Analysis of Different Design Approaches to Favor the Growth of polyhydroxybutyrate (PHB) producers in A Methane Utilizing Mixed Culture Community

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Abstract

Environmental protection and wellbeing have become a major concern in recent years. Currently, one of the most commonly studied environmental topic is plastic disposal and the extent to which it poses high-risk threats due to its subsequent accumulation in the environment. Petroleum based plastics are non-biodegradable and have very long half-lives, estimated to be hundreds of years, which leads to severe ecological repercussions. Since the environmental ramifications of the use of petroleum-based plastic has outweighed the challenges facing the industrialization of bioplastics, PHA biopolymer has gained broad interest in recent years.

This thesis offers an efficient approach for maximizing PHB polymer production in methanotrophic bacteria through using the methane gas produced in the anaerobic digestion process in wastewater treatment plants (WWTPS) as carbon substrate and electron donor for the enrichment the of methanotrophic bacteria. This is achieved through offering an innovative selection approach for PHB producers including type II methanotrophs whilst enabling other PHB accumulators to grow simultaneously. The values for PHB accumulation reached around 60% accumulation in ammonia mineral salt (AMS) media and 55% in nitrate mineral salt (NMS), which is higher than what has been reached in the literature for both nitrogen sources. Furthermore, characterization of the PHB polymer was performed using high-end confocal microscope to observe stained PHB granules within the cell and ^{1}H and ^{13}C NMR to characterize the chemical structure of the extracted polymer. Optimization of the operational parameters; Temperature, time, Carbon to oxygen ratio and ammonia concentration, was performed using central composite design (CCD) to assess the maximum accumulation percentages and acquire practical information about factors and their interactions.

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Chapter 1: Introduction

1.1. Background

Environmental protection and wellbeing have become a major concern in recent years. Currently, one of the most commonly studied environmental topic is plastic disposal and the extent to which it poses high-risk threats due to its subsequent accumulation into the environment [1]. Petroleum based plastics are non-biodegradable and have very long half-lives, estimated to be hundreds of years, which leads to severe ecological repercussions such as the intoxication of living organisms including humans, and the disruption of marine ecosystem either by entanglement in plastic materials or ingestion of microplastic particles [2]. In addition to its detrimental impact on aquatic life, plastics pose equivalent risks on terrestrial life as well, for instance it was reported that plastic pieces have been detected in more than 90% of seabirds [3], [4].

Despite the successful advancement in plastic manufacturing, it still has a crucial environmental drawback which is, resistance to degradation. Consequently, the existing routes of plastic disposal are inefficient, as it is mainly disposed into landfills or to aquatic surfaces, and as a result these synthetic polymers can persist in the environment for thousands of years [5]. Furthermore, remaining routes for plastic disposal are either recycling the plastic by down cycling it to a lower value polymer or by incineration, however this route is associated with toxic chemical emissions into the atmosphere such as; sulfur oxides, hydrogen chloride, and arsenic [6], [7].

Thus, there has been an evolutionary approach to find a more suitable eco-friendly biodegradable polymer alternative, i.e. bio-based plastic which can be synthesized from nature acquired resources such as the plant-based poly lactic acid (PLA) and the bacteria based polyhydroxy-alkanoate

(PHA). When produced by bacteria, PHA serve as both a source of energy and a carbon store [8]. PHA polymers can be produced using sustainable renewable waste materials such as whey and molasses waste unlike petroleum-based plastics which are produced from non-renewable fossil fuels [9]. PHAs are microbially synthesized hydrophobic inclusions of polyesters that are stored intracellularly under stress conditions of phosphorus, nitrogen, and oxygen limitations accompanied by carbon abundance [10]. These polymers are non-toxic, biocompatible, and insoluble in water, they tend to have a high polymerization rate, crystallinity and high molecular mass which makes them comparable to petroleum-based plastics [11]. Moreover, PHAs completely break down to water and carbon dioxide under aerobic conditions or methane under anaerobic conditions, which make them safe to dispose into the environment without any dangerous or unpredictable outcomes [7]. There are various types of PHA polymers due to the identification of over 150 monomers differing in composition that can be co-polymerized together to create compounds with varying range of different properties. The most studied type of PHA is polyhydroxy-butyrate (PHB), having only one methyl group attached to the polymeric chain.

Interestingly, it has been found that a certain type of Methanotrophic bacteria have the ability to accumulate PHB through the oxidation of methane gas [12]. Methane is produced as a waste product in the anaerobic digestion process in wastewater treatment plants. The use of methanotrophs to mitigate the methane released, would cut the production cost while averting its harmful emission to the atmosphere [13], [14]. The amount of methane wasted as flared gas only from wastewater treatment plants is estimated to be 21 million tons each year [15]. Furthermore, the predicted annual share of the PHB produced from biogas sources was around 20%–30% of the entire plastics market [16], [17].

1.2. Problem Statement

Owing to the fact that PHAs offer an alternative solution to overcome the aforementioned problems associated with petroleum-based plastics, they can replace petroleum-based plastics in the near future [18]. However, there are some limitations facing the process of PHA industrialization or upscaling, with the most being related to its production cost [19]. In fact, the production cost of 1 kg of polyhydroxy butyrate (PHB) is estimated to be around 15 to 30 USD which is 20 to 40 times higher than that of producing the same weight of polypropylene estimated to be around 0.7 USD [20].The cost intensive process of synthesizing the PHA polymer lies in the high cost of its carbon feed-stock, design of culturing operational conditions, and the polymer extraction process [21]. Studies on PHA production have been using expensive carbon substrates like glucose sugar which comprises over 40% of the production cost (Son et al., 1996). Furthermore, studies have also proved that culturing strategies of high temperature requirement, high reactor cost along with time intensive PHB extraction, pricey chemicals and advanced equipment requirement have comprised the rest of the 60% of the cost [22], [23]. Hence, further studies should focus on lowering the production cost of PHAs through exploring the usage of waste renewable feedstock for PHA accumulation, designing systems with lower energy requirements, using recombinant more efficient microbial strains, using less expensive chemicals and more economical PHA recovery methods.

Moreover, as mentioned previously, a specific type of methanotrophic bacteria (type II methanotrophs), present in wastewater treatment plants (WWTPs), has the ability to accumulate PHB using methane produced from the anaerobic digestion process as their carbon source. Conventionally, the selection for type II methanotrophs i.e., PHB accumulators, was established through using ammonia as a selective pressure also known as media-based selection [24]. This

selection strategy is based on the fact that ammonia (NH_4) has a chemical structure that resembles that of methane's (CH_4), thus, when used as the nitrogen source, it acts as a competitive substrate with methane and eventually intoxicate the cell [25]. However, unlike Type I methanotrophs, Type II methanotrophs have the proper genes to overcome the toxicity of hydroxylamine and nitrite produced by methane co-oxidation, which makes ammonia an optimum candidate for type II selection due to its ability to metabolize these chemical byproducts. Hence, the carbon substrate conflict would no longer exist for type II methanotrophs [26]. However, this culturing strategy has been proven to reach a threshold accumulation of maximum of 40- 51% of strains for example studies [24], [27]. Furthermore, most studies in the literature has been using pure culture methanotrophic species for PHA accumulation, which isn't feasible in real life WWTPs due continuous exposure to contamination. Moreover, here it is worth mentioning that using nitrate as the nitrogen source requires less energy than ammonia because it's directly metabolized to the final product, however it does not impose any selection pressure for type II methanotrophs [26]. Thus, developing a new design-based strategy for selecting PHA accumulating bacteria able to strive in a methane fed environment while growing independently from the nitrogen source would result in higher energy savings.

Furthermore, there is a need for to implement different optimization models in order to study the effect of different variables on the PHB accumulation and their interaction on one another which will provide a more realistic assessment for the implementation of the experiment in different operational conditions. Mathematical models in for the production of PHB from methanotrophic bacteria has been lacking, even the studies that implement variable alterations by studying the effect of each variable at a time which lack variable interaction study and how they affect one another and gives missing information of real-life implementation.

1.3. Objectives

The overall objective of this research is to develop a robust and efficient system to produce an eco-friendly biodegradable polymer, i.e. PHA that can replace petroleum-based plastics. In the pursuit of this objective, methanotrophic bacteria have been selected due to their ability to accumulate PHB while using methane produced in the anaerobic digestion process in wastewater treatment plants as their carbon source which would cut the production cost while preventing methane harmful emission into the atmosphere. In order to achieve the overall objective and tackle the aforementioned problems, specific objectives have been identified as follows:

- Developing a new strategy for the selection of PHB accumulators, while maintaining high methane consumption and growth rates.
- Comparing different nitrogen sources effect on PHB production which will allow exploring culturing options using the new setup
- Characterizing the PHB extracted using staining technique and nuclear magnetic resonance.
- Optimizing the different parameters affecting PHB accumulation such as carbon to oxygen ratio, nitrogen source concentrations, incubation duration and temperature.

1.4. Thesis Layout

This thesis comprises five chapters. After an introduction in the first chapter, a comprehensive literature review including the microbial taxonomy metabolism and key enzymes, different products extracted from methanotrophic bacteria and their important applications, is presented in Chapter 2.

In Chapter 3, the detailed description of the materials and methodology of the experimental setup used to achieve high PHB accumulation process is provided. Additionally, the system performance results are presented and discussed the different parameters as well affecting the accumulation rate are investigated.

Chapter 4 focuses on the modelling and simulation of an optimization model using the variables of operational data and their effect on the accumulation by using the response surface methodology model of the central composite design. A designing approach specifically associated with design of experiment for different optimization purposes The detailed steps for the model calibration and validation are described as well the results and the corrolation between the model and the measured data are discussed.

Finally, chapter 5 assembles the research findings of this study and the suggestions for future work.

1.5. Thesis Contribution

This study provides an insight into different PHB accumulation strategies and varying selective pressure to induce the growth of PHB producers which will help in providing a more feasible approach for offering bacterial biomass of PHB producers a more diverse environment for multiplication. This study aimed at reaching a high PHB accumulation percentage using the new experimental setup that can compete with what has been reached in the literature. A novel strategy for PHB accumulator's selection has been developed to overcome the restrictions of only using ammonia as a nitrogen source by creating a nitrogen independent selection method. The novel strategy depends on recycling PHB producers into the system without the need to use a specific

nutrient to select type II methanotrophs while achieving a high methane consumption rate. The maintenance of this system for consecutive cycles has proved its sustainability and its potential application in wastewater treatment plants. The use of methane as a substrate for PHB accumulation provides a cheaper approach for bioplastic production while investing the methane used in wastewater treatment plants into a useful application instead of flaring it into the atmosphere, contributing to climate change and superseding a high carbon footprint.

Chapter 2: Literature Review

2.1. Introduction

Wastewater treatment plants (WWTPs) comprise a valuable asset for environmental and economical contributions, thus they are currently regarded not only for their known function as a remediation facility, but as facilities encompassing an important revenue stream in resource recovery. Nowadays, WWTPS are perceived as biorefinery facilities loaded with organic resources that can be consumed as microbial substrates in order to sustainably generate electricity, remove contaminants, and recover valuable products [28]. In fact, it is estimated that the theoretical potential recoverable energy from 1 gm COD is around 14.7- 17.8 KJ [29]. Whereas, the average energy consumption of conventional WWTPs is 3.24 KJ/ gCOD which means that the potential energy in wastewater is 4.5 to 5.5 times higher than the energy consumed during its treatment [30]. Moreover, it was estimated that the yearly methane production from WWTPs is 21 million tons in the form of biogas produced from anaerobic digestion process which might be crucial for the energy portfolio if properly used [31]. However, methane is a gas and has a boiling temperature of -160 ºC which makes it hard to store, transport and distribute and if compressed or liquified it needs high energy input and capital cost. Thus, it is preferable to find an alternative way to use the biomethane produced form WWTPs that does not require high energy or large costs. Fortunately, WWTPS are characterized with a large array of a biodiverse microbial population that has the ability to produce an assortment of products using different substrates [32], [33]. One of these biodiverse microbial populations is methanotrophs, which can use the methane produced as their energy source and convert it to liquid fuels such as methanol or store it under specific conditions within their cells in the form of Polyhydroxyalkanoate (PHA). In this chapter, the microbial

characteristics of methanotrophs will be thoroughly reviewed including their genotypes, phenotypes, ecophysiology, and the factors affecting their growth. Moreover, an illustration of a comprehensive overview of the enzymatic pathways exerted by their cellular machinery will be discussed including the PHA production pathway which is the main focus of this thesis. Afterwards, the characterization and quantification techniques of the produced PHA will be described. Finally, methanotrophs role in the recovery of multiple diverse products within WWTPs will be reviewed and their potential industrial applications.

2.2. Methanotrophs

2.2.1. Methanotrophic bacteria role in methane mitigation

Methanotrophic bacteria has a unique metabolism that is fairly simple since it only relies on one carbon substrate which is methane. However, it has the ability to manufacture a mixture of value-added organic compounds such as methanol, Polyhydroxybutyrate (PHB), and Ectoine [34]. Methanotrophs utilize methane gas as an electron donor in order to generate sufficient energy required for cellular growth and the biosynthesis of secondary metabolites. Methanotrophs fascinating ability to perform multiple roles simultaneously, begins with the methane oxidation pathway which begins with the conversion of methane gas to produce methanol, a sustainable renewable biofuel [17]. Moreover, it has a crucial role in methane mitigation, which is one of the Greenhouse gases (GHGs) that contribute to global warming. According to the environmental defense fund (EDF), methane comes second after carbon dioxide as a major contributor to global greenhouse gases emissions, yet it is 84 times more potent than carbon dioxide due its higher ability of heat trapping through the absorbance of infrared radiations [35], [36]. Methane is released into the atmosphere either by anthropogenic activities or natural origins like

methanogenic bacteria and archaea in landfills which collectively represent more than 140 billion cubic meters of flared methane [37]. Moreover, methane is produced within WWTPs in the form of biogas through anaerobic digestion process. Thus, a pool of methane gas is created and is readily available for methanotrophs. Hence, methanotrophic bacteria are crucial in the global methane cycle due to its ability to mitigate this endothermic hydrocarbon. It is noteworthy that WWTPs is responsible for an estimate of 4% of the global methane production in the anaerobic digestion treatment process, this gas is normally flared into the atmosphere contributing to global warming [38].

2.2.2. Methanotrophs Genotypes and Phenotypes

Methanotrophs are mesophilic gram-negative bacteria which represents a subgroup of a broader bacterial group known as methylotrophs. Whereas, methylotrophs have the potential to utilize different single and multi-carbon substrate, while methanotrophs exclusively utilize methane substrate as sole carbon and energy [39] . unlike. Most methanotrophs are aerobic with the exception of *M.oxyfera* which is the only isolated anaerobic methanotrophic bacteria [40]. Thus, its metabolism relies on the role of oxygen to oxidize the methane substrate to form carbon dioxide (CO_2) and water. Aerobic methanotrophs are taxonomically classified based on phenotype, ability of spore formation, possession of specific membrane bound proteins, and their metabolic properties [38]. Thus, there are three main groups of methane oxidizing bacteria type I, II and X each of which undertakes a unique enzymatic pathway. Type I methanotrophs use the rubulose monophosphate (RuMP) pathway and belong to (gamma-proteobacteria) residing in families *methylococcaceae* and *methylothermaceae*, and their key genera are *Methylomonas, Methylobacter, Methylococcus*. Whereas, type II methanotrophs use the Serine pathway and belong to (alpha-proteobacteria) in family *methylocystaceae* and *beijerinckiaceae*, and their key

genera are *Methylosinus* and *Methylocystis.* Finally, Type X methanotrophs use Calvin-Benson-Bassaham (CBB) cycle and descends from *methylacidiphilaceae* family and only comprise two genera *Methylacidiphilum* and *Methylamicrobiales* [38]. Furthermore, family Type X methanotrophs differs from type I methanotrophs as they have low concentrations of enzymes of the serine pathway ribulose- bisphosphate carboxylase, in addition they are considered extremophilic bacteria as they live in high temperatures [41].

Important genetic biomarkers are used to recognize the phylogenetic identity of methanotrophs within a bacterial culture. For example, the detection of the pMMo is considered a universal marker for methanotrophic bacteria as all groups of methanotrophs possess this gene responsible for the production of methane mono-oxygenase enzymes, which exits inside the Intracytoplasmic reticulum (ICM) [15]. On the other hand, the sMMO gene is used to identify the type II methanotrophs where it is usually found inside the cytoplasm [42]. Other genes involved in the production of PHB polymer phaA, phaB and most importantly phaC are inspected for their expression to assess the extent to which enzymes responsible for PHB accumulation are translated to catalyze polymer production[43]. Furthermore, the presence of archaeal methanotrophs is confirmed with the detection of the archaeal gene 16S rRNA as illustrated in **Table 2-1**. These analytical methods usually takes place with the help of a Polymerase chain reaction (PCR) instrument used for primer amplification for further investigation with gel electrophoresis [44].

In terms of phenotypic characteristics, methanotrophs show red color with gram stain have varying morphology. For instant, type X are mainly cocci and found in pairs, type II are crescent shaped rods and can occur in rosettes, while type I can be found as either cocci or rods found singularly,

under light microscope [38]. Type I usually have a distinct pinkish pigmentation while type I and X occur in a white color colony [45].

Table 2-1: Genetic biomarkers of methanotrophic bacteria

Gene Target	Gene Name	Primer Name	5'-3' Sequence
Particulate methane monooxygenase	(pmoA)	A189f A682r	5'-GGNGACTGGGACTTCTGG-3' 5'-GAASGCNGAGAAGAASGC-3'
Soluble methane monooxygenase	(mmoX)	mmo882f mmo1403r	5'-GGCTCCAAGTTCA GGTCGAGC-3' 5'-TGGCACTCGTAGCGCTCCGGCTCG-3'
Gene for archaea	16S rRNA	Arc349f Arc806r	5'-GYGCASCAGKCGMGAAW-3' 5'-GGACTACVSGGGTATCTAAT-3'
16s rRna gene for bacterial	16S rRNA	Bac349f Uni806R	5'-AGGCAGCAGTDRGGAAT-3' 5'-GGACTACYVGGGTATCTAAT-3'
PHB synthase	phaC	phaCF phaCR	5′-ATCAAYAARTTCTACRTBCTCGAYCT-3′ 5′-ATGTAATTGTTGAYGAMRWAGGWCCA-3′

2.2.2. Methanotrophs Eco-Physiology

Methanotrophic bacteria inhabit oxic zones where oxygen is present as an electron acceptor and organic carbon, methane, is present for cellular biosynthesis such as soils and freshwaters, rice paddies, and WWTPs sludge [46]. In addition, most methanotrophs are mesophilic and prefer neutral pH. However, some genera that are thermophilic inhabit areas with high methane profusion and high temperature range like volcanoes and soil paddies [47]–[49]. Moreover, few psychrophilic species in temperature between 4 and 10°C mainly belonging to type I methanotrophs have been reported in arctic regions [37], [50], [51]. Furthermore, methanotrophs acidophilic and halophilic which means that they can tolerate high acidity and salt concentration [52].

