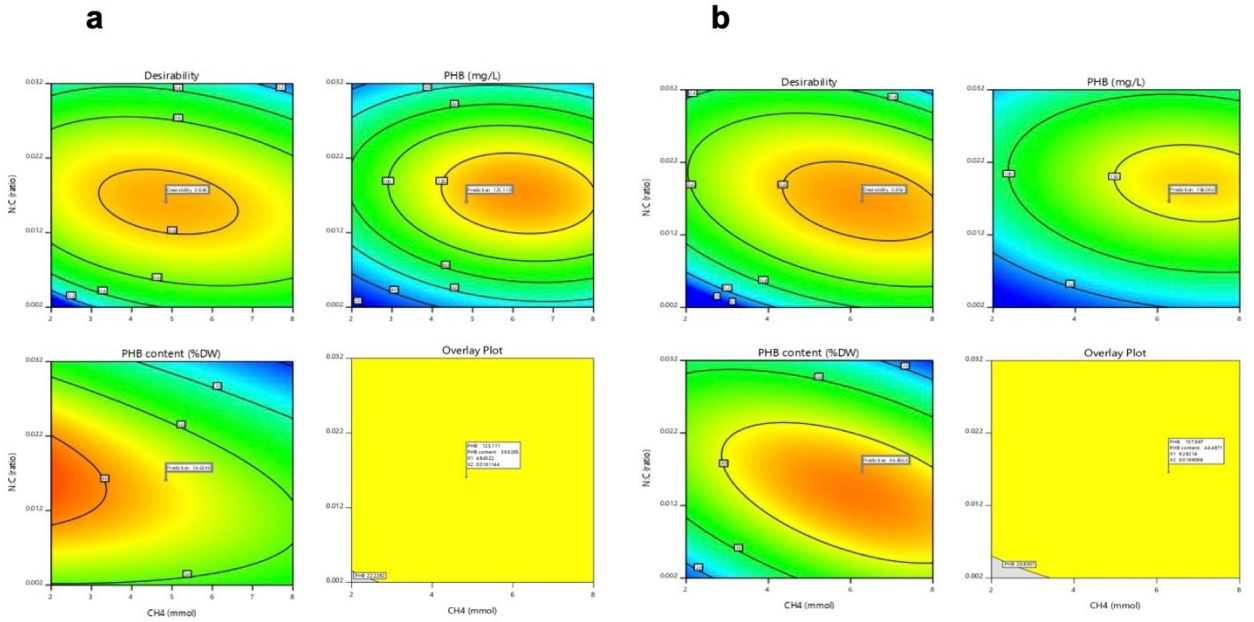
Supplementary Fig. A-3. Comparison of regression models between predicted and actual values for PHB concentration (a) and %PHB cell content (b) when *Methylocystis* sp. Rockwell cultures were provided with various amounts of methane in NMS medium. The colour points represent the square root values. The square root transformation was used to normalize the data, reduce heteroscedasticity and for clearer visualization. The R^2 value represents the fit of the regression model.



Supplementary Fig. A-4. One-way ANOVA followed by post hoc Tukey's test of PHB production. Cultures were grown with the indicated amounts of methane and N:C ratios with either nitrate (a) or ammonium (b) as N-source. Error bars indicate standard deviation ($n = 3$). Conditions such as- 6 mmol methane with N:C of 0.012 for nitrate (a), 6 mmol of methane with N:C of 0.022 and 8 mmol methane with N:C of 0.012 for ammonium (b) showed significantly high levels of PHB were indicated by asterisk sign (*).



Supplementary Fig. A-5. Model prediction for multi-objectives optimal conditions (MOOC) to balance higher PHB yield and %PHB cell content. The model predicted N:C ratio of 0.016 for both nitrate (a) and ammonium (b) as N-sources was subjected to experimental validation.



Supp. Tables A1-A4. Effect of methane amount and N:C ratio on PHB production from *Methylocystis* sp. Rockwell when ammonium is used as the N-source.