2.2.2.1. Methanotrophs key metabolic pathways

As previously mentioned, there are three types of aerobic methanotrophs that differs phylogenetically, i.e. type I, II, and X), each of which follows a distinct metabolic pathway. As shown in **Figure (2-1)**, all three types share a common methane oxidation pathway to produce formaldehyde, thereafter, each group of Methanotrophs carry on in one of these enzymatic pathway; (i) rubulose monophosphate (RuMP), (ii) Serine, and (iv) Calvin-Benson-Bassaham (CBB) [53]. On a similar note, this common metabolic pathway relies on the action of a series of oxidizing enzymes to convert methane to methanol then to formaldehyde carried out by methane monooxygenase (MMO) and methanol dehydrogenase (MDH) enzymes, respectively. The first enzyme MMO comprises two types, (i) membrane bound and copper reliant particulate (pMMO) and (ii) soluble (sMMO). pMMO is considered as a universal marker for methanotrophic bacteria and is expressed by nearly all types of methanotrophs whereas sMMO is only expressed by type II methanotrophs and some of type X methanotrophs in the conditions of copper scarcity and in that case it utilizes iron instead [54]. Following the common metabolic pathway of methane oxidation to formaldehyde, type I methanotrophs undergo the RuMP cycle which is responsible for formaldehyde assimilation and detoxification. In the RuMP cycle, formaldehyde is fixed with ribulose 5-phsosphate to form 3-hexulose 6-phophate sugar which is then converted to glyceraldehyde-3-phosphate to finally generate one Pyruvate molecule. Simultaneously, another pathway called pentose monophosphate pathway which is a sugar phosphate-based pathway takes place to ensure the regeneration of ribulose 5-phsosphate required for formaldehyde fixation in the RuMP cycle [55]. These two pathways are crucial precursors of amino acids, nucleotide, and lipids biosynthesis. Afterwards, pyruvate is further dissimilated into one acetyl-CoA molecule for further incorporation into the tricarboxylic acid (TCA) cycle and the electron transport chain in nutrient

sufficient conditions for energy generation [56]. On the other hand, in type II methanotrophs formaldehyde produced from the common metabolic pathway is further oxidized to form `1ate and then converted to methylene THF to form serine amino acid which yields phosphoglycerate in several stepwise reaction called serine cycle. The serine pathway requires 3 Adenosine triphosphate (ATP) and 2 Nicotinamide adenine dinucleotide (NADH) for activation unlike the RuMP cycle which only requires 1 ATP. The serine pathway is followed by the ethyl malonyl Co-A (EMC) pathway which includes several CoA derivatives which are important originator for creating high-end products [57]. Furthermore, an intermediary cycle for glyoxylate recycling called Glyoxylate regeneration cycle (GRC) which links the serine cycle with the TCA and EMC cycle in type II methanotrophs[58], [59]. Thus, the serine, EMC, and GRC pathways are the core trails, which leads to mapping the derivatization of the secondary metabolites in type II methanotrophs. In the case of nutrient deficient conditions, an additional pathway to three previously mentioned pathway is introduced to store an energy supply in the form of PHB granules as will be described in the following section [60]. Whereas, the heterotrophic group of methanotrophs, type X, possess the ability to assimilate carbon and undergo the CBB cycle. The CBB cycle begins with carbon dioxide (CO_2) fixation resulting from the conversion of formate to CO_2 by the action of formate dehydrogenase (FDH) and ultimately use this pathway for biomass generation [61]. Furthermore, Type X from Type II are uniquely different than type I since they are believed to perform an incomplete serine cycle or have reduced concentrations of enzymes involved in the serine cycle [53]. On the other hand, they are able to survive in elevated temperatures than type I and type II methanotrophs [62].

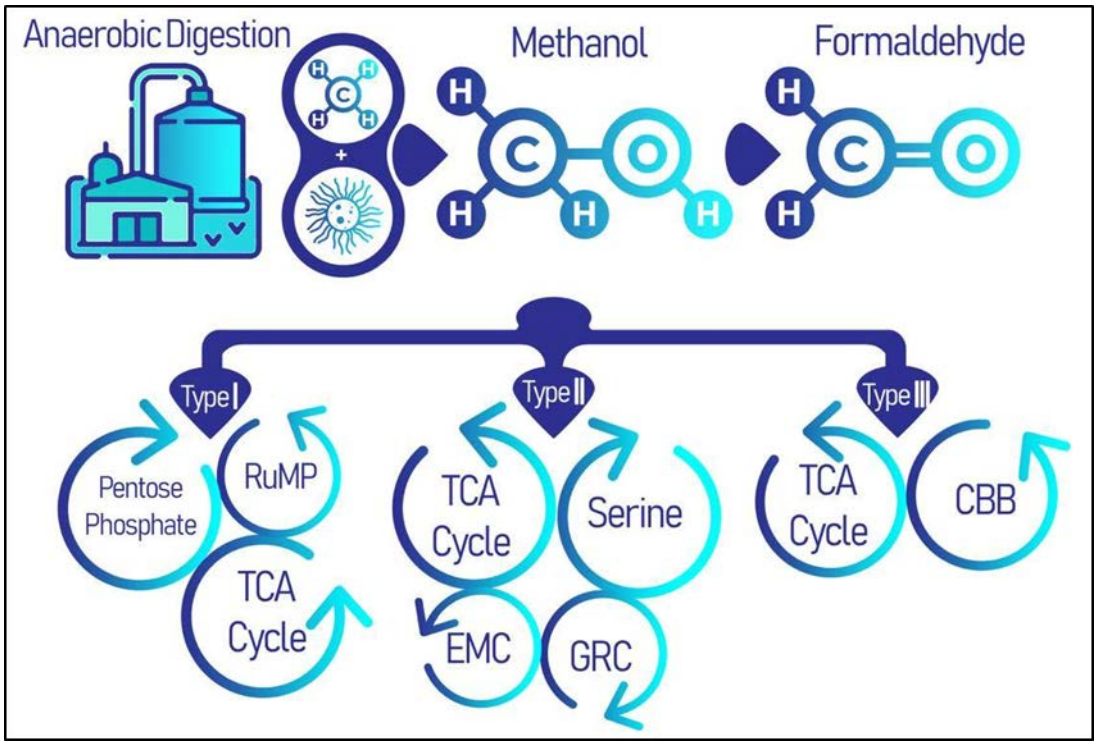

Figure 2-1: Metabolic Pathways for type I, II, and X methanotrophs

2.2.2.2. Type II methanotrophs PHB production pathway

In type II methanotrophic bacteria, PHB is usually produced through the combination of different enzymatic pathways in the case of nutrient deficient conditions. It is estimated that the production of one PHB monomer ($C_4H_6O_2$) requires the oxidation of 8 moles of methane (CH_4) by 12 moles of oxygen and the reduction of one mole of flavoprotein (FP) to its reduced counterpart (FPH_2) while generating 4 moles of carbon dioxide and 12 moles of ATP as shown in **Equation (2.1)** [63].

$$8\,CH_4 + 12\,O_2 + FP \rightarrow C_4H_6O_2 + 4\,CO_2 + 12\,ATP + FPH_2 \quad \textbf{(2.1)}$$

To break down the PHB production pathway, first methane is oxidized to formaldehyde, which then unites with glycine to form L-serine marking the start of the serine cycle. The serine cycle consists of transamination of the serine to form a hydroxy-pyruvate molecule catalyzed by the

action of serine- glyoxylate transaminase. The enzyme hydroxy-pyruvate reductase is responsible for the reduction of hydroxy-pyruvate into (R)glycerate that is further phosphorylated into 2-phospho-D-glycerate which is an important intermediate in the central metabolic pathway (CMP) responsible for biomass production [64]. In the right conditions following the phosphorylate of pyruvate, phosphoenolpyruvate is produced by phosphopyruvate hydratase, followed by the addition of carbon atom with the action of carboxylase to generate oxaloacetate. Malate dehydrogenase then reduce the oxaloacetate into (S)-malate. Finally, acetyl CoA and glyoxylate are formed subsequently after the formation of (S) Malyl-CoA catalyzed by the enzymes malyl CoA ligase and malate CoA lyase. The next step relies on the presence of the enzyme isocitrate lyase where it catalyzes the initiation of glyoxylate regeneration pathway (GRC) to consistently provide the serine pathway with the substrate glycine to ensure continuity of the cycle [59]. Furthermore, there are cases where isocitrate enzymes is missing which triggers the initiation of the EMC cycle instead through the generation of glyoxylate. These metabolic reactions yield 1 mol of 3-phospho-D-glycerate ($C_3H_7O_7P$), which is used in the CMP, after the consumption of two moles of formaldehyde (CH_2O) with 1 mole of carbon dioxide at the expense of 3 moles ATP and 3 moles of NADH as shown in **Equation (2.2)** [34].

$$2\,CH_2O + CO_2 + 3\,ATP + 3\,NADH \rightarrow 3\,C_3H_7O_7P + 3\,ADP + P_i + 3\,NAD \qquad \textbf{(2.2)}$$

Furthermore, the PHB cycle is mediated by the acetyl CoA substrate which under nutrient limitation begins the process of carbon storage through PHB synthases which is the terminal enzyme involved in the process of moderate final monomer formation as shown in **Figure (2-2).** It is considered as a part of a group of enzymes; encoded by important genetic markers phaA, phaB and phaC [65].An important group of PHA associated proteins have been found to modulate the

accumulation of PHB polymer by playing an important role in the activation of the translation of the aforementioned genetic codes [10], [43]. Type II methanotrophs has been found to produce high yield of PHB accumulation up to 55% of the cell dry weight (CDW) and have vital role in the industrial accumulation of PHB [8], [66].

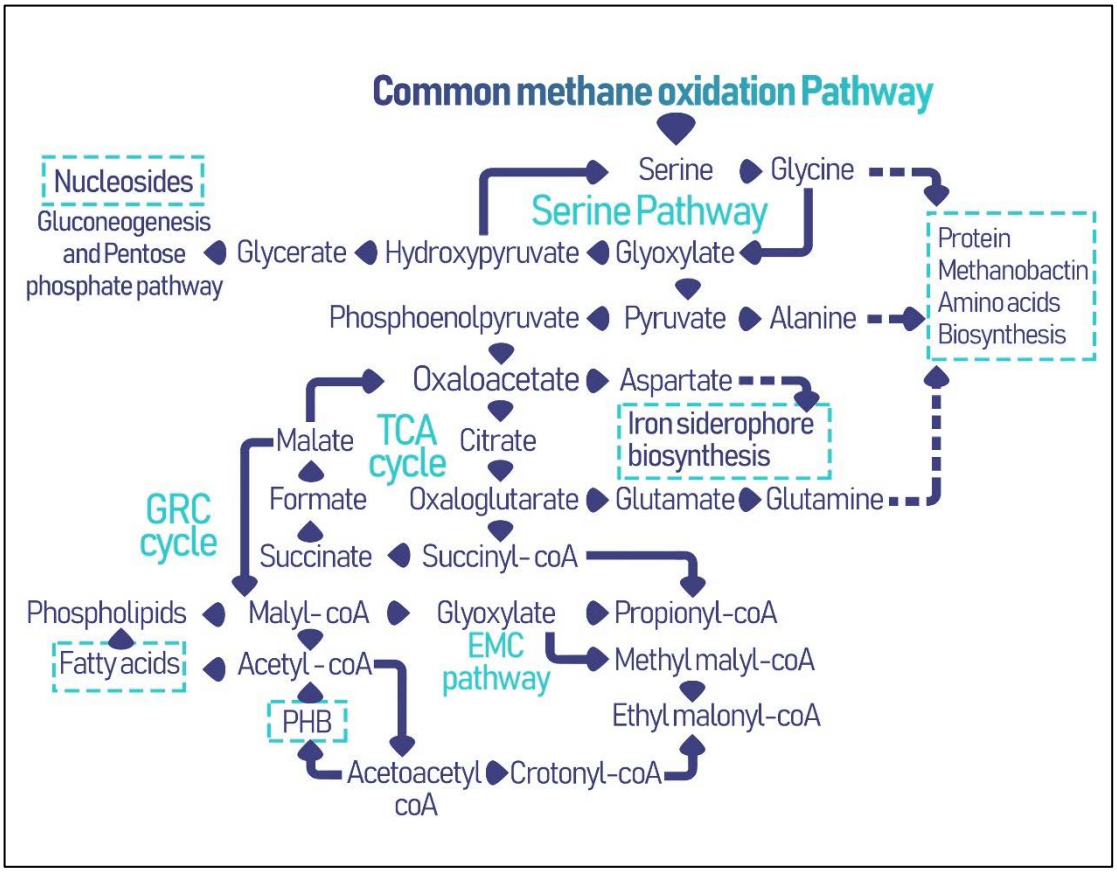

Figure 2-2: Comprehensive illustration of the metabolic reactions within the PHB production pathway

2.2.3. Factors affecting Methanotrophs growth

Extensive research has been performed to investigate the effect of factors such as pH, salinity, temperature, nutrient availability in terms of type and concentration, such as (phosphorus, Copper, and Nitrogen source), and finally carbon to oxygen ratio on the growth of methanotrophs and the extent to which these factors impact its metabolic reactions and the secondary metabolites

generation [38]. These factors were also manipulated to favor the growth of one type of methanotrophic bacteria over the others in order to sway the reactions in the intended pathways which ultimately influences the resultant products. For example, many strategies have controlled these parameters in a way that favor the growth of type I methanotrophs for the purpose of the production of several products such as; Methanobactins, Exopolysaccharide and nucleosides. Other strategies have been employed to inhibit the growth of type I methanotrophs and favor type II methanotrophs to benefit from their ability to produce high value products such as PHA, Ectoine and Fatty acids. The following sections will discuss the effect of the operational parameters on methanotrophs growth and will focus on how to manipulate these parameters to favor type II methanotrophs growth to ultimately produce PHB.

2.2.3.1. pH

The great majority of methanotrophic bacteria grow optimally at a pH range of 5.5 to 8 with an exception of several strains of acidophilic methanotrophs such as, *methylacidiphilum fumariolicum* (strain SoIV) and *methylacidimicrobium tartarophylax sp*. which grow at pH levels as low as 0.5 [67]. While, species such as *methylomicrobim* prefer a more alkaline environment with reported pH as high as 10 [68]. However, it was reported that the selection of type II methanotrophs was directly proportional with increasing CO_2 dissolution in the culture medium, stimulating the serine cycle, which might be achieved through reducing the pH to 5 [47]

2.2.3.2. Salinity

Most methanotrophic bacteria are able to withstand salty environments with up to 10% NaCl concentrations. Some studies proved tolerance to higher salinity concentrations in methanotrophic communities, other species such as *methylosoma* species has been proven to be sensitive to high

salinity [69]–[71]. Furthermore, a study demonstrated the stability of the growth rate and the methane consumption in a salinity concentration up to 7 mg/L, these values witnessed noticeable fluctuations when exceeding beyond this concentration [72].

2.2.3.3. Nitrogen source

The effect of different nitrogen sources such as atmospheric Nitrogen, ammonia, and nitrate on methanotrophic communities has been investigated thoroughly in different studies in the literature [26], [42], [43], [73], [74]. In terms of atmospheric nitrogen, it has been reported that oxygen sensitive nitrogenase enzyme catalyzes its fixation in all type II methanotrophs as well as some species of type I methanotrophs such as: *methylococcus, methyloglobulus, methyloprofundus, and methylobacter* [9], [67]. On the other hand, ammonia and nitrate have been used as selection pressures for type II and type I methanotrophs respectively, since it was observed that type I methanotrophs were dominant in high oxygen and nitrate concentrations while type II were dominant in relatively low oxygen and nitrate concentrations [42], [75], [76]. Subsequently, this observation was elucidated by the metabolic behavior of both ammonia and nitrate as shown in **Equation (2.3) and (2.4)**.

Biomass yield using ammonia:

$$29.5\ CH_4 + 44.25\ O_2 + NH_3 + FP \rightarrow 3\ C_4H_8O_2N + 17.5\ CO_2 + 18\ ATP + 4\ FPH_2 \quad \textbf{(2.3)}$$

Biomass yield using nitrate:

$$41.5\ CH_4 + 62.25\ O_2 + 3HNO_3 + FP \rightarrow 3\ C_4H_8O_2N + 39.5\ CO_2 + 42\ ATP + 4\ FPH_2 \quad \textbf{(2.4)}$$

Normally, methane is oxidized by the action of MMO enzyme, however in the presence of ammonia, it beats the methane substrate for the attachment to the active site of the MMO enzyme due to the structural similarity between the ammonia and methane molecules and perform what is known as

competitive inhibition of methane substrate **(Figure 2-3)**. Hence, this will result in the oxidation of the ammonia instead of methane and yield the formation of hydroxylamine and nitrite derivatives, which usually cause inhibition of bacterial growth due to cellular intoxication which leads to the inhibition of type I methanotrophs. However, in the case of Type II methanotrophs, these bacterial group possess genes that enable them to cometabolite these contaminants and reallocate them back into cellular metabolism. It is noteworthy that supplementing bacterial culture with nitrate would result in a higher enrichment of a more diverse and more dense community however it is not selective for type II methanotrophs associated with nutrient imbalance conditions to stimulate PHB production [77]. Hence, the dominant type of methanotrophic bacteria within the culture medium is greatly influenced by the type and concentration of the nitrogen substrate. [78].

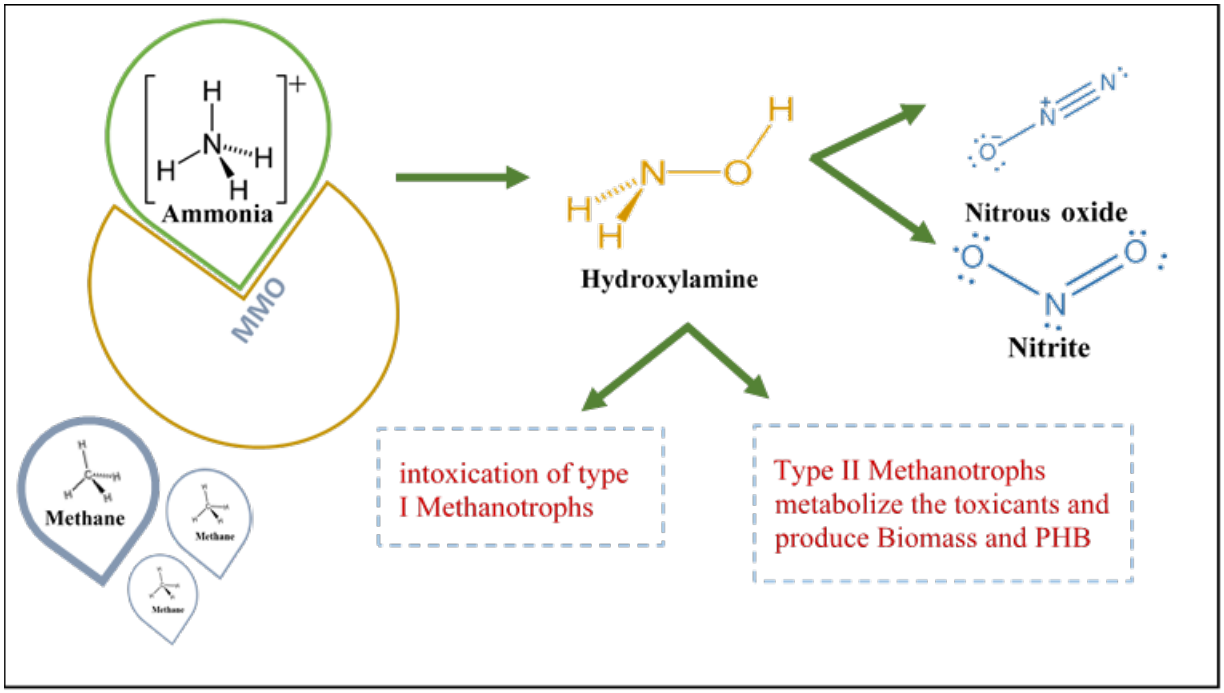

Figure 2-3: An illustration of the competitive inhibition between ammonia and methane

Moreover, nitrogen source and concentration have shown diverse effects on PHB accumulation in methanotrophic communities. Hence, when employing a phase of nitrogen sufficiency followed by nitrogen deficiency called growth phase and PHB accumulation phase, different strains have

dissimilar reactions to these exposures. For example, the strain *Methylosinus trichosporium OB3b* was reported to produce 50% and 38% PHB after exposure to 20 mmol and 10 mmol of nitrate supplementation in growth phase respectively [43], [79]. On the other hand, in a different study performed on the same strain, the addition of 10 mmol of ammonium supplementation generated 13% PHB accumulation [80]. Furthermore, *Methylocystis parvus OBBP* pure culture reached an accumulation as high as 60% and 36% PHB accumulation with ammonium and nitrate enrichment respectively [43], [80]. Whereas, *Methylocystis hirsute* and *Methlocystis GB25* both reached 51% PHB in ammonium supplemented medium [81], [82]. The strains *Methylosinus trichosporium IMV3011* and *Methylobacterium organophilum CZ-2* when exposed to 10 mmol of nitrate concentrations accumulated 47% and 38% PHB respectively [83], [84].