Supp. Table A-1. ANOVA for quadratic model in response to PHB concentration. The model F-value of 9.30, R^2 of 0.823 (from Figure A-2a) and $p < 0.05$ implies the model is appropriate. In this case A, B, and B^2, highlighted in bold, are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	112.85	5	22.57	9.30	**0.0016**
A-CH4	25.02	1	25.02	10.31	**0.0093**
B-N:C	23.47	1	23.47	9.67	**0.0111**
AB	2.20	1	2.20	0.9080	0.3631
A^2	4.91	1	4.91	2.02	0.1853
B^2	57.25	1	57.25	23.59	**0.0007**
Residual	24.27	10	2.43		
Total	137.12	15			

Supp. Table A-2. Final equation for sqrt. and quadratic model in response to PHB concentration.

Coded equation	Parameter	Actual equation	Parameter
+12.02		-2.81	Intercept
+1.68	A	+2.20	CH4
+1.62	B	+825.67	N:C
-0.6680	AB	-14.84	CH4 * N:C
-1.25	A^2	-0.14	$CH4^2$
-4.26	B^2	-18915	$N:C^2$

The correlation among the controlled factors (methane amount and N:C ratio) and response measures (PHB concentration and %PHB cell content) for cultures grown with ammonium as N-source are obtained using the regression analysis technique. The equations obtained for the quadratic model using square root transformation are shown in the following equations.

For PHB concentration, the complete regression was:

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_2 \bullet B) + (a_{12} \bullet A \bullet B) + (a_{11} \bullet A^2) + (a_{22} \bullet B^2) \quad (1)$$

$$= -2.81 + (2.20 \bullet A) + (825.67 \bullet B) - (14.84 \bullet A \bullet B) - (0.14 \bullet A^2) - (18915 \bullet B^2)$$

The final regression, taking into consideration the statistical significance of parameters (Table A-2).

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_2 \bullet B) + (a_{22} \bullet B^2) \quad (2)$$

$$= -2.81 + (2.20 \bullet A) + (825.67 \bullet B) - (18915 \bullet B^2)$$

Supp. Table A-3. ANOVA for quadratic model in response to %PHB cell content. The model F-value of 6.55, R^2 of 0.766 (Figure A-2b) and $p < 0.05$ implies the model is appropriate. In this case AB, B^2 highlighted in bold are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	11.46	5	2.30	6.55	**0.0060**
A-CH4	0.25	1	0.25	0.7068	0.4202
B-N:C	0.70	1	0.70	1.97	0.1912
AB	3.82	1	3.82	10.90	**0.0080**
A^2	0.80	1	0.80	2.24	0.1653
B^2	5.92	1	5.92	16.91	**0.0021**
Residual	3.50	10	0.35		
Total	14.96	15			

Supp. Table A-4. Final equation for sqrt. and quadratic model in response to %PHB cell content.

Coded equation	Parameter	Actual equation	Parameter
+6.65		+1.90	Intercept
+0.16	A	+0.94	CH4
-0.28	B	+286	N:C
-0.88	AB	-19.54	CH4 * N:C
-0.50	A^2	-0.05	$CH4^2$
-1.37	B^2	-6084.20	$N:C^2$

The linear regression model for %PHB cell content

$$(\hat{y}') = b_0 + (b_1 \bullet A) + (b_2 \bullet B) + (b_{12} \bullet A \bullet B) + (b_{11} \bullet A^2) + (b_{22} \bullet B^2) \quad (3)$$

$$= 1.90 + (0.94 \bullet A) + (286 \bullet B) - (19.54 \bullet A \bullet B) - (0.05 \bullet A^2) - (6084.20 \bullet B^2)$$

And the final version of the regression model, taking into account the statistical significance of each term is:

$$(\hat{y}') = b_0 + (b_{12} \bullet A \bullet B) + (b_{22} \bullet B^2) \quad (4)$$

$$= 1.90 - (19.54 \bullet A \bullet B) - (6084.20 \bullet B^2)$$

Supp. Tables A5-A8. Effect of methane amount and N:C ratio on PHB production from *Methylocystis* sp. Rockwell when nitrate is used as the N-source.

Supp. Table A-5. ANOVA for quadratic model in response to PHB concentration. The model F-value of 9.05, R^2 of 0.819 (from Figure A-3a) and $p < 0.05$ implies the model is appropriate. In this case A and B^2 highlighted in bold are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	70.07	5	14.01	9.05	**0.0018**
A-CH4	8.85	1	8.85	5.72	**0.0379**
B-N:C	1.31	1	1.31	0.8492	0.3785
AB	1.92	1	1.92	1.24	0.2915
A^2	5.25	1	5.25	3.39	0.0954
B^2	52.74	1	52.74	34.06	**0.0002**
Residual	15.48	10	1.55		
Total	85.55	15			

Supp. Table A-6. Final equation for sqrt. and quadratic model in response to PHB concentration.

Coded equation	Parameter	Actual equation	Parameter
+11.21		-0.90	Intercept
+0.9978	A	+2	CH4
+0.3846	B	+712.20	N:C
-0.6235	AB	-13.85	CH4 * N:C
-1.29	A^2	-0.14	$CH4^2$
-4.08	B^2	-18155	$N:C^2$

The correlation among the control factors (methane amount and N:C ratio) and response measures (PHB concentration and %PHB cell content) for cultures grown with nitrate as N-source are obtained using the regression analysis technique. The equations obtained for the quadratic model using square root transformation are shown in the following equations.

For PHB concentration, the complete regression was:

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_2 \bullet B) + (a_{12} \bullet A \bullet B) + (a_{11} \bullet A^2) + (a_{22} \bullet B^2) \quad (5)$$

$$= -0.90 + (2 \bullet A) + (712.20 \bullet B) - (13.85 \bullet A \bullet B) - (0.14 \bullet A^2) - (18155 \bullet B^2)$$

The final regression, taking into consideration the statistical significance of parameters (Table A-6).

$$(\hat{y}) = a_0 + (a_1 \bullet A) + (a_{22} \bullet B^2) \quad (6)$$

$= -0.90 + (2 \bullet A) - (18155 \bullet B^2)$

Supp. Table A-7. ANOVA for quadratic model in response to PHB cell content. ANOVA for quadratic model in response to %PHB cell content. The model F-value of 5.6, R^2 of 0.737 (Figure A-2b) and $p < 0.05$ implies the model is appropriate. In this case A, B, and B^2 highlighted in bold are significant contributors to the model ($p < 0.05$).

Source	Sum of squares	DF	Mean square	F-value	p-value
Model	12.60	5	2.52	5.60	**0.0102**
A-CH4	3.40	1	3.40	7.55	**0.0206**
B-N:C	3.35	1	3.35	7.44	**0.0213**
AB	1.05	1	1.05	2.33	0.1576
A^2	0.02	1	0.02	0.0548	0.8196
B^2	4.77	1	4.77	10.61	**0.0086**
Residual	4.50	10	0.45		
Total	17.10	15			

Supp. Table A-8. Final equation for sqrt. and quadratic model in response to %PHB cell content.

Coded equation	Parameter	Actual equation	Parameter
+5.95		+5	Intercept
-0.62	A	+0.07	CH4
-0.61	B	+196.04	N:C
-0.50	AB	-10.25	CH4 * N:C
-0.10	A^2	-0.01	$CH4^2$
-1.23	B^2	-5461.76	$N:C^2$

The linear regression model for %PHB cell content

$$(\hat{y}') = b_0 + (b_1 \bullet A) + (b_2 \bullet B) + (b_{12} \bullet A \bullet B) + (b_{11} \bullet A^2) + (b_{22} \bullet B^2) \quad (7)$$

$$= 5 + (0.07 \bullet A) + (196.04 \bullet B) - (10.25 \bullet A \bullet B) - (0.01 \bullet A^2) - (5461.76 \bullet B^2)$$