2.2.3.4. Methane to oxygen ratio

Methane and oxygen concentrations are important determinants of the dominant type of bacteria and their metabolic activity. For example, an excess concentration of methane in the headspace above 35% was found to generate a high formaldehyde concentration which cause an inhibitory effect on methanotrophs [85]. On the other hand, a methane concentration above 1% is enough to form a stable methanotrophic community [86]. Moreover, the dominance of type II methanotrophs in a bacterial culture in different methane and oxygen concentrations showed more tolerance to lower atmospheric pressure. For example, type I *Methylosinus trichosporium OB3b* achieved a PHB accumulation of 45% at 0.2 atm while type II *Methylocystis parvus OBBP* PHB accumulation at 0.3 atm, was 60% [87]. Furthermore, most studies indicated that the lower methane concentration was linked to high abundance of type II methanotrophs. Thus, in the presence of ammonium and low methane, the ammonium oxidation process is enhanced and hydroxylamine formation is increased which necessitates the cell to accumulate PHB to use it as an electron donor

in order to assist in methane consumption. Moreover, another theory was proposed that entails the hindrance of methane co-oxidation due to the presence of high oxygen concentration which affects the activity of the oxygen sensitive nitrogenase enzyme [88].

Furthermore, a study performed to assess the methane oxidation activity in a lake showed that methanotrophic community was most active in the presence of high nitrogen concentration regardless of oxygen concentrations. However, in the case of nitrogen limitation, low oxygen was crucial to maintain the oxidation of methane which may be associated with the necessity of nitrogen fixation [87]. In accordance, another study reported that an oxygen concentration lower than 4% was crucial for nitrogen fixation in pure culture methanotrophs [78]. This ability was further linked to type II and X methanotrophs in low oxygen saturated environments [38]. As a conclusion, the ratio of methane to oxygen can be defining to the dominance of either type of methanotrophic bacteria in a way that high methane to oxygen ratio is associated with type I methanotrophs while low methane to oxygen is associated with type II dominance [38], [89].

2.2.3.5. Temperature

Despite the fact that most of type I species (*methylosphaera, methyloprofundus, methyloglobulus, methylovulum,* and some strains of *methylobacter, methylosarcina, methylomonas, methylomicrobium*) and all type II species can withstand a temperature as low as 5°C , studies performed on methanotrophic cultures were performed in a range of 20 to 40 °C for its convenience for application in industry [67], [90]. Furthermore, a decrease in pMMO and sMMO activity was observed when temperature rise above 45°C for the former and 30 °C for the latter in the *Methylosinus trichosporium* OB3b strain[91]. On the other hand, higher temperature are more suitable for some species like methylothermus and methylacidiphilum, belonging to type I and X

respectively, here they grow optimally at temperatures higher than 55 ºC and lower than 60 ºC [67], [71], [92]. While methylococcus and methylocaldum genus, considered thermophilic methanotrophs, have optimal growth at 42-55 ºC, unlike psychrophilic methanotrophs methylosphaera grow optimally at 10-15 ºC [93]. It is noteworthy that type I methanotrophs showed higher tolerance to colder temperatures [55]. However, the effect of temperature change on the accumulation of PHB polymer studies are lacking in many PHB concerned studies.

2.3. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are optically active microbial synthesized polyesters of hydroxyacids repeating monomers. Moreover, these polymers are accumulated intracellularly as hydrophobic inclusion bodies of diameter ranging from 0.2–0.5 μm in the cytoplasm that can be obsereved with transmission electron microscope. Furthermore, these hydrophobic inclusions are stored as energy reserve material and can reach as high as 90% of the dry cell weight in some species such as *Bacillus Megateruim* which are accumulated under stress limitation conditions of phosphorus, nitrogen, and oxygen and/or excess carbon [9], [94]. Additionally, they have comparable property with nowadays regular plastic like Polypropylene as they are non-toxic, biocompatible, isotactic and insoluble in water they tend to have a high polymerization rate, crystallinity and high molecular mass which is (PP) [11]. Additionally, PHAs completely break down to water and carbon dioxide under aerobic conditions or methane under anaerobic conditions. Thus, PHAs are completely safe for human with the potential to replace the everyday plastic without the major drawbacks and health hazardous impact [94].

Interestingly, more than 300 bacterial species have been investigated for its PHA accumulation capabilities [14]. Whereas, PHA has been classified into more than 150 different types based on

the large number of different hydroxy-alkanoic acid monomeric structure with varying side chain length [94], [95]. the main precursors in catalyzing the final production of the fatty acid monomer in many types of bacteria were found to be the PHA synthases enzymes responsible for final monomer formation and control the length of the monomeric side chain [58]. Whereas, The length of monomer is defined by the type of the bacterial strain and the substrate within the culture media [96]. Thus, the polymeric chain is classified into 3 categories; short chain length (SCL) c3-c5 hydroxy-acids monomers such as poly 3-hydroxybutyrate (P3HB) and poly hydroxy-valerate (PHV) or medium chain length (MCL) c6-c14 such as polyhydroxy-octanoates and long chain length (LCL), characterized with more than 15 carbons [9]. Interestingly, each category is characterized with uniquely different chemical and physical properties as well as designated industrial applications [97]. For instance, the MCL PHAs have lower crystallinity than SCL PHB or PHV and are more flexible [98]. Moreover, PHA can be produced either using Gram-negative bacteria or Gram-positive bacteria [99]. However, it is noteworthy that most of the PHA production researches have been focusing on Gram-negative bacteria even though Gram-positive bacteria, especially Bacillus spp., have long been found to produce large amount of PHB [100]. Moreover, Gram negative microorganisms have a lipid bilayer that isn’t biocompatible and trigger immune defense response which makes gram-positive bacteria are better suited for medical applications [66]. In addition, The cost intensive process of synthesizing the PHA polymer is mainly because of the carbon source, however some domain of applications medical and biotechnical such as, wound handling, vascular systems, orthopedic area, drug capsules, urological valves, tissue regeneration in cases of gum inflammation and ultrasound and tomography, are willing to invest such cost to minimize the risk factor associated with non-biocompatibility [20].

Due to their similar properties, PHA polymers could replace Petroleum based plastics in the near future, which are known to have deleterious effects on the environment as well as hazardous to human health [101]. It is noteworthy that 40% of the 75 billion pounds per year of plastics produced is disposed into landfills, as well as several hundred thousand tons are released into marine environments every year and accumulate in oceanic regions [102] . Additionally, other options for plastic disposal are incineration, however it is associated with the release sulfur oxides, hydrogen chloride, cadmium, lead, zinc, and arsenic to the atmosphere or to be down cycled to lower value products [103]. Furthermore, petroleum-based plastics are produced from fossil fuels which are non-renewable unlike the PHA polymers which can be produced using renewable carbon source. Thus, PHA polymers are considered as one of the green alternatives to overcome the aforementioned problems associated with petroleum-based plastics [104].

Generally, The SCL PHB is considered the most widely studied PHA for its mechanochemical properties and along with lower production cost which is due to its uncomplicated structure that makes it simpler to manufacture. For example, it is predicted to compete with conventional plastic in many industrial applications as it has high crystallinity ≈70%, impermeability to gas (water/vapor pressure of 560 g.µm/m2/day), high elasticity modulus of ≈ 3 GPa, and tensile strength at break of 25 MPa which makes it is less likely to break and remarkably way more durable than PolyPropylene (PP) and polyethylene (PE) [63], [105]. Nevertheless, there is a need for the development of PHB for industrial application as upon storage it is thermal stability is very low which makes it breakable [106]. Hence, research is studying the co-polymerization of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate [P(HB-co-HV)], which was found to be more durable and flexible than PHB. These characteristics makes it a great candidate to be used to in various products, such as films, compost bags, disposable food containers and molded products

[11], [107]. Furthermore, PHB has been used in heart surgeries and constructing synthetic blood [108].

Applications: PHA is high value market product as it is actively replacing many ongoing industries. For instance, it is replacing regular PP and PE, and PET due to high quality mechanical and chemical properties of PHA available in many applications for example; PHA latexes is being integrated in the paper manufacturing sector as surface coating component [102]. Applications of PHA comprise many sectors; food packaging, pharmaceuticals, drugs, cosmetics, and agriculture [41]. It is estimated that the global market share for PHA will reach around $98 million USD by year 2024, with 5% yearly incremental growth. Multinational companies are already producing PHA such as Mirel in USA, Bio-on in Italy and Hydal in Czech Republic with yearly production reaching up to 10,000 tons [34], [109]–[111]. Examples of food and packaging applications include; containers, foils, netting utensils, hygienic products, one-use tableware, and fabric threads [112]. While PHA is offered as an answer to petroleum based plastic problems, it is as well presented with the cost obstacle [94]. Therefore, it is most widely encouraged in medical applications where the risk factor of using non biocompatible material outweigh the high cost of PHA production. Some examples of areas of medical applications of PHA are; sutures, wound handling, vascular systems, orthopedic area, drug capsules, urological valves, also used in tissue regeneration in cases of gum inflammation heart surgeries and constructing synthetic blood [100].

2.3.1. Characterization techniques

PHAs can be identified and quantified using a number of techniques and staining methods, since PHAs are biolipids, which means that lipophilic stains are best suitable for their detection such as Sudan black B dye and Nile red [113]. Both stains are used to identify intracellular lipid inclusions

by interacting with the ester functional group, the staining processes is fairly simple consisting of directly applying the dye onto the bacterial [114]. Furthermore, scanning transmission electron microscope has been used to both identify and quantify these intracellular granules using analytical computer software [99].

Furthermore, FT-IR and H^1-NMR are also used for polymer identification, by observing designated distinct peaks at specific retention time that are only unique to PHAs while, gas chromatography GC is often used for PHA quantification [115].

2.3.1.1. Plate assay method

When dealing with mixed culture consortium, there is usually a pool of different bacteria making it very challenging to identify PHA accumulators. Therefore, a rapid detection and isolation method has been developed to qualitatively characterize the PHA producing bacteria and eliminate the non-PHA producers. The viable colony method of screening using Sudan Black B dye has been developed to narrow down the dominant producers instead of using other time, effort, and money consuming methods [116], [117]. This technique consists of applying 0.02% alcoholic solution of Sudan Black B to stain bacterial isolates on grown sterilized modified nutrient agar plates supplemented with suitable substrate. Afterwards, The plates are first divided to equal parts, then the bacterial isolates are spotted into the designated parts [113]. Next, bacteria are incubated and allowed enough time to grow and form colonies. Subsequently, the Sudan black B dye is poured onto the plate until colonies are submerged and covered completely and left unshaken for a half an hour. Finally, Excess dye is decanted, and the colonies are rinsed with 96% ethanol to eliminate any remaining dye. Generally, The dark blue colonies indicated dye absorption, hence a positive result for PHA accumulation [98].

2.3.1.2. Sudan black B staining method

For further confirmation of PHA accumulation, a glass slide of a bacterial smear is prepared for microscopic observation. Sudan black B is applied with a counter stain to create a contrast allowing PHA observation with light microscope with oil immersion lens 1000X magnification. First, after heat fixation of bacterial isolate [113]. The slide is submerged with Sudan black solution (0.3% Sudan black B w/v in 60% ethanol). After 10 mins the slide is rinsed with distilled water and the (0.5%) safranin counterstain is applied as well and left undisturbed for another 5 mins. Positive results is indicated by the observation of scattered dark blue spots within bacterial cell [118].

2.3.1.3. Staining with Nile red and Nile blue dyes A

Also known as viable colony staining, is also used for bacterial lipid stored inclusions [119]. This method has an advantage of using viable colonies without the need to kill the bacteria with toxic solvents or dyes -hence the name- which provide a tool to observe the accumulation of PHB during different growth phases in the presence of the dye. This method simply consists of exposing the bacterial cells to an ultraviolet light (UV). Afterwards, the oxazine Nile blue A and its fluorescent form oxazone Nile Red are applied to the culture media the intensity of the colonies luminescence reflects the density and the amount of the stored. This fluorescent dye can be fairly expensive, however, it is a highly sensitive staining method as only few micrograms are needed for the detection of different amount of PHA unlike other dyes such as the carbol fushin dye and the Sudan black dyes where a much bigger volume is needed while being less accurate in small amount detection [101]. For preparation a very small amount of Nile red or blue is added to di-methyl-sulfoxide (DMSO) to give final concentration of 0.5 µg dye/ml medium. Then, The culture medium is incubated to allow the growth of bacterial isolates. Lastly, the colonies of interest are

exposed to UV light (312nm) where fluorescence of bacterial culture indicates lipid polymer accumulation [77], [101], [119].

2.3.1.4. Fourier transform infrared spectroscopy

Infrared spectroscopy (IR) is usually as a confirmation tool for the presence of PHA polymer. However, it cannot be used for structure identification of the PHA molecule, where for determination of the type of PHA or quantification purposes another NMR and GC are more suitable. Nevertheless, some methods have been developed using the peak area and intensity to quantify the PHA content using a computer software, however this method hasn't been proven very efficient in terms of reliability and time consumption [120].

For PHA characterization using IR, the PHA polymer was first extracted. The purified samples are characterized by the FT-IR spectroscopy and ground with infrared quality KBr (1:10), pressed into discs under vacuum. PHA peaks are expected to be observed between 4000-400 cm^{-1} on the IR spectra which signifies the presence of important functional groups **(Table 2-2)** [76], [82], [99].

Table 2-2: Designated PHB peaks in infrared spectroscopy (IR) spectrum

Functional Group	**Wavelength (cm^{-1})**
-OH	3420
CH_3, Asymmetric	2955
CH_2, Asymmetric	2925
CH_3, Symmetric	2855
C=O	1741
CH_3, Terminal	1378
C-O-C, Asymmetric	1259

2.3.1.5. Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is usually used to determine the chemical structure of the extracted polymer. NMR are recorded at ambient temperatures. Tetramethyl silane (TMS) or CDCl are used as internal reference and its chemical shifts reported as ppm. It shows different types of protons in the compound when using proton (^{1}H-NMR) spectrum and carbon (^{13}C-NMR) shows different carbon within the compound correlated with designated peaks. For example, in ^{1}H-NMR spectrum reports chemical shifts associated with PHB polymer at δ 0.80 (t, 3H, CH_3), 1.14-1.20 (m, 6H, 3 CH_2), 1.47-1.81 (2 m, 2H, $\underline{CH_2}$-CH), 2.40-2.45 (m, 2H, $\underline{CH_2}$-CO), 5.06 (quintet, 1H, CH), multiplicity (s = singlet, d = doublet, t = triplet, q = quadruplet, quint = quintuplet, m = multiplet, mc = multiplet centered at, dd = doublet of doublet, td = triplet of doublet, etc.) 2.5 ppm and a triplet peak at 5.2 ppm is assigned to CH-CO proton. Peak at 1.6 ppm is assigned to methylene protons adjacent to the CH carbon in the sidechains. Peak at 1.3 ppm and triplet peak number 5 at 0.9 ppm are assigned to the methylene protons and terminal methyl proton of the side-chains respectively [99], [111]. The ^{13}C-NM is used to identify the carbonyl group in the PHB polymer that is overseen in the case of ^{1}H-NMR which appears around 169 ppm. When all of this peaks appear simultaneously on an NMR spectra it confirms the structure of the PHB polymer as elucidated in **Figure 2-4** [106], [121], [122].

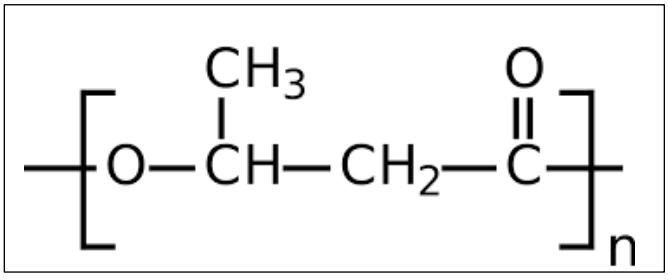

Figure 2-4: Expected PHB chemical structure characterized by NMR

2.3.1.6. Transmission electron microscope (TEM)

Scanning transmission electron microscope (TEM) shows an enhanced image of the bacterial cells with the PHB granular stored within. It is the most widely used due to its reliability to identify PHA granules, and has been developed to be used in quantification by calculating the percentage polymer occupation in ratio with the cell area [123]. It is mostly performed through primary and secondary fixation of bacterial cells using 3% glutaraldehyde and 1% osmium tetroxide respectively. Afterwards, the cells are suspended in molten agar and dissected with a scalpel. The sample are then dehydrated and submerged in propylene oxide in order to be polymerized. Afterwards, the cell resins are incubated at 70 ºC for 48 hr in 100% spurr. Next, the ultra-thins microtomed layers are moved to a copper grid. Finally, before observation 2% uranyl acetate and lead citrate solution stains are employed for contrast [76], [99].

2.3.2. Quantification techniques

2.3.2.1. Gas Chromatography

One of the most commonly used method for quantification is the gas chromatography for its ability of accurate and precise detection of the smallest PHB concentrations. For quantification, a pre-extraction step is required which can compromise the results accuracy if not performed properly due to technical errors. The extraction step usually consists of lysing the bacterial cell wall with sodium hypochlorite or hydrochloric acid, washing of the cell debris, dissolving the PHB in boiling chloroform and filtering the dissolved polymer, lastly, deionized water is sometimes added for phase separation to obtain high purity compound. the PHB concentration is assessed by injecting a sample from organic layer, internal standard to ensure accuracy.[82], [99], [124], [125]. The

concentration is calculated through quotient of by the area under the peak of PHB acid and benzoic acid[125]

2.3.2.2. Spectrophotometric method

The fastest and most widely used method to assess PHB content inside the cell is the spectrophotometric estimation of the of optical density (OD). First, the biomass density is measured at OD_{600} to calculate the CDW in correlation to the calibration curve. Following the same aforementioned extraction steps, however, the chloroform is left to evaporate and the PHB powder is transferred to a glass vial. Afterwards, the glass vial containing PHB powder and concentrated sulfuric acid are boiled in order to convert the PHB into the brown colored crotonic acid. The crotonic acid color intensity signifies the concentration of the PHB polymer which is measured at 235 nm using UV-spectrophotometer against a sulfuric acid blank, after calibration curve is constructed with standard PHB [126][116] [82].

2.4. Importance of Methanotrophic bacteria as multiple product producer

In the last 50 years, methanotrophs ability to perform multiple roles have been investigated as they own a unique metabolism that enables them to first, mitigate harmful greenhouse gas methane, remove harmful contaminants such as ammonia and nitrate found in water systems. Furthermore, they have the potential to produce valuable bioactive derivatives through methane uptake in order to form important products through phosphorylation pathways such as: single cell protein, biopolymer, S-layer, the copper binding protein (methanobactin), methanol biogas, organic acids, ectoine, vitamin B 12, enzymes catalysts, and foreseen. These products have been found valuable for advancement in many industrial fields as shown in **Figure 2-5**.

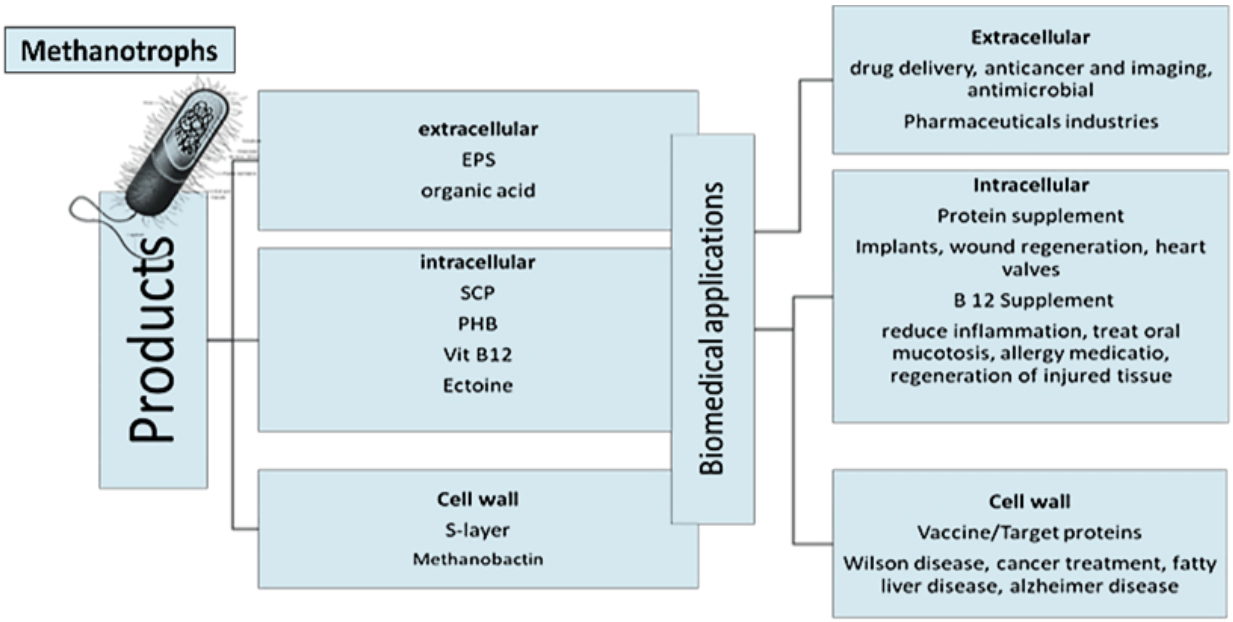

Figure 2-5: Illustration of the diverse potential products synthesized by methanotrophs and their industrial applications

The biotechnological revolution has taken place in the 21st century as biotechnology, nowadays, comes hand in hand with all other research scientific areas; engineering, agriculture, astronomy and biomedicine. Hence, taking advantage of available tools and assets present within bioremediation facilities systems is the next generation technology. The relationship between those areas and WWTPs seems weak at first glance, however this following section illustrates an inter-correlated relationship between the two.

2.4.1. Exopolysaccharide

The extra-polymeric substance or exopolysaccharide (EPS) is a biocompatible, non-toxic, and decomposable product, it is a high Molecular weight polymer with a sugar backbone [127]. EPS constitutes biofilms in environments that is produced as a result of low nutrient availability and high contamination in order to protect bacterial cells from environmental toxicity [128]. They are mainly formed by polysaccharide and protein integration where low amount of DNA and lipids are also detected [128]. Moreover, EPS perform many roles as they ensure structural stability of

the bacterial biofilm and act as filter to allow the passage of certain nutrients while blocking the entrance of other molecules [129]. EPS production in methanotrophs was found to be associated with high carbon to oxygen and nitrogen ratio thus methane rich environments were found to possess the thickest EPS biofilms such as soil interfaces [128]. Since, The EPS is carbon based it was assumed that its role is carbon assimilation in environments where nitrogen is depleted as type I MOB are unable to fix nitrogen, thus EPS is produced through the RuMP pathway (sugar based pathway) to produce EPS as a carbon reservoir [129]. On the other hand, type II methanotrophs has been known to occasionally produce the EPS polymer to catalyze nitrogen fixation by limiting oxygen penetration which in turn causes oxygen depletion this will in turn trigger enzymatic initiation [60].

Applications; includes Hydrogels dextran solutions as plasma expanders, pharmaceutical excipients (e.g., xanthan – as suspension stabilizer, or pullulan – in capsules and oral care products), drug carriers [130].

2.4.2. Surface layers

Surface layers are microbially synthesized polymeric protein that cover the outside of microbial cell, they are of special importance since they comprise about 15 % of the total protein content of the cell [131]. These crystalline structures are characterized with an amorphous layout that is mostly hydrophobic molecules, acidic amino acids, and lysine [132]. This nanoscale defined lattice matrix is characterized with identical pore sizes possess which allows it to self-assemble in a variety of environments [133]. This negatively charged two-dimensional crystalline protein or glycoprotein lattice embody the complex network of proteins involved in vital cellular functions such as synthesis, excretion, and the layout of membrane bound proteins [134]. Whereas, The

specific role of S- layer has been controversial, however some studies illustrates that S-layer might be serving different function for different bacterial groups, as part of an adaptation evolutionary mechanism, in a diversity of eco-niches [134]. For example, (i) S-layer serve as protective membrane against high osmotic pressure where the porous membrane have the ability to counteract the external pressure in halophilic bacteria. (ii) Its ability to withstand high temperature and mechanical stress has been linked to maintenance of cell shape and structural integrity for archaeal groups [128]. (iii) Provides gram positive bacteria with a periplasmic space [135]. (iv) it Guarantees the presence of binding sites for different Exoenzymes for example; provide linkage sites for the thermostable protease that allows it to tolerate thermophilic environments, the ability to incorporate enzymes like exoamylase in *B. stearothermophilus* [127]. (v) Provide Cell adhesion capability [129]. (vi)The Selective Semipermeable membrane protect from lyases activity and prevent parasite and toxic penetration while, at the same time modulate the passage of certain essential molecules and some important enzymes. (vii) Retaining organic substrates and metal compounds bound to cellular membrane [133]. (viii) As for methanotrophs; they have been linked with conveying copper ions to particulate methane monooxygenase pMMO which will help maintain equilibrium in regard of copper concentrations [128], [134].

A little over four hundred different species of prokarya have indicated possess gene sequences responsible for expression of S-Layer [136]. It's is noteworthy of mentioning, that S-layer has little to no structural similarities for different taxonomical groups [133]. Furthermore, short sequence of Post- translational mRNA is usually responsible for transcription of a single protein that make up the S-layer crystalline matrix. The promoter's sequences responsible for translation of S protein have dented long end 5' untranslated region (UTR) which protects the protein from RNase activity dissimilative activity. S- proteins are characterized with longer half- lives, that is why is stringently

modulated to be synthesized in the stationary phase rather than the log phase that is achieved through regulatory repressive gene sequences to stop the translation of the S protein in the growth phase namely, splA in *thermus thermophilus*. Hence, some species possess different genes responsible for each phase of cell synthesis [45].

Moreover, in gram-positive bacteria, they are most commonly attached through the SLH motif to the peptidoglycan layer. On the other hand, in gram-negative bacteria, S-layers are attached to the outer membrane lipid bilayer by ionically binding to the lipopolysaccharide or covalently joining N-terminal end to the S-layer, also by hydrophobic interactions as it can be joined by the van der Waals forces with lipids. Accordingly, most methanotrophic bacteria have an encapsulating S-layer such as *Methylococcus*, *Methylothermus*, and *Methylomicrobium* cells. Furtheremore, structure of the S-layer varies greatly between methanotrophs [131].

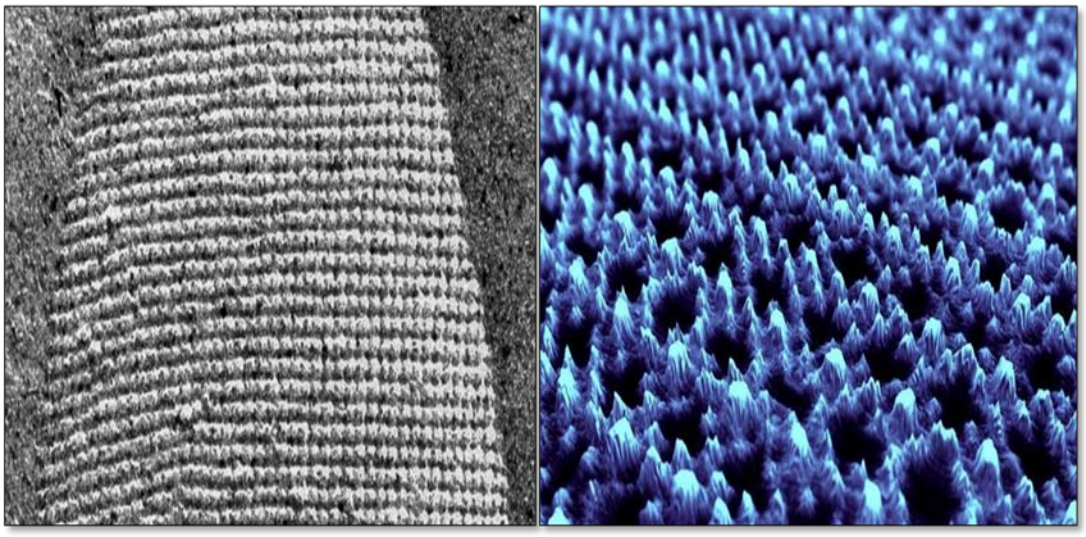

Figure 2-6: TEM micrograph of an S-layer carrying bacterial cell and AFM image of an S-layer on a silicon wafer *(Adopted from: https://boku.ac.at/en/nano/biophysics/research/s-schichtproteine)*

Applications include nanotechnology to manufacture nano-sized ultrafiltration membranes, biomedicine vaccines, immunoactivity reagents and target therapy, and synthetic biology; as it is

used in the synthesis of pH biosensors, biocatalysts, and treatment of heavy metal contamination [135], [137].

2.4.3. Methanobactin

Methanobactin (Mbns) are peptide chalkophores due to their copper binding capability which is needed for sell synthesis, Mbns are also involved in copper weathering [138]. They were originally characterized in the methanotrophic bacterial species *Methylococcus capsulatus* and were found to be imperative for the functioning of the methane oxidizing ezyme pMMO [134]. It is transported outside of the cell to trap and bind to copper ions Cu (I) [139]. This chelating agent also reduces Cu (II) to Cu (I) in copper deficient environments, since Cu (II) is known to be toxic for the bacterial cell [140]. Methanobactin cupper complex on its turn return back to the cell through active transport channels to convey copper for important metabolic functions in methanotrophic bacteria, where the nitrogen heterocyclic ring embedded in the peptide chains ensure Copper binding [70]. The backbone of the peptide chain is composed of an amino acid sequence which differs within for each bacterial strain [141]. Mbns form a modified ribosomal RNA that regulate its post translation [142].

Applications: Acts as chelating agent treat Wilson disease, cancer treatment, fatty liver disease, alzheimer disease[70], [143], [144].

2.4.4. Antibacterial proteins

Methylocystis minimus and Methylobacter luteus were found to produce a thermostable proteins capable of killing pathogenic bacteria [145]. The Bacteria encodes genes that produce peptidase

enzyme that is found to function as bacteriocin and now is under research for possible application in biomedical industry as an antibiotic [145].

Applications: antibiotic medications [146].

2.4.5. Single-cell Protein

It is a protein derived from microbial cells that feed on a range of organic carbon sources. Many microorganisms are suitable for this process, including algae, blue-green fungi and bacteria and may comprise up to 80% protein content [33]. The currently known expression of Single Cell Protein (SCP) was first used in 1966 substituting the old term Microbial proteins that was used to describe the dried cells of microbial organisms that served as an ingredient or a substitute for protein-rich foods [15]. In view of the insufficient world food supply, the use of biomass produced by industrially scaled reactors would provide a resource for SCP recovery [53]. Single Cell Protein is of great nutritional value because of its high protein, vitamin and lipid content and for its essential amino acids [147]. Methanotrophs are a well-known source for SCP production where the proteinaceous substance within MOBs is estimated to be 60-65% [53].

Applications: important agricultural applications as it is used as natural fertilizer and live feedstock which relieves stress on land conversion [28]. Furthermore, it has been associated with eyes and skin health since it is used a protein supplement especially for undernourished children [147]. Whereas, this supplement offers a source of vitamins, amino acids, minerals, and crude fibers [148]. Nowadays, Spiruilina tablets are prescribed as enriched vitamin as it lowers blood sugar level of diabetics due to the presence of gamma-linolenic acid and prevents the accumulation of cholesterol in human body [53].

2.4.6. Ectoine

Ectoine $C_6H_{10}N_2O_2$ and its derivative hydroxyectoine $C_6H_{10}N_2O_3$ are high value compatible solutes [149]. This water-soluble white powder is secreted intracellularly by microbial cells in order to overcome the environment hyper-salinity and balance the osmotic pressure [15]. It is noteworthy that, this osmoregulator can also be excreted to the extracellular environment as a response to hypo-salinity [150]. Furthermore, ectoine synthesis was found to be escorted with many amino acids, lipids and proteins, which ensures their structural stability [45]. Thus, The cyclic imino acids possess many medical and biotechnological applications and considered as an expensive ingredient which currently retails for 1000$/kg [151].Interestingly, in halophilic environments, methanotrophic bacteria synthesize ectoine by the conversion of oxaloacetate produced by theTCA cycle to aspartate followed by activation of cascade reactions catalyzed by diaminobutyric acid (DABA) aminotransferase (EctB), DABA acetyltransferase (EctA), and ectoine synthase (EctC) as shown in **Figure 2-7** [152].

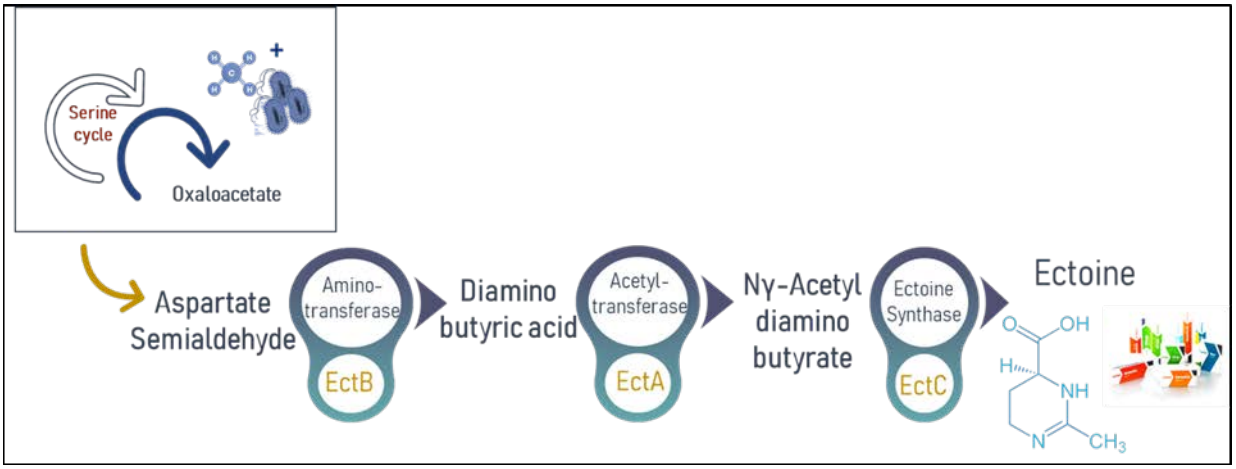

Figure 2-7: An illustration of the reactions involved in the synthesis of ectoine

Applications: eye drops, skin care products, and creams since it acts as antihistaminic agent, allergy medication, regeneration of injured body tissue, and DNA stabilizing agent which is used to treat oral muctosis in cancer patients [149], [151], [153].

2.5. Conclusion

Nowadays, WWTPS are functioning as biorefinery facilities, thus Methanotrophs are considered as a valuable asset due to its ability to perform multiple roles varying from contaminants removal to the biosynthesis of valuable products. Furthermore, PHA is considered the next generation alternative to the plastic pollution crisis due to its ecofriendly nature and comparable physicochemical properties with conventional plastics. Therefore, the current research study is designed in an attempt to selectively enrich this bacterial group present in WWTPs to assess its PHA accumulation capability and methane mitigation rate as well as optimize the operational parameters to provide an input for later upscaling purposes.

Chapter 3: A comparison between different design approaches to favor the growth of PHB accumulators in a methane utilizing mixed culture

3.1. Introduction

Plastic pollution is causing major repercussions ranging from leaching toxic chemicals into environments to pilling up in oceans interrupting natural ecological balance. In addition, plastic manufacturing process is energy intensive as it is estimated that the production of 140 million tons of plastics necessitates 150 million tons of fossil fuel causing a huge carbon footprint.

As an alternative, PHA polymer is especially unique due to its ability to overcome the adverse impact associated with the use of petroleum-based plastic as it represents a biodegradable ecofriendly sustainable solution. This polymeric biocompatible material can be integrated into many industries as it has similar physical and chemical properties with petroleum-based plastics. Methanotrophic bacteria present in WWTPs are able to accumulate relatively high amount of PHB within their cells and represent an important candidate for industrial applications due to their numerous abilities. Methanotrophic bacteria use a common methane oxidation pathway catalyzed by the action of methane monooxygenase enzyme to convert methane to methanol. Afterwards, methanol dehydrogenase (MDH) catalyze the conversion of methanol to formaldehyde which is oxidized to formate. Next, formate is in turn assimilated in the ribulose monophosphate pathway RuMP by type I beta-proteobacteria to generate carbon dioxide and biomass. On the other hand, type II methanotrophs belonging to gamma-proteobacteria use the serine pathway to subsequently store PHB as end product in nutrient deficient conditions [61].

Conventionally, the selection for type II methanotrophs i.e., PHB accumulators, was established through using ammonia as a selective pressure or media-based selection [24]. However, this method has been proven to reach a threshold accumulation of maximum of 40- 51% of strains [27], [154], [155]. Therefore, this chapter presents new method for selecting PHA accumulating bacteria that consists of recycling back PHA accumulators into the system while growing independently from the nitrogen source. This is achieved through designing a PHB based selection system instead in order to specifically stimulate the growth of PHA producers while eliminating other bacterial strains that might otherwise affect their growth.

This strategy is established through biomass exposure to consecutive cycles of nitrogen availability followed by nitrogen starvation in a fed batch system since, nitrogen source has been proven to be a precursor that greatly affect the accumulation percentage [156].. Moreover, ammonia (NH_4) is typically used as an inhibitor to type I methanotrophs as it has a chemical structure that resembles that of methane's (CH_4), thus, it acts as a competitive substrate with methane and eventually intoxicate the cell [25]. Unlike Type I methanotrophs, Type II methanotrophs have the proper genes to overcome the toxicity of hydroxylamine and nitrite produced by methane co-oxidation, which makes ammonia an optimum candidate for type II selection due to its ability to metabolize these chemical byproducts. Hence, the carbon substrate conflict would no longer exists for type II methanotrophs [26]. Moreover, it has been reported in the literature that the degree to which different species withstands ammonia or nitrate toxicity depends greatly on type of bacteria for example, *M. trichosporium OB3b* that had higher growth yield than *M. album BG8* in nitrate starvation [78]. However, nitrate as a substrate require less energy than ammonia because it's directly metabolized to the final product [157] Therefore, it is important to screen for PHB producers from different bacterial groups and provide a setting where they grow simultaneously

with those of methanotrophic bacteria without any limitations that the nitrogen source can induce while removing the nitrogen source as a factor contributing to the production cost.

Thus, the main focus of this chapter is to apply a different selection pressure to favor the growth of type II methanotrophs other than the nitrogen source; which is the design of experiment. Hence constructing the experimental set-up in a way that allows bacterial strains which can withstand the stress conditions of nutrient depletion to be constantly recycled back into the systems would ensure that PHB accumulators have dominance within the culture media. Hereafter, the design-based selection is hypothesized to allow a broad array of diverse micro-organism to co-exist with the type II methanotrophic community, thus achieving higher PHB accumulation percentage. Furthermore, applying a comparison study between the novel design-based selection and nitrogen-based selection in order to measure; the growth rate, biomass yield, gas consumption and rate of PHB accumulation within the two systems. Furthermore, it compares the effect of different nitrogen source ammonium and nitrate enrichments on the selection of a dynamic bacterial consortium that is able to both effectively mitigate methane while enhancing PHB accumulation.

This chapter aims to study the effect of experimental setup and nitrogen on Growth of Methanotrophs to: (1) compare between the PHB accumulation in different environments, (2) measure the growth rate, biomass yield, gas consumption and PHB accumulation of both new and conventional set-up using different nitrogen sources, and (3) determine whether changes in these conditions affects rate of key enzyme expression. Further characterization techniques are described in using staining techniques and spectrophotometric methods and physical characteristics assessment of the acquired polymer.

3.2. Materials and methods

3.2.1. Setup and Operational condition

All the experiments were conducted in 250 ml culture bottles with a butyl rubber cap to enable gas vacuuming and subsequent injection. In each bottle, 10 mL of seed was added to 40 ml of mineral salt medium (MSM) for culture enrichment. The bottles were vacuumed using a vacuum pump every 24 hours, followed by the injection of a daily 200 ml of methane and oxygen feed in a 1:1 ratio. For all the culture enrichments, methane was used as the only carbon source in order to selectively target methane oxidizing bacteria enrichment. The enrichment culture bottles were incubated in an orbital shaker incubator operated at a temperature of 25 °C and a mixing speed of 160 rpm. pH was controlled between 6-7 using 10 NaOH%. The experiment was monitored by regularly measuring optical density (OD), gas consumption, and PHB accumulation after each stage of growth and PHB phase.