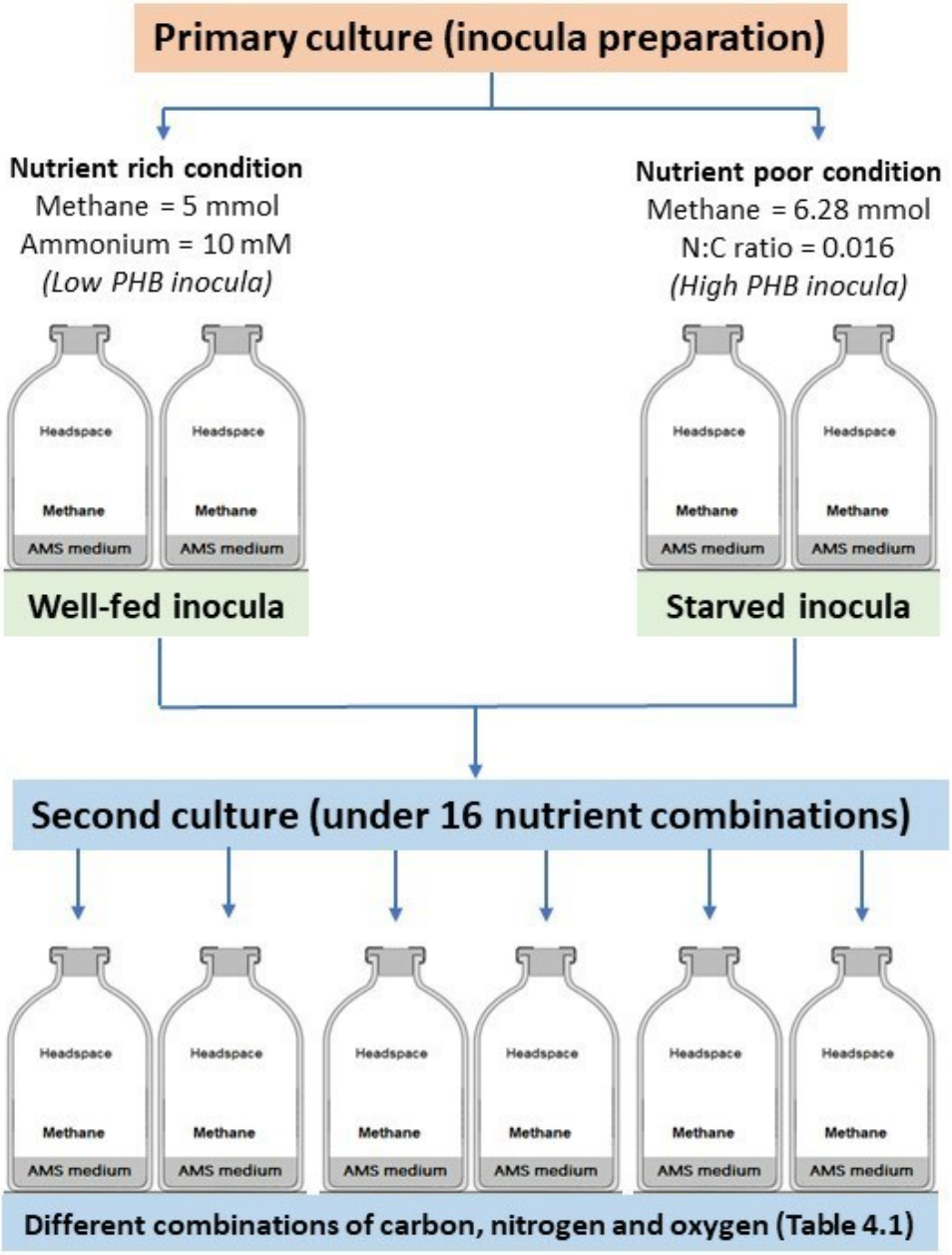
And the final version of the regression model, taking into account the statistical significance of each term is:

$$(\hat{y}') = b_0 + (b_1 \bullet A) + (b_2 \bullet B) + (b_{22} \bullet B^2) \quad (8)$$

$$= 5 + (0.07 \bullet A) + (196.04 \bullet B) - (5461.76 \bullet B^2)$$

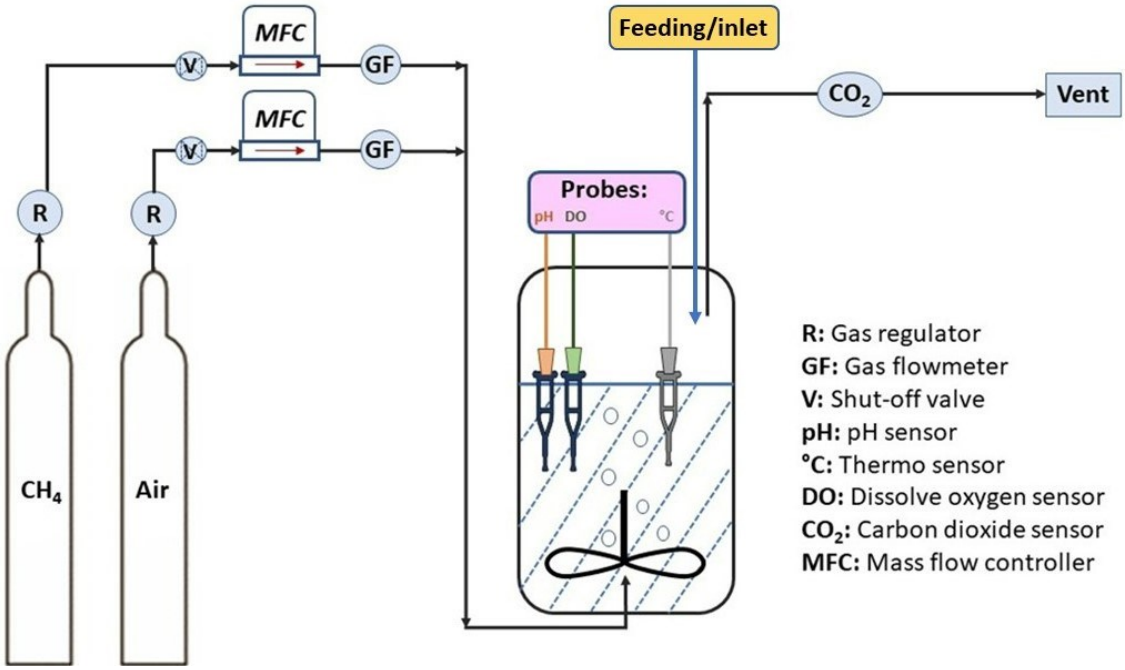
B. Supplementary information for chapter 4

Supplementary Fig. B-1. Schematic of experimental workflow. Well-fed and starved inocula were prepared first under specified culture conditions (see method, section 4.3) followed by re-culturing them under specified nutrient combinations (see Table 4.1). Each nutrient combination had 9 replicate cultures and sacrifice 3 replicates at 48, 96, and 144 hours for analysis.