Consecutive cycles of fed-batch mode were implemented along this study to investigate different parameters affecting the methane fed culture, and prospective PHB accumulating consortium. Four sets of culture bottles each in triplicates were used in this study. All sets consisted of a 96 h cycle duration comprising two equal duration phases of growth phase and PHB accumulation phase each of which lasts for 48 h. The growth phase media contained the nitrogen source in order to create a nutrient sufficient stage aimed for bacterial enrichment. Afterwards, the biomass was transferred to the PHB accumulation phase which was devoid of any nitrogen source to create a nutrient deficient stage in order to enable the initiation of the serine enzymatic pathway and start the selection of PHB accumulators. The first two sets were operated as control and followed a conventional set-up. In the conventional set-up, the cycle started by inoculating the biomass at the

beginning of the growth phase then, the enriched culture was split into two portions. Around 42 mL is transferred to the second stage to initiate PHB phase while the remaining portion is used as a seed and recycled back into the growth phase with an OD of around 0.3 as shown in **Figure 3-1 (a)**. The other two sets were operated using the suggested new strategy which followed the same nutrient sufficient and deficient alternating settings as well, of nitrogen starvation namely, feast famine. However, in this novel set-up, the whole biomass was transferred after the growth phase into the PHB phase and instead of recycling a portion of the biomass back after the growth phase like in the conventional set-up, all the biomass was recycled after the PHB accumulation phase back to the growth phase as shown in **Figure 3-1 (b)**. The proposed strategy applied is to prove that the design of experiment can be used as a selective pressure to preference the growth of PHB accumulators and eliminate non producers. Furthermore, this strategy allows different PHB producers from different strains to exist in synergy with the methane oxidizing bacteria, due the removal of nitrogen restriction. Additionally, the difference between the two sets in each setup was the type of the nitrogen source, with ammonia being used in set A and nitrate in set B.

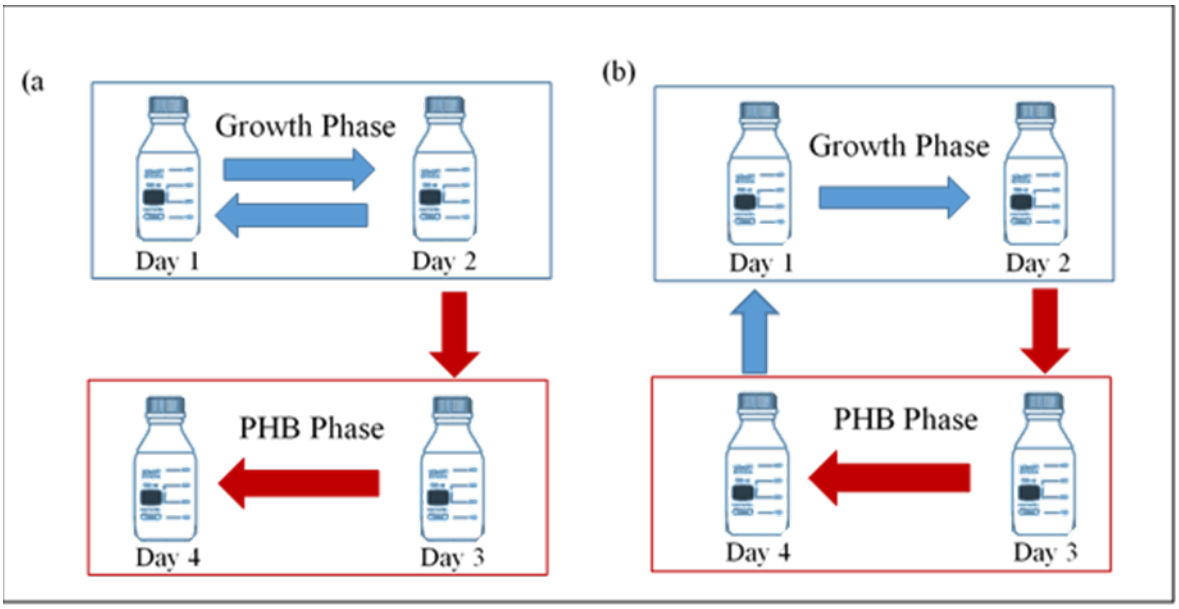

Figure 3-1: Schematic of the experimental setup: (a) Conventional Set-up (recycling of biomass after PHB phase and (b) New set-up (recycling of biomass after growth phase)

3.2.2. Media composition (inoculum and synthetic media composition)

A sample of recycled activated sludge (RAS) was collected from Humber wastewater treatment plant situated in Toronto, Canada. The collected RAS was filtered using 100 μm cell filter then, centrifuged and used as a seed for culture enrichment and added to 40 mL of the mineral salt media (MSM) in each of the culture bottles.

The mineral salt media (MSM) was prepared using the following concentrations as shown in **Table 3-1** (Bowman 2014); 1000 mg $MgSO_4.7\ H_2O$/L, 200 mg $CaCl_2.H_2O$/L, 272 mg KH_2PO_4/L, 610 mg K_2HPO_4/L, 4 mg Fe-EDTA. For each bottle with 50 mL MSM media, 1 mL of trace metal solution and 250 μl of $CuSO_4$ solution (corresponding to a final concentration of 1000mg/L) were added. The trace metal solution contained the following concentrations (mg/L): 10 $ZnSO_4.7H_2O$, 3 $MnCl_2.4H_2O$, 30 H_3BO_3, 3 $Na2MoO_4.2H_2O$, 200 $FeSO_4.7H_2O$, 2 $NiCl_2.6H_2O$ and 20 $CoCl_2.6H_2O$. Two different MSM were prepared to investigate the effect of nitrogen source i.e., Ammonia salt media (ASM) for A sets and Nitrite salt media (NSM) for B sets. In the ASM,

270 mg/L of ammonium chloride (NH_4Cl) were added to the MSM to achieve a final concentration of 5 mM, whereas in the NSM, 1700 mg/L of sodium nitrate ($NaNO_3$) was added corresponding to a final concentration of 20 mM.

Table 3-1: Chemical composition of mineral salt medium (MSM) and Trace elements

Mineral Salt Medium (MSM)		**Trace Elements**	
Compound	**mg/L**	**Compound**	**mg/L**
$MgSO_4.7\ H_2O$	1000	$ZnSO_4.7H_2O$	10
$CaCl_2.H_2O$	200	$MnCl_2.4H_2O$	3
KH_2PO_4	272	H_3BO_3	30
K_2HPO_4	610	$Na2MoO_4.2H_2O$	3
Fe-EDTA	4	$FeSO_4.7H_2O$	200
NH_4Cl	270	$NiCl_2.6H_2O$	2
$NaNO_3$	1700	$CoCl_2.6H_2O$	20

3.2.3. Analytical techniques

3.2.3.1. OD measurements

A periodic measurement of the optical density (OD) for monitoring the growth of the bacterial culture in both of the growth and PHB accumulation phase was performed using DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA) at (OD_{600}). A calibration curve was created to create a correlation equation between the volatile suspended solids (VSS)- assessed following standard protocol- and the OD. The VSS was further used to calculate the specific growth rate (μ) and the biomass yield (Y) using **Eqs. (3.1) and (3.2),** respectively (AlSayed et al., 2018)

$$\mu = \frac{\frac{(VSS_f - VSS_i)}{t}}{VSS_{av.}} \quad \textbf{(3.1)}$$

Where VSS_f is the final volatile suspended solids in (mg), VSS_i is the initial volatile suspended solids in (mg), $DCW_{av.}$ is the average of the initial and final volatile suspended solids in (mg) and t *is time in* (hr.).

$$Y = \frac{(VSS_f - VSS_i)}{Methane\ Consumption} \quad \textbf{(3.2)}$$

3.2.3.2. Gas Consumption

SRI 8610C gas chromatograph (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD) and molecular sieve column (Restek, Bellefonte, PA) was used to measure methane, oxygen, nitrogen and carbon dioxide concentrations. A tight glass gas syringe was used to take a gas sample from the bottles headspace which was then injected into the gas chromatograph. The program temperature for the injector, oven, and thermal conductivity detector (TCD) were set at 60, 80, and 80 °C, respectively. Helium gas with a flow rate of 15 mL min^{-1} was used as the carrier gas. External calibration curves were established using a known specific concentrations of gases mixture in order to calculate the area under the peak and convert it into gases concentrations. The calibration was performed through vacuuming the headspace of three 250-ml serum bottles filled with 50 ml water followed by the injection of 200 ml of different gas mixture with specific methane, oxygen, nitrogen concentrations. Afterward, a 1 mL sample was taken from the headspace and injected in the GC instrument which on its turn illustrated designated peaks used to perform calibration.

3.2.3.3. PHB extraction

PHB was extracted from cellular biomass and quantified using gas chromatography following the protocol described in (Teeka al., 2012) with some modification as follows [99]. First, 10 ml of culture media after growth phase and after PHB accumulation phase, was centrifuged at 8,000 g for 20 min at 20°C, then the supernatant was decanted. Afterwards, 10 ml of 5% sodium hypochlorite was added to the pellets, vortexed and incubated for one hour at 40°C in a shaker incubator, then the hypochlorite mixture was again centrifuged, and the supernatant was decanted. Subsequently, the previous sequence of centrifuging, decanting, adding liquid and vortexing was repeated using 10 ml of water, acetone, and ethanol, respectively. Thereupon, boiling Chloroform was added to the remaining pellets and filtered through a 0.5 µm syringe filter, followed by water addition to collect any impurities from the sample and then the mixture was further incubated for 2 hours at 55°C in a shaker incubator. Finally, the chloroform clear layer was transferred to another container and kept undisturbed overnight at room temperature to evaporate. Eventually, a white powdered film of the PHB polymer formed which was then used for further analysis.

3.2.3.4. PHB quantification

The PHB acquired from culture bottles was quantified using gas chromatography. First, PHB was dissolved 2 ml chloroform and transferred to glass vial. Next, the PHB solution was kept at 100 ∘C for 3.5 h in digester. Afterwards, 1mL of deionized water was added and the mixture was vortexed for 30 seconds two phases appeared; an aqueous top layer and a lipophilic bottom layer. Subsequently, 1 mL was withdrawn from bottom layer and injected into the GC for PHB quantification using SRI gas chromatography equipped with a flame ionization detector (SRI instrumentation, Torrance, USA) and MXT-wax column (Restek, Bellefonte, PA.). The

temperature program was as follows: 1 min 80 °C, 100 °Cmin-1, 180 °C for 4min. a standard curve was constructed using external standard with known concentrations of 5, 10, 20 mg of sodium hydroxybutyrate standard purchased from purity 99.9 % purity (Sigma Aldrich). Benzoic acid was used as an internal standard to improve results accuracy.

3.3. Microbial analysis

Two samples were taken from the enrichment during the cultivation to ensure the dominance of type II in the mixed culture. The first sample was taken after 9 cycles of cultivation while the second sample was taken after 16 cycles. The RNA purification and the amplification of the V4 region of the 16S SSU rRNA were performed with the Earth Microbiome Project benchmarked protocols. The RNA purification procedure was performed with the help of the RNeasy mini-kit by QIAGEN and a clean-up step with MoBio PowerMag soil DNA isolation kit as per the manufacturer protocol.

In summary, PCR amplification was carried out with a 25µl PCR combination of; deionized water, Hot Start PCR Master enzyme, 1 µl of the Template RNA, 0.5 ml of forward primer (10 µM), 0.5 ml of reverse primer (10 µM). The cycle program consisted of 94°C for 2 min, 94°C for 30 s, 50°C for 30°C, 72°C for 30 s, with a final polymerization step at 72°C for 10 min. The amplified RNA was then quantified with Quant-KiT PicoGreen dsDNA Assay Kit (ThermoFisher, USA). Afterwards, an amount of 240 ng from each sample was mixed into one tube and the clean- up process was carried out using MoBio UltraClean PCR clean-up kit. Next, Nanodrop was used to estimate final nucleic acid concentration to guarantee that the concentration isn’t too little or too much ranging from 1.8-2 to ensure proper sequencing.

Finally, sequencing was performed using the Illumina MiSeq personal sequencer (Illumina Incorporated, USA) at the McMaster Genomics Facility, Ontario, Canada. Filtration was performed with Cutadapt and trim adapter sequences and PCR primers from the preliminary read with a quality value of at least 30 and read length of at least 100bp. Sequence variants were then resolved from the trimmed raw reads using DADA2 to determine sequence variants. DNA sequence reads were filtered and trimmed, and taxonomy was given using the RDP classifier against the SILVA database version 1.2.8.

3.4. Characterization techniques

3.4.1. Sudan Black B staining

Sudan black B staining method. For further confirmation of PHA accumulation, Sudan black B is with a counter stain used to create a contrast allowing PHA observation with light microscope with oil immersion lens 1000X magnification. First, after heat fixation of bacterial isolate. The slide is submerged with Sudan black solution (0.3% Sudan black B w/v in 60% ethanol). After 10 mins the slide is rinsed with distilled water and the (0.5%) safranin counterstain is applied as well and left undisturbed for another 5 mins. A positive result for PHA accumulation shows dark blue scattered spots under phase contrast microscope.

3.4.2. Nucleic magnetic resonance (NMR)

The observed NMR spectra of 0.7 mL sample of 16 mg of acquired PHB sample dissolved in deuterated chloroform (CDCl3) used as a solvent at a final concentration of 10 g/L using a Bruker DXR 600 spectrometer at 24 °C with a 5-mm ^{1}H-probe, and. The ^{1}H-NMR and ^{13}C-NMR spectrum

for PHB was recorded at 600 MHz, they are used to show correlation between proton and carbon atoms.

3.5. Results and Discussion

3.5.1. Methanotrophs enrichment

A methane oxidizing community have been established after using a diluted sludge sample added to a mineral salt medium while providing methane as the sole carbon source. After 4 cycles of operation (start-up period), a consistent white color biomass dominated, and methane consumption have been observed. Gene expression has also been performed for further confirmation. Previous studies have focused on selecting only type II methanotrophs as the sole PHB producers relying on high ammonium concentrations [158]. However, these studies have proved that methanotrophic bacteria on its own are incapable to achieve accumulation percentage that can compete with other PHB producers such as *Azotobacter vinelandii* and *Pseudomonas fluorescens* of 85% and 70% respectively [159], [160]. This new setup aims to increase biomass diversity in order to allow for PHB producers to co-exist with the methane oxidizing bacteria and achieve higher accumulation rates through adding a screening PHB accumulation phase that constantly recycle back PHB producers into the system.

3.5.2. Growth Rate

The growth rates result for both AMS and NMS using conventional and new setup in the growth phase were substantially higher than those in PHB accumulation phase as shown in **Figure 3-2**. This observation is in accordance with literature due to the presence of all the necessary nutrients for bacterial growth unlike in the PHB accumulation phase where nitrogen deficient environment

was created which in its turn hindered normal bacterial growth [161]. Moreover, it was observed that NMS sets had higher growth rates than those of AMS in both setups. The maximum growth rates for the NMS sets were 15% and 40% higher than those of AMS sets in the conventional and new setup, respectively. The previous results can be attributed to the fact that ammonia exhibits competitive inhibition behavior with methane substrate, which allows a smaller array of bacteria to be enriched in comparison to nitrate [26]. The methane monooxygenase (MMO) enzyme activity is reduced in ammonia's presence due to the structural similarity that exists between ammonia and methane. Ammonia molecule compete with the methane molecule and attaches to the enzyme's active site which diminish its effectiveness in metabolizing the main substrate methane [74]. Moreover, the co-oxidation of ammonia carried out by MMO enzyme results in the production of hydroxylamine and nitrite byproducts, which can cause cellular toxicity [162]. However, this toxicity occurs only in type I methanotrophs since type II methanotrophs have the ability to metabolize these byproducts through the nitrification of the hydroxylamine to nitrous oxide through the action nitric oxide reductase N_2O from the oxidation of hydroxylamine [163]. Consequently, the relatively lower abundance of the fast-growing type I methanotrophs caused by the ammonia intoxication results in lower growth rates in the AMS sets. On the other hand, the presence of nitrate do not exert a similar inhibitory effect, in contrast it has been reported that nitrate requires less energy since it is directly metabolized to the final product which entails both type I and type II methanotrophs to metabolize it, hence, nitrate can be considered as a more efficient nitrogen source for bacterial growth [164]. Furthermore, the conventional set up had a slightly higher maximum growth rates of 0.187 hr^{-1} and 0.163 hr^{-1} for NMS and AMS, respectively compared to 0.145 hr^{-1} and 0.103 hr^{-1} in the new set-up **as shown if Figure 3-2 (a) and (b).** This is explained by the fact that in the conventional set up bacteria are recycled after the growth phase

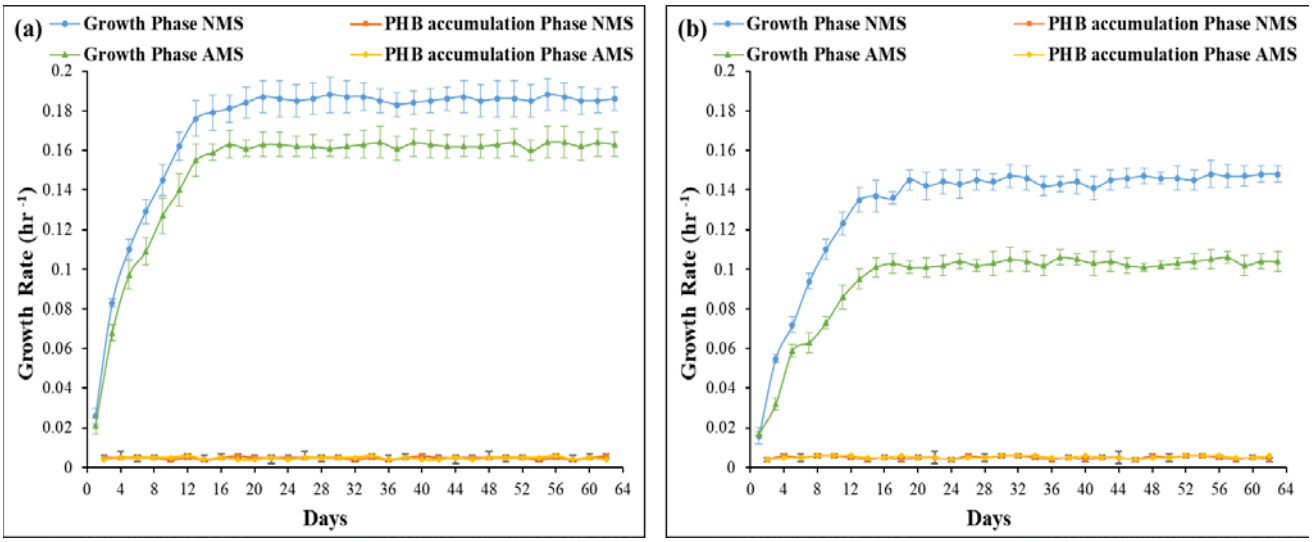

Figure 3-2: Growth Rate curves for, (a) Conventional Set-up (recycling of biomass after PHB phase and (b) New set-up (recycling of biomass after growth phase)

while they are still in the exponential phase unlike in the new setup where biomass is recycled after they reach the stationary phase, which indicates that more time is required to re-establish a high growth rate. As shown in **Figure 3-2 (a)**, the highest maximum growth rate was reached in the NMS sets of the conventional system, which can be associated with the dominance of fast-growing type I methanotrophs in the biomass due to the absence of the of the competitive inhibition caused by ammonia. This abundance was confirmed by the observation of the significant pink color change in the culture media attributed to the presence of type I methanotrophs [165]. The growth rate of mesophilic methanotrophs reported in the literature depends on the type of bacterial strain present in the culture media and have a wide range of 0.02 to 0.2 hr^{-1} which is an accordance with the values obtained in this study [48], [166], [167].

3.5.3. Methane uptake and Biomass yield

After the start-up period, a high methane uptake rate was recorded for all bacterial culture, which indicates the presence of methanotrophic bacteria. As shown in **Figure 3-3,** high methane uptake

rates were observed for NMS sets in the growth phase for both setups, which corresponds to their higher growth rates due to the absence of the previously explained competitive inhibition behavior when nitrate is being used as the nitrogen source. As such, the NMS sets' methane uptake rates were 2.63 ± 0.06 and 2.08 ± 0.09 mg CH_4/ hr, for conventional and new setup, respectively. In comparison, AMS sets had lower uptakes rate of 2.16 ± 0.09 and 1.77 ± 0.08 mg CH_4/ hr, for conventional and new setup, respectively which was as well in accordance with the observed growth rates. Moreover, it can be observed from the previous results that in the growth phase, the overall methane uptake rates in both sets of the conventional setup were higher than those of the new setup, which may be referred to the recycling strategy, which enabled bacterial biomass to consistently stay in boosting growth conditions. The previous results were in the same range reported in the literature of 2.25-2.375 mg CH_4/hr [168].