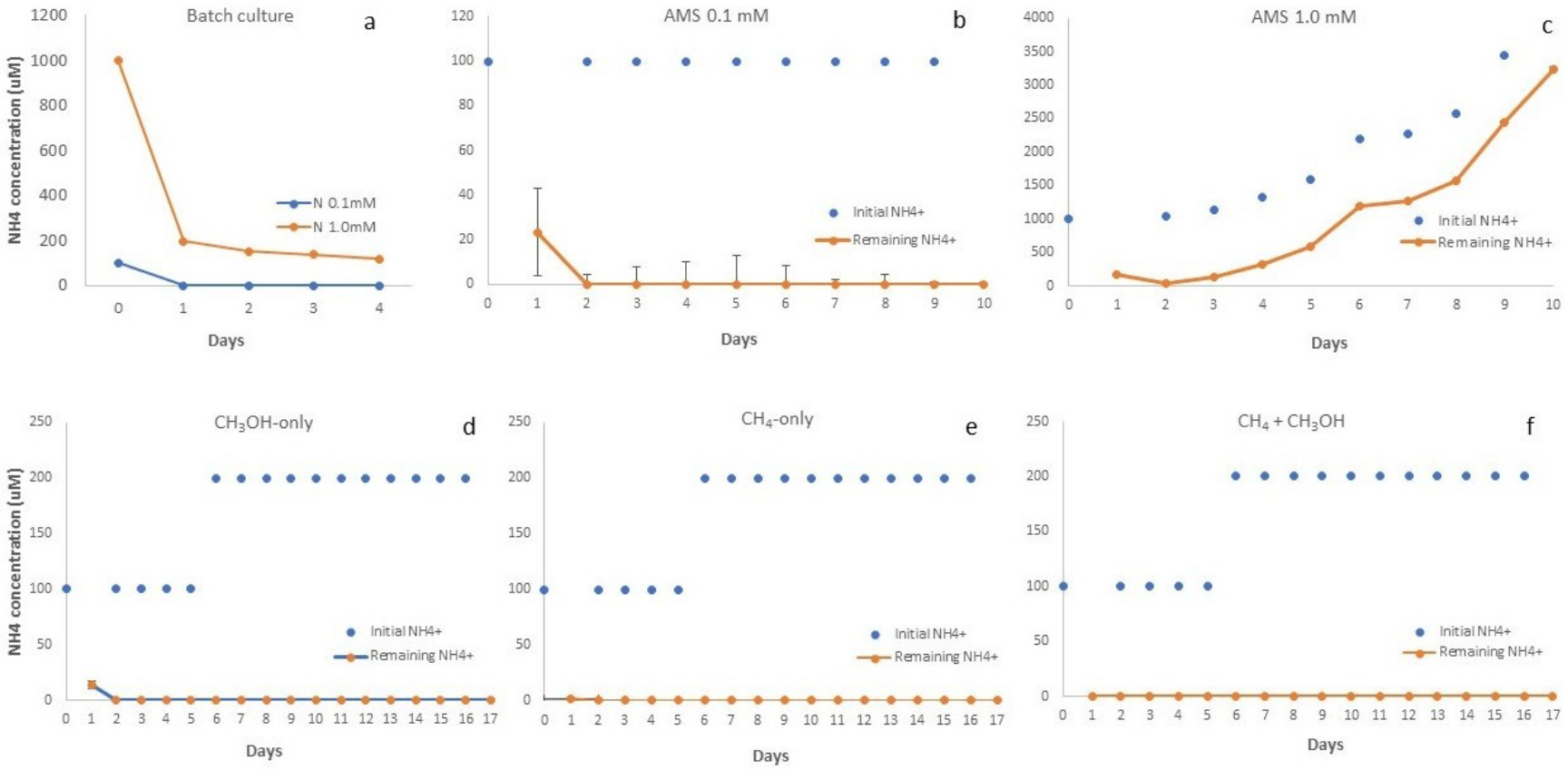


C. Supplementary information for chapter 5

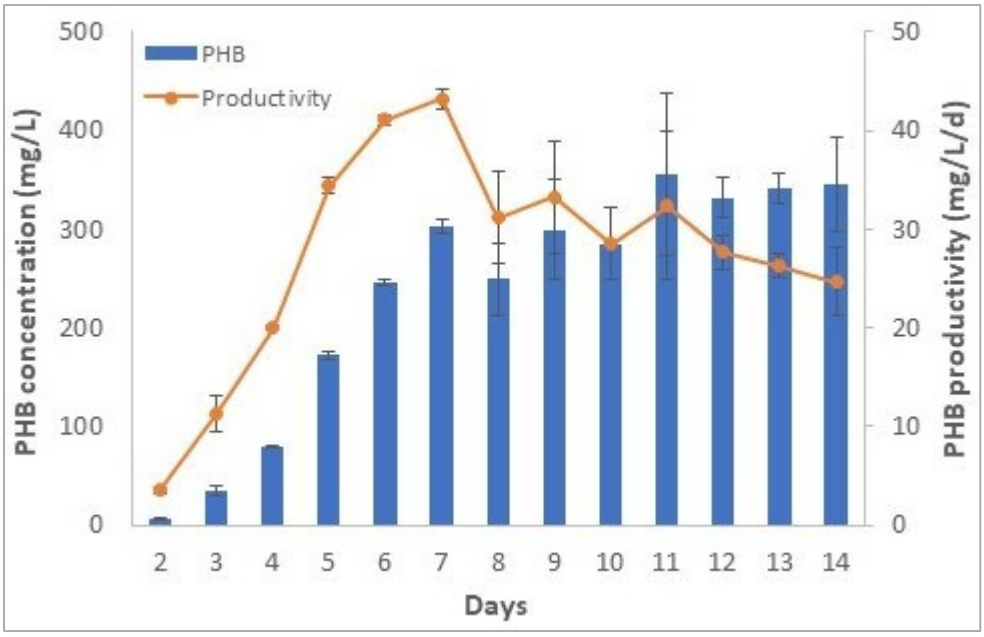
Supplementary Fig. C-1. Schematic of bioreactor setup. The flow of methane and air from a separate tank is regulated by a mass flow controller before entering to the reactor vessel. The excess of gases and carbon dioxide vented to the fume hood.



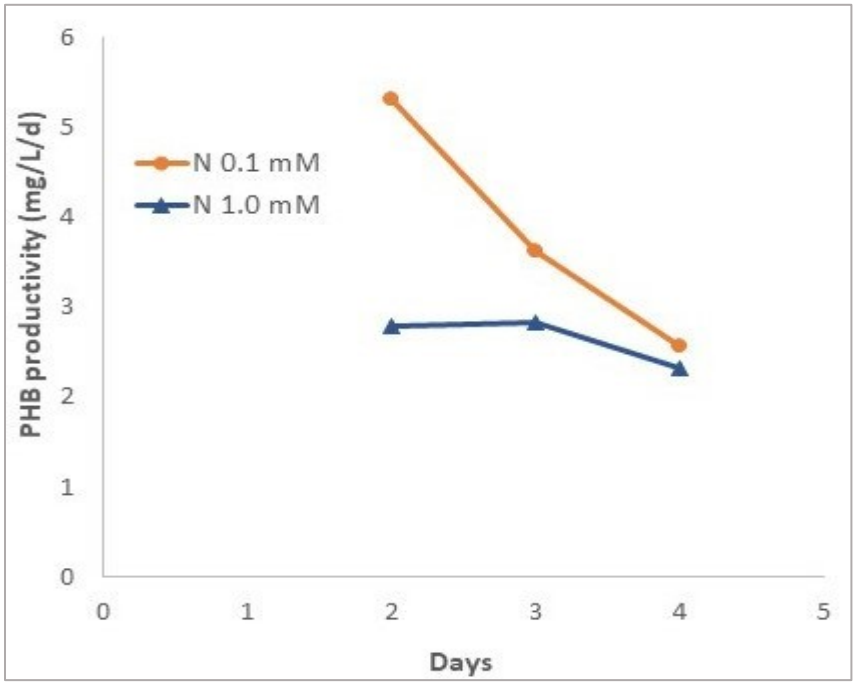
Supplementary Fig. C-2. Ammonium assay of batch and fed-batch cultivation. Ammonium assay of methanol batch culture using 0.1 mM or 1.0 mM of ammonium (a). Ammonium assay of fed-batch cultivation for determining methanol toxicity using 0.1 mM of ammonium (b) and 1.0 mM of ammonium (c). Ammonium assay of fed-batch cultivation using methanol-only (d), batch cultivation using methane-only (e) and fed-batch of methane + methanol combination (f) using 0.1-0.2 mM of ammonium. Error bars represent SD (n = 2).



Supplementary Fig. C-3. PHB production of *Methylocystis* sp. Rockwell in fed-batch cultivation with methanol and 0.1 mM ammonium feedings. A separate expt. runs with Pulse feeding of methanol (1 to 15 mM) and 0.1 mM of ammonium per day resulted in higher PHB concentration and productivity on day 7. While PHB concentration remain at the similar level (about 300 mg/L), the PHB productivity decreased after day 7 over the experimental duration.



Supplementary Fig. C-4. PHB productivity of *Methylocystis* sp. Rockwell in batch cultivation with 15 mM of methanol and 0.1 or 1.0 mM of ammonium.



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