On the other hand, in the PHB accumulation phase, methane uptake rates were not in correlation with the observed growth rates as high methane uptake rates were measured implying that the methane was consumed through a different carbon assimilation pathway other than cellular growth. One potential pathway for methane consumption is its storage inside the bacterial cell as energy storage in the form of PHB which usually occur in nutrients deficient environments which is the case in the PHB accumulation phase. Commonly, the abundance of nutrients triggers the initiation of the tricarboxylic acid (TCA) cycle for both type I and II methanotrophs either after the completion of RuMP pathway for the former or serine pathway for the latter which in turns results in biomass growth. On the other hand, in the case of nutrient deficient conditions, type II methanotrophs has the ability to further oxidize the glycerate resulting from the serine cycle and convert it to carbon based intracellular polymer i.e., PHB through the PHB cycle. Furthermore, lower methane uptake rates were observed in the PHB accumulation for the conventional setup

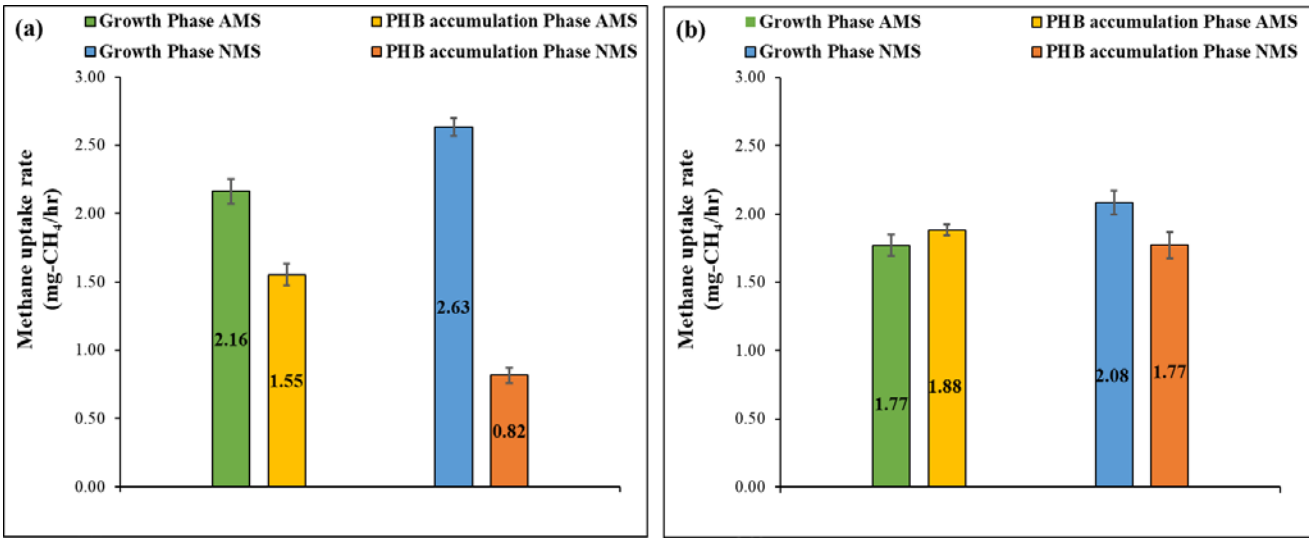

Figure 3-3: Methane uptake rate for, (a) Conventional Set-up (recycling of biomass after PHB phase and (b) New set-up (recycling of biomass after growth phase)

compared to those of the new setup. The previous observation may be correlated to the difference in the selection criteria applied. In the new setup the recycling technique, as previously mentioned, hinders the multiplication of type I methanotrophs non PHB accumulators and stimulate the growth of type II methanotrophs, which in turn consume the methane for the purpose of PHB accumulation in unfavorable conditions. On the other hand, in the conventional setup the lack of selection pressure for PHB accumulators reflects on the reduced methane uptake in the PHB accumulation phase since the type of bacteria present in the system do not have the ability either to grow or store PHB in nutrient deficient environment. This is reflected in the consumption rates where methane uptake rates of 1.88 and 1.76 mg CH_4/hr were observed in the new setup compared to 1.55 and 0.82 mg CH_4/hr in the conventional setup for AMS and NMS, respectively. Moreover, it was noteworthy that NMS set in the conventional setup resulted in considerably lower uptakes rates compared to all the other sets which is referred to the absence of any PHB accumulators selection criteria resulting from using nitrate as a nitrogen source (no nitrogen based selection) and recycling after the growth phase (no design based selection).

In terms of yield, it illustrates the relationship between the growth rate and methane uptake rate as shown in **Figure 3-4.** In the growth phase the biomass yield for the four sets were comparable and ranging between 0.5-0.6 mg VSS/mg CH_4 which is in accordance with the values reported in the literature for methanotrophic bacteria, of around 0.4 to 0.6 mg VSS/mg CH_4 [166], [169], [170]. On a similar note, the biomass yield in the growth phase was substantially higher than the PHB accumulation phase due to the low growth rate in the accumulation phase compared to the relatively high methane uptake rate. The previous observation highlights the aforementioned discussed deduction of the methane utilization in a different pathway other than growth, i.e. PHB accumulation in the accumulation phase. On the other hand, high biomass yield was observed in the PHB accumulation phase was in the case of NMS set of the conventional setup only which is referred to the corresponding low methane rate due to the absence of PHB accumulator selection where nitrate enabled bacterial growth.

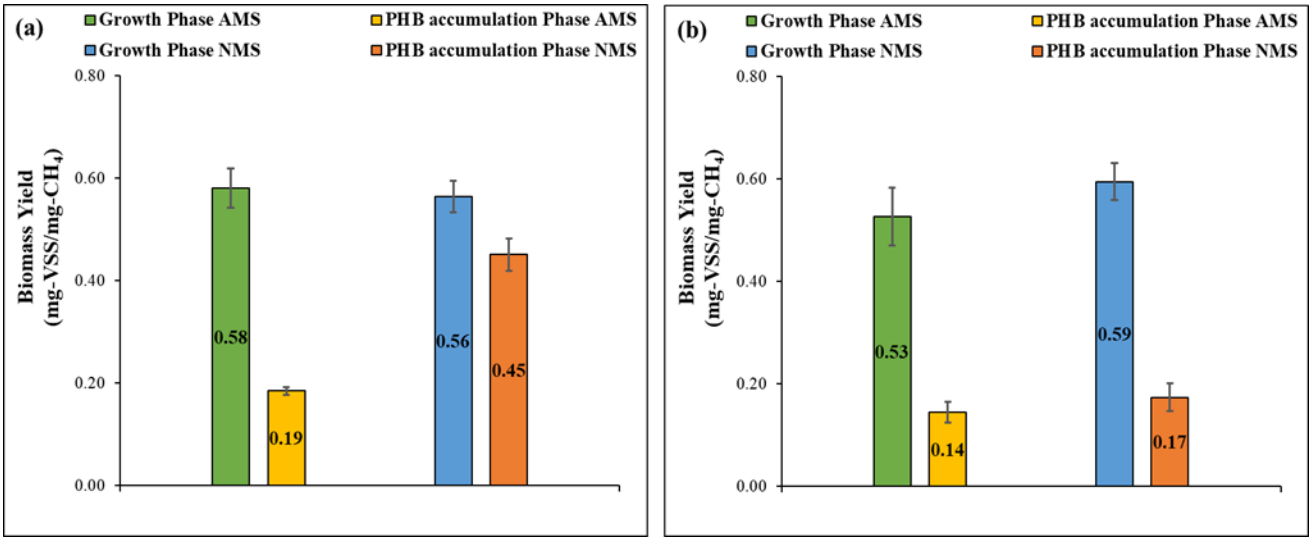

Figure 3-4: Biomass Yield for, (a) Conventional Set-up (recycling of biomass after PHB phase and (b) New set-up (recycling of biomass after growth phase)

3.5.4. PHB accumulation

As the experiment progressed, a gradual increase in PHB accumulation measurements was noticed in all sets. After the 5th cycle of operation the extracted polymer amount stabilized and was in a constant range within the subsequent cycles, this corresponded to the observation of a distinct whitish color in all sets except for the NMS conventional set where a slightly pinkish color was observed. The polymer storage was initiated as a result of the stress conditions of nitrogen deficiency applied in the PHB phase, which explains the very low accumulation percentage recorded in the growth phase where nitrogen was abundant. The PHB cycle usually is intercorrelated with the serine cycle in type II methanotrophs. PHB accumulation is normally initiated with a spontaneous reaction occurring by conversion of formaldehyde through a designated pathway into 5,10-methylene-tetrahydrofolate (MTF) which units with one carbon dioxide molecule to produce the amino acid serine [43], [171]. Through a set of orchestrated phosphorylation reactions, serine is further converted into glycerate, phosphoenolpyruvate, malate, malyl co-A and finally glyoxylate [38]. It is noteworthy of mentioning that the glyoxylate regeneration cycle (GRC) links the serine cycle with the TCA and PHB cycle by constantly converting and regenerating the acetyl coenzyme A to glyoxylate and constantly feed it back into the serine cycle [59], [171]. The glyoxylate and malyl-CoA are incorporated in the tricarboxylic acid cycle TCA cycle by conversion to isocitrate of the former and acetyl-CoA of the latter, which are considered terminal products of the serine cycle. In the case of the absence of nutrients essential for TCA cycle completion, the PHB cycle is triggered to store an alternative supply of energy that acts as a reducing potential for carbon assimilation when nitrogen is depleted [14]. Simultaneously, an important carbon assimilatory pathway is integrated with PHB cycle which is ethyl malonyl-CoA (EMC) pathway which encompasses several CoA derivatives to deliver awith 3-

hydroxybutyryl-CoA molecule, where coenzyme A is released through a stepwise catalytic pathway take place in order to form the hydroxybutyrate monomer with the help of a set of enzymes expressed by some specifically PHB related genetic codes called phaA, phaB, and phaC that express the enzymes; β-ketothiolase, acetoacetyl-coA reductase, and PHB synthase respectively [58]. Thus, the 3- hydroxy-butyryl (HB) monomer is accordingly added to the polymeric chain and stored intracellular in a granular form. On the other hand, in nutrient sufficient conditions after the serine cycle, instead of taking the PHB pathway the acetyl Co-A produced combine with another acetyl Co-A molecule to produce citrate which marks the start of tricarboxylic acid cycle to produce energy for biomass synthesis through the electron transport chain.

A gradual increase in polymer accumulation was observed especially in AMS in the first few cycles almost no Polymer accumulation was detected after the 5^{th} cycle a 25% accumulation was detected, and a 10% increase of accumulation was observed until a constant result was reached. High accumulation percentage was measured in ammonia enriched culture of around 59% using the new design as opposed to almost 38% using the conventional setup which is in accordance to what was found in the literature [27]. Another finding of great importance is that it was found that a 450% increase in PHB accumulation when comparing the new set up to the conventional in NMS medium, which confirms the success of the tested hypothesis, that PHA accumulating methanotrophic consortium could grow without the restriction of a specific nitrogen source and relying on cellular toxicity.

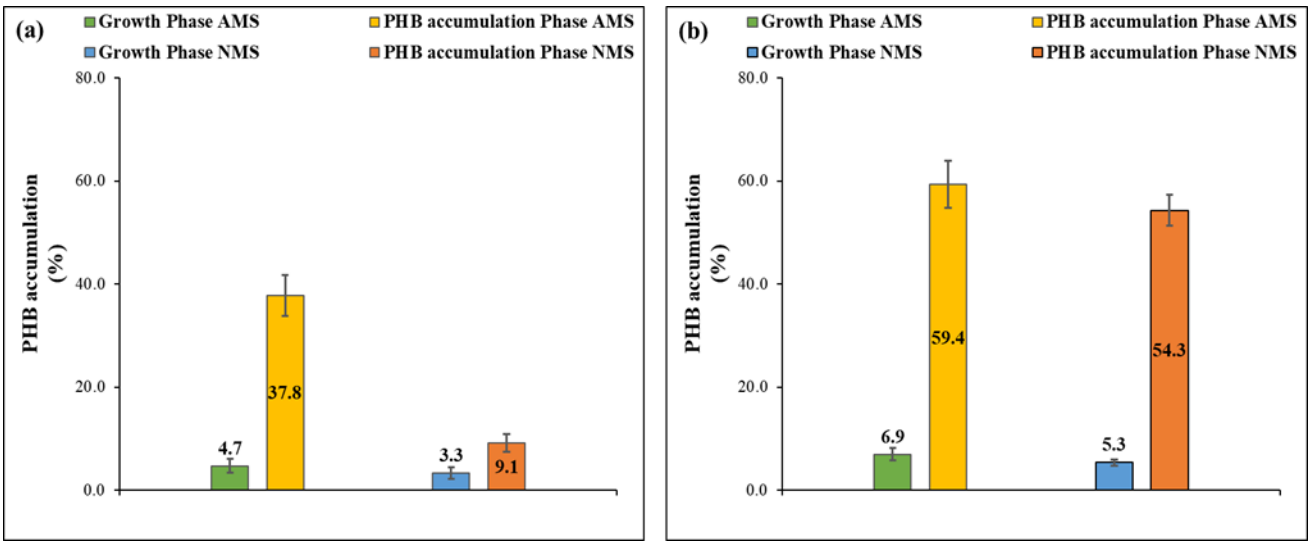

Figure 3-5: PHB accumulation percentage for, (a) Conventional Set-up (recycling of biomass after PHB phase and (b) New set-up (recycling of biomass after growth phase)

3.5.5. Microbial analysis

Two samples of nitrate-enriched culture were sent for a microbial analysis at McMaster University after 3 and 9 weeks of experimentation. The microbial analysis of the first sample demonstrated a dominance of non PHB-accumulating bacteria, type I methanotrophic bacteria of around 39% *methylophilus* species indicated by mint green color, while type II methanotrophs only comprised 15% *methylocystis* of the total microbial community as shown in **Figure 3-6 (a)**. After 9 weeks of experimentation which is equivalent to 16 operational cycles, a shift in the microbial community has occurred, from a dominance of gamma-proteobacteria of type I methanotrophs to a dominance by *pseudomonas* genus ≈ 31% in the yellow color, as illustrated in **Figure 3-6 (b).** Pseudomonas bacteria is able to feed on a variety of carbon substrates and is well known for PHB accumulation capability as it can accumulate more than 80% of their cell dry weight [172], [173]. This substantial increase in Pseudomonas can in turn explain the high PHB accumulation percentage acquired in the last experiment. Furthermore, type II methanotrophs have as well witnessed around a 50%

increase reaching around 21% *methylocystis* species. On the other hand, the increase of PHA producers was accompanied by a decrease in type I methanotrophs from ≈ 40% to only 6% of the total microbial community. This shift confirms that the newly proposed experimental design has successfully screened for the PHB accumulators, where type II methanotrophs were accompanied by other PHB accumulators that co-exist in this methane rich environment while uptaking metabolic byproducts of methanotrophic bacteria as substrate without relying on ammonia toxicity.

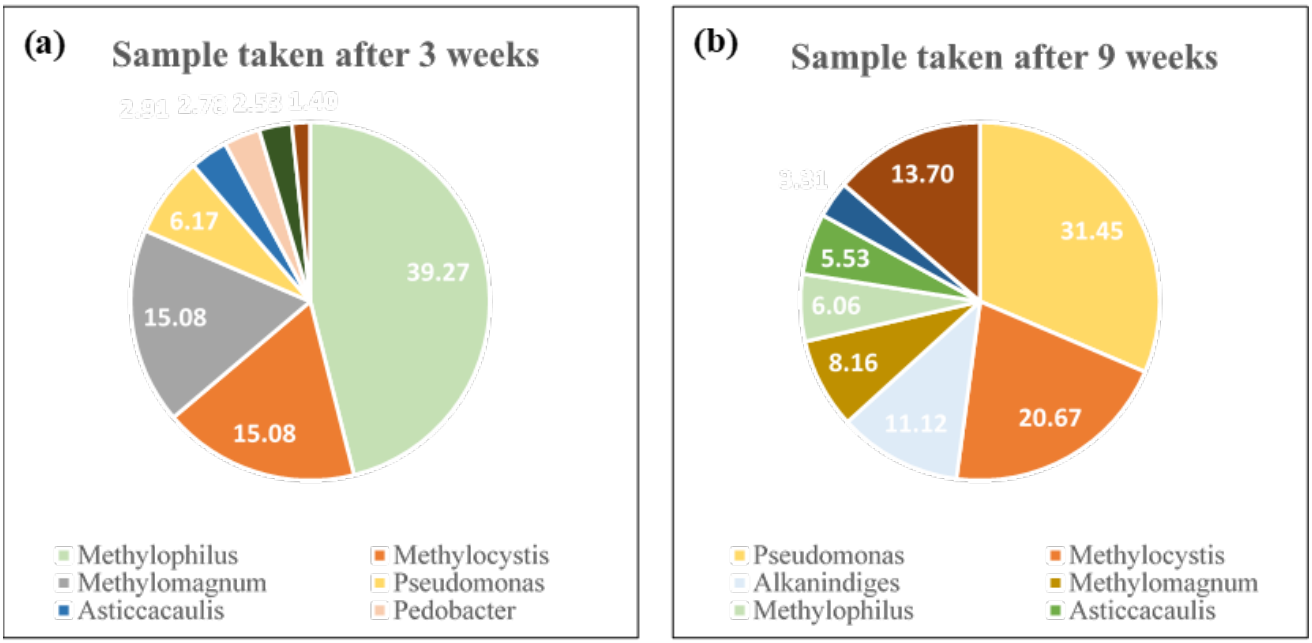

Figure 3-6: Microbial analysis for samples taken from the NMS enriched biomass of the new setup: (a) sample taken after 3 weeks of enrichment, and (b) sample taken after 9 weeks of enrichment

3.5.6. PHB Characterization

3.5.6.1. Sudan black and extracted powder

Sudan Black B dye is a lipophilic dye that attaches to the ester bond in the polymeric chain of the PHB granule and is used for the preliminary detection of PHA lipid inclusions [113]. The

absorption of this lipophilic dye is reflected by the observation of scattered dark blue spots, which confirms the presence of PHA granules within the microbial cells as shown **Figure 3-7 (a)**.

PHA extraction was carried out with sodium hypochlorite protocol, which is crucial for further characterization of the biopolymer with other analytical techniques [99]. High purity extraction was achieved and confirmed by the NMR spectra by adding multiple steps, washing, and polymer filtration. The extracted polymer is in the form of a white powder that is prone to crystallization under specific conditions as shown in **Figure 3-7 (b)**.

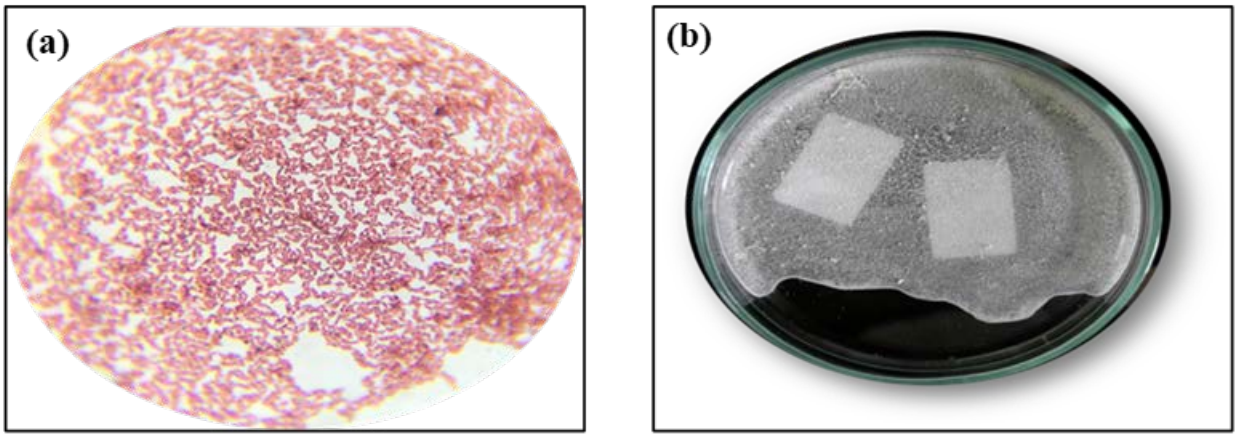

Figure 3-7: PHB granules (a) observed under confocal microscope using Sudan Black B dye (b) extracted polymer powder with sodium hypochlorite method

3.5.6.2. Nuclear magnetic resonance (NMR)

The proton and the carbon NMR indicate the presence of different functional groups at varying chemical shifts as shown in **Figure 3-8** (C=O, CH, CH_2 and CH_3). The NMR spectra for PHB polymer was in accordance with PHB spectra recorded by Jiang et al. (2008) which confirms the structural formula for the acquired polymer [160].

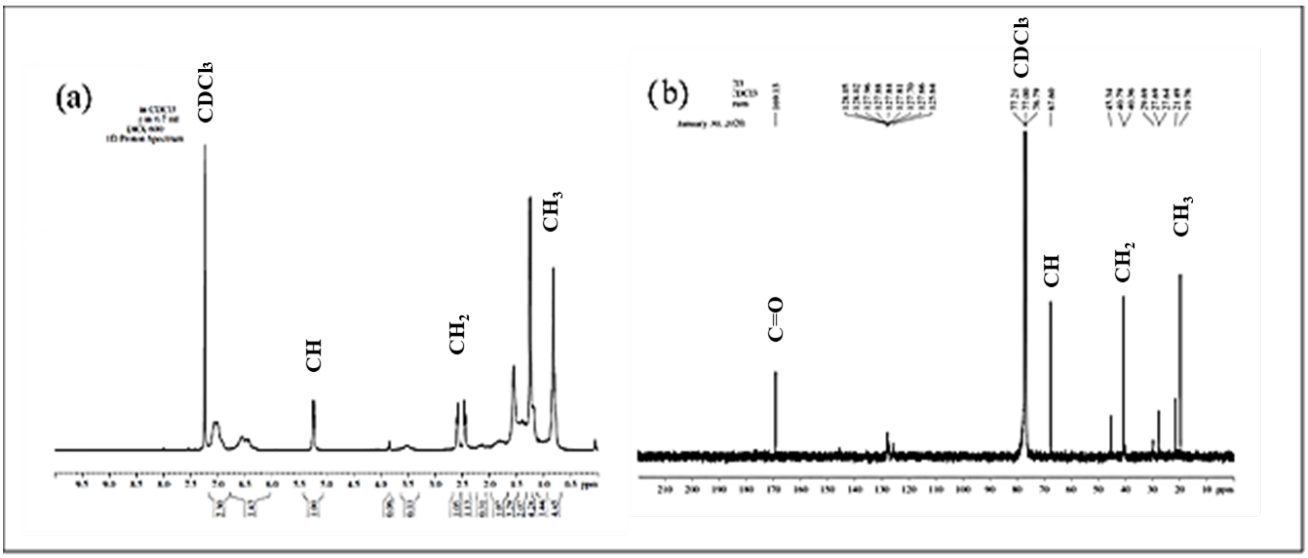

Figure 3- 8: NMR for extracted PHB polymer, (a) Proton NMR and (b) carbon NMR

The peak number 1 was observed at 1.2 ppm and 19.76 for ^{1}H- NMR and ^{13}C- NMR respectively and was assigned to terminal methyl proton **CH_3**. The multiple peak number 2 at the range 2.4-2.6 ppm and 40.79 which were assigned to methylene protons **CH_2** adjacent to the **CH** carbon in the sidechain. Peak number 3 at 5.2 ppm and 67.6ppm was assigned to **CH=CO proton**. The Peak number 4 at 169.13 in ppm ^{13}C- NMR which was assigned to the carbonyl group **C=O** as shown in **Table 3-2.**

Table 3-2: The chemical groups and their chemical shift signals for ^{13}C NMR and ^{1}H NMR spectra

Functional groups	^{13}C NMR (ppm)	^{1}H NMR (ppm)
1. CH_3	19.76	1.2
2. CH_2	4.79	2.4-2.6
3. CH	67.60	5.2
4. C=O	169.13	-

3.5. Conclusion

The main objective of this chapter is to develop a comprehensive comparison between two experimental set-ups for favoring the growth of PHB accumulators: the conventional ammonia dependent setup and the newly proposed setup which is ammonia independent and rely solely on the recycling technique. In this experiment nitrogen limitation was the main precursor for the initiation of PHB accumulation process, which was achieved through two cultivation stages: the growth phase (nitrogen rich) and the PHB accumulation phase (nitrogen limited). Furthermore, two nitrogen sources were employed since the main objective was to attain high PHA accumulation percentage that is analogous to that of ammonia's enforcing the concept that ammonia as a nitrogen source is not essential for the selection of type II PHB accumulators. The success of the newly proposed setup was confirmed by achieving the highest PHB accumulation percentage recorded for a mixed culture community in both AMS and NMS media of 59.4% and 54.3%, respectively, compared to 37.8% and 9.1% for the conventional setup. The growth rate in the new setup of 0.103 hr^{-1} and 0.145 hr^{-1} for AMS and NMS respectively was lower than that of the conventional setup 0.187 hr^{-1} and 0.163 hr^{-1}. A steady methane uptake rate was also achieved throughout this experiment for both setups measuring in NMS 2.63 ± 0.06 and 2.08 ± 0.09 mg CH_4/ hr while AMS sets measured 2.16 ± 0.09 and 1.77 ± 0.08 mg CH_4/ hr where the former is the new setup and the latter is the conventional setup. Furthermore, the biomass yield illustrated the relationship between the growth rate and the methane consumption rate where the high consumption rate in the PHB phase was not correlated with a high growth rate in the PHB phase similar to the growth phase. The biomass yield was in the range of 0.5-0.6 mg VSS/mg CH_4 almost in all sets except NMS of the conventional setup in the PHB phase where low growth rate, low methane consumption accompanied by low PHB accumulation percentage was observed, this was

associated with the lack of any selection pressure to favor the growth of PHB accumulators as no ammonia was provided and the recycling technique didn't screen for PHB accumulators. Finally, the microbial sequencing of microbial samples showed a significant increase in the ratio of type II methanotrophic bacteria after 9 weeks of experimentation. Furthermore, an interesting finding was observed in NMS sets of the new setup where microbial analysis showed that after 3 weeks of experimentation the presence of a methanotrophic community did not favor PHB accumulators since it was dominated by type I methanotrophs. However, after 9 weeks of experimentation a microbial shift towards PHB accumulators was established through the enrichment of other PHB producer that co-existed with type II methanotrophs. This interesting finding opens the door towards the enrichment of a diversity of microbial groups that can support the presence of methanotrophic bacteria either by removing toxic byproducts or enhance their metabolic activities or by accumulating PHB within their own cells which can help supersede the threshold accumulation recorded for methanotrophic bacteria.

Chapter 4: Response Surface methodology model using central composite design optimization of a mixed culture methanotrophic community

4.1. Introduction

The constant use of petroleum-based energy is replenishing fossil fuel reserves and creating major environmental ramifications [174]. The emission of toxic chemicals resulting from fossil fuel burning, such as sulfur dioxide and heavy metals generated from coal combustion cause phenomena such as acid rain and many life-threatening diseases such as respiratory illness and cancer. In addition, the emission of gaseous particles such as carbon dioxide and methane are resulting in a rise in global temperatures due to their heat trapping capability, which can alter ecological cycles and cause extinction of important species. For instance, plastic production process is comprising between 2.5% to 3.6% of oil and natural gas of global consumption valued as 270 million tons in 2008 only [175]. This number is expected to witness an incremental increase to reach 20% by the year 2050 leaving behind around an estimate of 15% of the global carbon foot print [176]. In addition, the accumulation of these petroleum based polymers in the environment is producing more damage to the different living organisms [155]. Therefore, many industrial entities are currently choosing bioplastics manufacturing over petroleum-based plastic as a sustainable solution to the adverse impact associated with conventional plastic. In fact, it is predicted that global bioplastic manufacturing and production will increase by 300% within the next few years. However, the production cost, limited recycling, and poor disposal practices represent the main drawbacks for the globalization of bioplastics [97]. Hence, one of the viable bioplastics product is PHA, a microbially synthesized polymer, which is offered as an alternative

since it is a biodegradable, compatible and can be produced using renewable feedstock [177]. Nevertheless, it has been estimated that operational expenses alone comprise around 11% of the total production cost. Moreover, maintenance of a pure culture community and PHA marketing increase the total production cost by 400% to 900% in comparison with petroleum based. Thus, the establishment of a stable mixed culture PHA producing community has become imperative for cost reduction, as well as process optimization. The convenience of feedstock availability within WWTPs ensured the feasibility of the prospective process of PHA production systems in separate treatment tanks following anaerobic digestion, where process design relies on the availability of PHA producing bacteria within wastewater treatment sludge. However, process optimization is crucial to obtain information about the relationship between different factors and how they can be manipulated to enhance PHA production, which can help reduce operational cost.

4.2. Optimization techniques

The implementation of Design of experiment (DOE) is the most promising method to recognize and adjust the most influential variables in order to obtain a fully comprehensive overview of an operational system. Moreover, DOE relies on statistical analysis to generate a factorial design that evaluates the influence of different factorial values on the process output in order to achieve successful experimentation. DOE is characterized with having two or more independent variables that are tested simultaneously unlike in an unpremeditated experiments where only one independent variable is tested at a time [178]. Therefore, DOE can be considered as a methodology technique to analyze and decipher a certain predicament since it is used to gather information and to scrutinize these inputs to acquire valuable outputs. Consequently, analysis of the response exerted by the factors' interaction will maximize information and minimize time and money

invested in the process analysis [179]. Furthermore, optimization is a form of DOE that establish the optimal parameters of a process. There are various optimization designs that can be fitted into different operational scenarios such as: Taguchi design, central composite design (CCD), Box–Behnken design (BBD), and Full factorial design (FFD). In order to choose a design that fits each experimental need, the DOE must be adapted to the problem, not the opposite. Afterwards, a DOE is associated to the model itself, then the main effects followed by the interaction effects and finally the quadratic effects **(Figure 4-1).**

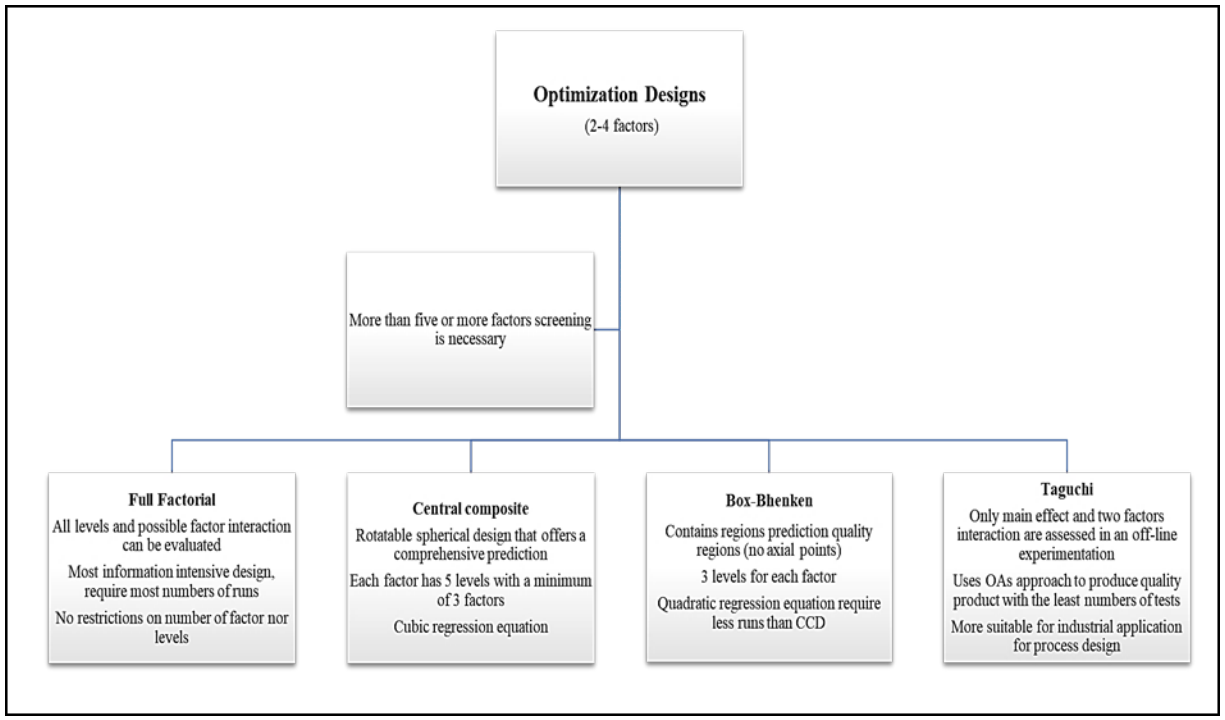

Figure 4-1: Different types of optimization designs employed in DOE

The Taguchi design is a set of design stages where the intrinsic variability of constituents and manufacturing stages have to be considered at the design phase [65]. As such, it is considered as an off-line type of fractional factorial design that uniquely uses Orthogonal Arrays (OAs) approach to guarantee that all levels of all factors are reflected equally [180]. Taguchi design relies on three stages: (i) concept, (ii) parameter and (iii) tolerance design, where concept design is applied to

identify the appropriate values of the design levels, while parameter design is implemented for a more dynamic response, lastly, the tolerance design is used for determining the results of the parameter design through constriction of the tolerance of the variables [181]. Therefore, OAs method allows the evaluation of both controlled factors and noise factors caused by increased variability in the design parameters, which is usually ignored in most types of other factorial designs, in order to minimize the discrepancy in response values [65]. Therefore as a result of using the Taguchi techniques, the industries are saving both time and money invested to increase product quality through process and design optimization [182]. However, the main disadvantage of the Taguchi method is that the results obtained are not absolute because factors interactions are not considered , and do not exactly indicate what parameter has the highest effect on the performance characteristic value where only the main effects and two-variable interactions are studied [183].

On the other hand, the Box-Behnken design (BBD) is a type of a quadratic model that resembles normal factorial models. However, it uses smaller number of required runs, since it analyses only three factorial levels, forming an incomplete block design [184]. The factorial level values points: -1,0,1, are always situated at the midpoints of the axes and represent the number of runs required for this design as shown in **Fig 4-2.** Whereas, the number of runs can be determined using **equation (4.1)**

$$\boldsymbol{N = 2\,x\,f\,x\,(f-1) + C_o} \qquad \textbf{(4.1)}$$

where $\boldsymbol{f}$ is the number of factors and $\boldsymbol{C_o}$ is the number of center points. It is worth mentioning that BBD does not account for the corner points in the quadratic model, which means that it does not test extreme points where there might be an influential effect outside of the tested area. Therefore,

the BBD model that cannot be employed in sequential experiments since it is an independent design and does not have any factorial matrix embedded within [185]. On the other hand, BBD provide high order estimation of the first and second order coefficients optimizable responses and is suitable for use where safe operating zones of a process are familiar, and where the axial points are not of interest. Moreover, BBD is less expensive to conduct than CCD and FFD because it requires fewer numbers of trials, since it avoids experiments, which are in extreme conditions [186].

Furthermore, Central composite designs (CCD) is a rotatable design that is considered as the most commonly used type of response surface experiment designs. CCDs are especially useful in sequential experiments because it allows building on previous factorial experiments by adding axial and center points. In addition, this quadratic model appears to be the most suitable replacement of a three level full factorial design since it offers analogous outcomes with fewer runs [184]. The advantage of using CCD design mainly lies in its robustness in evaluating first and second order equations while simulating a response variable with curvature by adding center and axial points to a previously established factorial design [187]. The axial design and the central design are established by testing five factorial levels for each variable, which allows predictions of extreme conditions [188]. CCD requires numbers of run according to **equation 4.2** as follows:

$$\boldsymbol{N = f^2 + 2f + C_o} \qquad \textbf{(4.2)}$$

Moreover, CCD is the most commonly used modelling approach that is employed in the response surface methodology (RSM), a frequently utilized optimization tool [186]. This statistical technique studies the interactions between several variables to determine the empirical model in order to modify and adjust process parameters. In addition, this mathematical model is efficient

and economical as it enables the experimentation of fewer runs than other models, which enable minimal resource utilization.

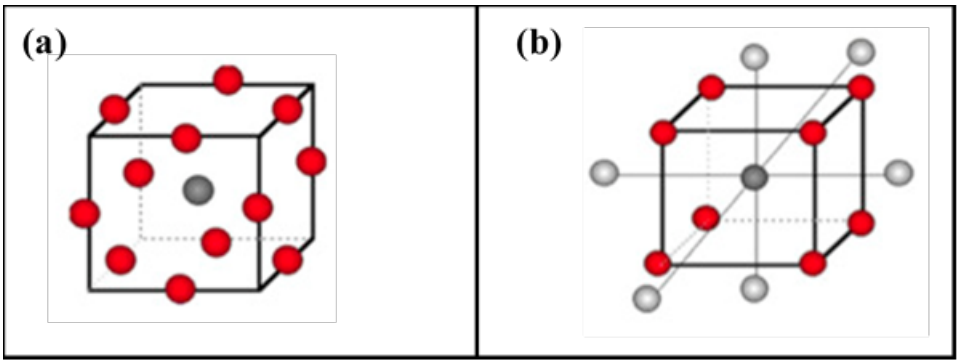

Figure 4-2: Number of runs required for the design model: (a) BBD, and (b) CCD

Another common experimental design is the full factorial design (FFD) where all variables customized at two levels: +1 and -1. The full factorial can test more than two factors where all potential combinations are evaluated [189]. This highly efficient design is very helpful to generate a detailed comprehensive understanding of variables interactions and process operation. However, the more factors to test the more runs and experimentation it requires [190]. Another disadvantage to the full factorial design is the need of accurate planning, as a small mistake in one of the levels, or in the overall process, will falsify enormous numbers of output data [81].

4.3. Materials and Methods

4.3.1. Setup and Operational condition

All the experiments were conducted in 250 ml culture bottles with a butyl rubber cap to enable gas vacuuming and subsequent injection. In each bottle, 10 mL of seed was added to 40 ml of mineral salt medium (MSM) for culture enrichment. Further details about experimental setup and operational conditions can be found in Chapter 3 section 3.2.1. This experiment aimed to discuss the effect of the initial methane to oxygen ratio on the growth of the enriched culture with ammonium as nitrogen source. In addition, to study if this ratio would have an impact on the ammonium co-oxidation. Four ratios were tested by changing the gases composition in the headspace after vacuuming every 24 hours and administer a daily feed of 1:3, 1:2, 1:1, 2:1, and 3:1 of methane to oxygen ratio while Helium was added to complete the headspace to 200 ml. All the bottles had an initial O.D of 0.3 ± 0.05. All other conditions were similar to the previous experiments. The enrichment culture bottles were incubated in an orbital shaker incubator operated at different temperatures of 15, 23, 25, 30, or 37 °C with a mixing speed of 160 rpm. The experiment was monitored by regularly measuring optical density (OD), and PHB accumulation after each stage of growth and PHB phase.

Consecutive cycles of fed-batch mode were implemented along this study to investigate different parameters affecting the methane fed culture, and prospective PHB accumulating consortium. All sets consisted of 48 hours for the growth phase and 12, 24, 36, 48, and 60 h PHB phase duration. The growth phase media contained an ammonia source in order to create a nutrient sufficient stage aimed for bacterial enrichment. Afterwards, the biomass was transferred to the PHB accumulation phase, which was devoid of any nitrogen source to create a nutrient deficient stage to enable the

initiation of the serine enzymatic pathway and start the selection of PHB accumulators. All sets were operated following the newly proposed intermittent cycling technique, which followed the nutrient sufficient and deficient alternating settings, of nitrogen sufficiency and starvation respectively.

4.3.2. Design of Experiment

The DOE applied in this experiment is the CCD design model, which is a type of response surface methods that gives information about factors interaction. CCD was used for studying the effect of multiple factors in order to optimize the response of PHA accumulation and growth rate. CCD is a type of response surface methodology (RSM), used for building second order (quadratic) equation for the response variable without needing to use a complete 3 level factorial design. The parameters known to affect PHA accumulation in methanotrophic culture were selected according to previous experiments and literature survey. As such, four key variables which are temperature, ammonia concentration, duration of PHB phase and carbon to oxygen ratio with five levels for each were tested in this experiment as shown in **Table 4-1.** Accordingly, 30 experiments were conducted containing six center points for estimating the experimental uncertainty variance.

Table 4-1: Variables tested in the CCD factorial design and the values of their 5 levels

Variables	**Units**	**Coded and real values of variables**				
		-2	**-1**	**0**	**1**	**2**
X_1 Carbon/Oxygen ratio	-	1:3	1:2	1	2:1	3:1
X_2 Nitrogen Concentration	mM	1	3	5	7	10
X_3 Temperature	ºC	15	23	25	30	37
X_4 Incubation Time	hr	12	24	36	48	60

The relationships and interrelationships of the variables were determined by fitting the second-degree polynomial equation to data obtained from 30 experiments using mean values of the number of cycles. Since four variables were studied, the polynomial equation was created to

estimate the interaction between the levels of different factors form a sphere that gives an overall prediction for the entire model and allows curvature calculation. Since four variables were studied, the polynomial equation was created using **equation (4.3)** as follows:

$$Y = B_o + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{14}X_1X_4 + B_{23}X_2X_3 + B_{24}X_2X_4 + B_{34}X_3X_4 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 + B_{44}X_4^2 \quad \textbf{(4.3)}$$

Where X_1, X_2, X_3, and X_4 are input variables; B_o is a constant; B_1, B_2, B_3, and B_4 are linear coefficients; B_{12}, B_{13}, B_{14}, B_{23}, B_{24}, **and** B_{34} are cross-product coefficients; B_{11}, B_{22}, B_{33}, and B_{44} are quadratic coefficients.

MINITAB software was used for regression and graphical analyses of the data obtained. The response surface and contour plots were generated to understand the interaction of various variables.

4.3.3. Media composition

A sample of recycled activated sludge (RAS) was collected from Humber wastewater treatment plant situated in Toronto, Canada. The mineral salt media (MSM) was prepared as described in Chapter 3 section 3.2.2 except for the nitrogen source concentration which was changed according to the desired ammonia concentration required for optimization. Thus, ammonium chloride (NH_4Cl) was added to the MSM with the following concentration of 54, 162, 270, 378, and 540 mg/L to correspond to a final ammonia concentration of 1, 3, 5, 7, and 10 mM.

4.3.4. Analytical techniques

4.3.4.1. OD measurements

A periodic measurement of the optical density (OD) for monitoring the growth of the bacterial culture in both of the growth and PHB accumulation phase was performed using DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA) at (OD_{600}). A calibration curve was created to create a correlation equation between the volatile suspended solids (VSS)- assessed following standard protocol- and the OD. The VSS was further used to calculate the specific growth rate (μ) and the biomass yield (Y) using **Eq. (3.1)**

4.3.4.2. PHB extraction

PHB was extracted from cellular biomass and quantified using gas chromatography following the protocol described in (Teeka et al., 2012) with some modification as follows [99]. First, 10 ml of culture media was centrifuged at 8,000 g for 20 min at 20°C, then the supernatant was decanted. Afterwards, 10 ml of 5% sodium hypochlorite was added to the pellets, vortexed and incubated for one hour at 40°C in a shaker incubator, then the hypochlorite mixture was again centrifuged, and the supernatant was decanted. Subsequently, the previous sequence of centrifuging, decanting, adding liquid and vortexing was repeated using 10 ml of water, acetone, and ethanol, respectively. Thereupon, boiling Chloroform was added to the remaining pellets and filtered through a 0.5 μm syringe filter, followed by water addition to collect any impurities from the sample and then the mixture was further incubated for 2 hours at 55∘C in a shaker incubator. Finally, the chloroform clear layer was transferred to another container and kept undisturbed overnight at room temperature to evaporate. Eventually, a white powdered film of the PHB polymer formed which was then used for further analysis.

4.3.4.3. PHB quantification

PHB was quantified using a widely used spectrophotometrically method which basically relies on the conversion of PHB to crotonic acid by the depolymerization action of sulfuric acid. Following the same aforementioned extraction steps, however, the chloroform is left to evaporate and the PHB powder is transferred to a glass vial. Afterwards, the glass vial containing PHB powder and concentrated sulfuric acid are boiled in order to convert the PHB into the brown colored crotonic acid. The crotonic acid color intensity signifies the concentration of the PHB polymer which is measured at 235 nm using UV-spectrophotometer against a sulfuric acid blank, after calibration curve is constructed with Sigma Aldrich PHB standard. PHB content is estimated in the units mgPHB/mgCDW. After measuring the biomass density at wavelength 600 and converting it to CDW using calibration curve [126][116] [82].

4.4. Results and discussion

A CCD was established for variables affecting the growth rate and PHB production of methanotrophic bacteria culture. A 2^4 CCD with 8 axial points (α=2) and 6 replications at center (n_o=0) leading to total number of 30 experiments was employed for optimization of the parameters. The CCD for four variables along with PHA production and growth rate values as response are presented in **Table 4-2**. In the following sections, the independent effect of those variables as well as the effect of the interaction between them on methanotrophs growth rate and PHB production will be discussed in detail.

Table 4-2: Central Composite Design matrix for 4 variables along with their observed responses

Run	X_1	X_2	X_3	X_4	PHB %	Growth Rate (hr^{-1})
1	-1	-1	-1	-1	46.7	0.080
2	-1	-1	-1	1	34.5	0.088
3	-1	-1	1	-1	54.6	0.117
4	-1	-1	1	1	49.2	0.129
5	-1	1	-1	-1	46.9	0.112
6	-1	1	-1	1	35.2	0.137
7	-1	1	1	-1	54.8	0.153
8	-1	1	1	1	49.8	0.156
9	1	-1	-1	-1	41.1	0.080
10	1	-1	-1	1	30.9	0.093
11	1	-1	1	-1	51.1	0.121
12	1	-1	1	1	46.0	0.142
13	1	1	-1	-1	41.5	0.132
14	1	1	-1	1	31.4	0.140
15	1	1	1	-1	51.5	0.128
16	1	1	1	1	46.3	0.149
17	-2	0	0	0	37.6	0.106
18	2	0	0	0	11.4	0.113
19	0	-2	0	0	19.2	0.073
20	0	2	0	0	41.8	0.135
21	0	0	-2	0	23.8	0.076
22	0	0	2	0	42.6	0.141
23	0	0	0	-2	25.4	0.085
24	0	0	0	2	13.1	0.112
25	0	0	0	0	44.9	0.106
26	0	0	0	0	44.7	0.105
27	0	0	0	0	44.3	0.105
28	0	0	0	0	44.5	0.106
29	0	0	0	0	44.1	0.107
30	0	0	0	0	44.3	0.107

4.4.1. Effect of Carbon to oxygen ratio (C:O)

As shown in **Figure 4-3,** PHB accumulation increased with the increase in C:O ratio, reaching a threshold accumulation at 3:2 carbon to oxygen ratio of around 45%. However, further increase in the ratio of the carbon to oxygen resulted in an incremental decrease of PHB accumulation to around 25% at 3:1. This was in accordance with the previously reported dominance of type II methanotrophs at lower carbon to oxygen ratios, while type I dominated in higher carbon to oxygen ratios [88]. The previous observation may be referred to the competitive inhibition exerted by ammonia at reduced methane concentrations which in turn selectively favor the growth of type II methanotrophs that are able to tolerate these conditions. Additionally, it was observed that the decrease in methane to oxygen ratio to 1:3 resulted in a decrease in the PHB accumulated which is attributed to the decrease in the food to microorganism ratio.

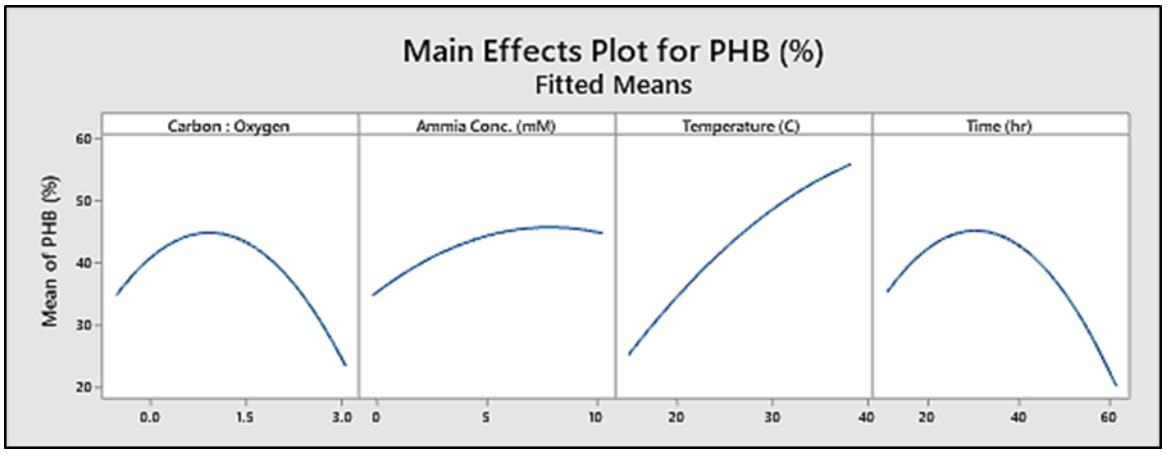

Figure 4-3: Separate main effect for each variable on PHB accumulation.

Other studies reported similar patterns for type II methanotrophs where higher PHB accumulation were associated with higher C:O ratio as shown in **Table 4-3.**

Table 4-3: PHB accumulation percentages that corresponds to different C:O ratios

Study	Temperature (°C)	C:O	Nitrogen source	PHB (%)	Reference
Doronina et al. 2008	30	1:3	Ammonium and Nitrate	30	[191]
Wendlandt et al. 2005	30	1:1	Ammonium	42.5	[82]
Rahnama et al. 2012	38	2:1	Ammonium	51	[81]

On the other hand, as illustrated in **Figure 4-4**, higher methane to oxygen concentrations supported the growth of type I methanotrophs specifically, however, both groups witnessed higher microbial growth rate and methane uptake rate values. In this study, a high carbon to oxygen 3:1, resulted in a higher overall growth rate of 0.13 ± 0.005 hr^{-1}, which was associated with the hinderance of the competition between methane and ammonium than in the lower methane to oxygen ratios where the growth rate dropped to 0.112 ± 0.006 hr^{-1}, since in latter case the biomass was affected by the toxicity resulting from the competitive inhibition exerted by the ammonia.

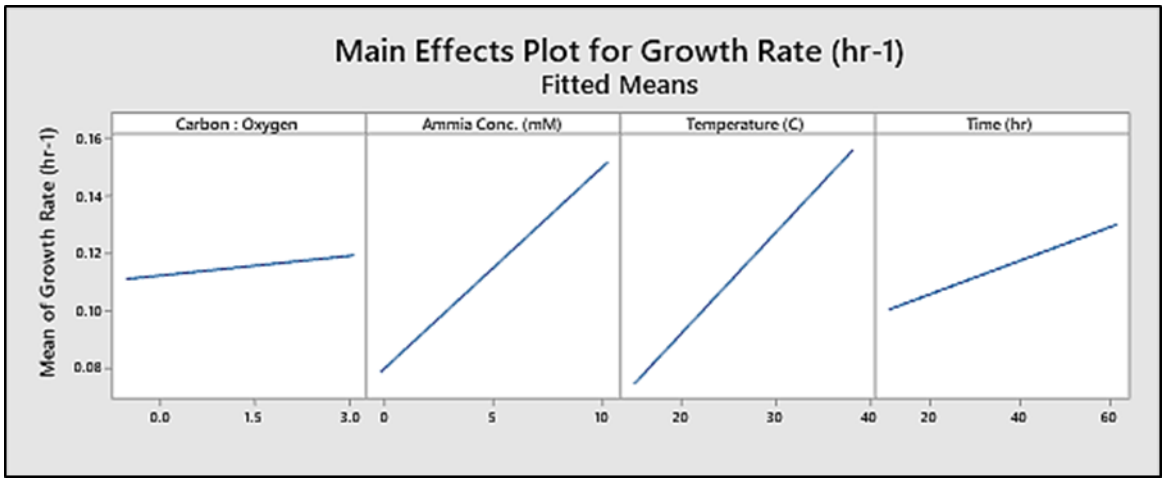

Figure 4-4: Separate main effect for each variable on the growth rate

4.4.2. Effect of Ammonia Concentration ($[NH_4]^+$)

As elucidated in **Figure 4-3**, the increase in ammonia concentration was accompanied by an increase in PHB accumulation reaching a maximum accumulation of around 46% at ammonia concentration of 7 mM. However, further increase in ammonia concentration did not affect the accumulation capacity, in contrast a 10 mM ammonia concentration achieved around 44% PHB accumulation. Nevertheless, the relatively high accumulation percentage at low ammonia concentration, proves that the newly proposed selection mechanism is nitrogen independent and it is not greatly affected by the ammonia concentration. However, very high ammonia concentration was reported to hinder the methane oxidation completely and cause cellular toxicity, hence a maximum concentration of 10mM was employed. As such, it was reported in the literature that a mixed culture of methanotrophic bacteria accumulated up to 52% PHB after growing on 5 mM of ammonium then transferred to deficiency conditions [12]. On another study, *Methylocystis* dominated the bacterial culture, growing on 14 mM ammonium and accumulated 44% PHB in nitrogen limitation conditions [192]. Furthermore, the strain *Methylosinus trichosporium* accumulated 47% and 30% PHB after growing on ammonium salt medium with concentrations of 10 and 16 mM respectively [154], [193], as shown in **Table 4-4**.

Table 4-4: Different PHB accumulation percentages at different ammonia concentrations

Study	Ammonia conc. (mM)	Culture type	PHB (%)	Reference
Fergala et al., 2018	5 mM	Mixed culture	52 %	[12]
Xin et al., 2006	10 mM	Pure culture *Methylosinus trichosporium*	47%	[193]
Helm et al., 2006	14 mM	Mixed culture	44%	[192]
Zhang et al., 2018	16 mM	Pure culture *M. trichosporium OB3b*	30 %	[154]

On the other hand, the relationship between the increase in ammonia concentration and the growth rate witnessed a linear increase which means that the excess ammonia in the media does not increase the capacity of PHB accumulation, however it stimulates the biomass to multiply. Moreover, it can be observed that higher ammonia concentration reflected both higher PHB and biomass yield which was linked to the reliable selective ability of ammonia to type II methanotrophs

4.4.3.Effect of Temperature (T)

As shown in **Figure 4-3 and 4-4**, temperature had a similar effect on both PHB accumulation and growth rate where lower temperature negatively affected the bacterial activity, its ability and accumulate PHB whereas methanotrophs growth rate and PHB production was enhanced with the temperature increase. As such, it can be observed that increasing temperature from 15 to 37 ºC resulted in an increase in the growth rate from 0.08 to 0.16 hr^{-1} as well as an increase in the PHB accumulation from 25 to 57%. Very few studies in the literature have been performed on assessing the relationship between PHB accumulation and temperature. It was reported in a study performed

on pure culture *Methylomonas rubra* that higher methane oxidation rate and by consequence growth rate were achieved in temperatures as high as 38°C [194]. Nevertheless, no study was conducted on a mixed culture methanotrophic community to assess the relationship between these variables.

4.4.4. Effect of Incubation Time (t)

The duration of incubation time in the PHB phase affects the amount of PHB accumulated within the cells as shown in **Figures 4-3** and **4-4**. The peak accumulation of PHB corresponded to 48% after 30 hr. while the growth rate was directly proportional with increasing time the PHB accumulation was inversely proportional with time after 30 hr. of incubation skewing the plotted curve to the right. Previous studies have found that a range of 24-48 hr. is the best incubation period for the nutrient deficient stage after which PHB begins to degrade in attempt to utilize this stored energy [80], [88], [158], [192]. However, the interaction between other independent variables and time was not studied before and what factors affect the speed of PHB accumulation which will be illustrated in the next section.

4.4.5. Interaction between different variables using CCD

In order to determine the maximum PHB production corresponding to the optimum levels of Carbon to oxygen ratio, ammonia concentration, temperature, and incubation time, a second order polynomial equation was applied to propose the optimum levels of these variables. The model explains the role of each variable and their second-order interaction in PHB production as shown in **equation (4.4)** as follows:

$$\boldsymbol{PHB}\ (\%) = 46.2 + 17.5\ \boldsymbol{C}\text{:}\boldsymbol{O} + 4.07\ [\boldsymbol{NH_4}]^{+}\ (mM) + 0.08\ \boldsymbol{T}\ (^{o}C) - 1.10\ \boldsymbol{t}\ (h) -$$

$$- 0.37\ \boldsymbol{C}\text{:}\boldsymbol{O}\ x\ [\boldsymbol{NH_4}]^{+}\ (mM) + 0.542\ \boldsymbol{C}\text{:}\boldsymbol{O}\ x\ \boldsymbol{T}\ (^{o}C) + 0.007\ \boldsymbol{C}\text{:}\boldsymbol{O}\ x\ \boldsymbol{t}\ (h) -$$

$$0.104\ [\boldsymbol{NH_4}]^{+}\ (mM)\ x\ \boldsymbol{T}\ (^{o}C) + 0.0023\ [\boldsymbol{NH_4}]^{+}\ (mM)\ x\ \boldsymbol{t}\ (h) + 0.0297\ \boldsymbol{T}\ (^{o}C)\ x\ \boldsymbol{t}\ (h) \quad \textbf{(4.4)}$$

The quadratic model in the previous equation contains 10 terms with four linear terms and six two level factorial interactions. The significance of each coefficient was determined by P value. The smaller the magnitude of P-value, the more significant is the corresponding coefficient. It is suggested that a variable with a P value of more than 0.05 can be considered influential on the response. As shown in **Table 4-5,** the first order main effect of Carbon : Oxygen, Temperature, and incubation time had P-values of 0.03, 0.02 and 0.032, respectively which implies that these variables had significant independent effects on PHB accumulation. Moreover, it can be observed that the interaction between the carbon to oxygen ration and temperature is significant on the response. Among the four variables tested, only Ammonia concentration had a P value > 0.05 implying that the levels tested did not exert a significant effect on the PHB accumulation. Furthermore, it can be observed that carbon to oxygen, ammonia concentration and temperature had a positive effect on PHA accumulation, whereas incubation time expressed a linear negative effect. **Table 4-5** shows the results of the second-order response surface model in the form of analysis of variance (ANOVA). The values of Fischer's (F) test, determination coefficient, and coefficient of variation show that the model is adequate for corresponding variables.

Table 4- 5: The results of the second-order response surface model in the form of analysis of variance (ANOVA)

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Linear	4	1412.33	353.08	6.57	0.002
Carbon: Oxygen	1	298.51	298.51	5.56	0.030
Ammonia Conc. (mM)	1	85.48	85.48	1.59	0.223
Temperature (ºC)	1	711.83	711.83	13.25	0.002
Time (h)	1	288.53	288.53	5.37	0.032
2-Way Interaction	6	78.08	13.01	0.24	0.956
Carbon: Oxygen x Ammonia Conc. (mM)	1	5.20	5.20	0.10	0.759
Carbon: Oxygen x Temperature (ºC)	1	36.28	36.28	0.68	0.042
Carbon: Oxygen x Time (h)	1	0.06	0.06	0.00	0.974
Ammonia Conc. (mM) x Temperature (ºC)	1	9.09	9.09	0.17	0.686
Ammonia Conc. (mM) x Time (h)	1	0.05	0.05	0.00	0.976
Temperature (ºC) x Time (h)	1	26.44	26.44	0.49	0.492

In order to develop a simpler and more fitted model equation, the above equation was modified by neglecting the least influential terms (P value > 0.05) as shown in **equation (4.5):**

$$\boldsymbol{PHB}\ (\%) = 46.2 + 17.5\ \boldsymbol{C}\text{:}\boldsymbol{O} + 0.08\ \boldsymbol{T}\ (^{o}C) - 1.10\ \boldsymbol{t}\ (h) + 0.542\ \boldsymbol{C}\text{:}\boldsymbol{O}\ x\ \boldsymbol{T}\ (^{o}C) \qquad \textbf{(4.5)}$$

4.4.5.1. Interaction between Carbon to oxygen ratio and Ammonia concentration

The effect of the interaction between the carbon to oxygen ratio and the ammonia concentration on PHB accumulation is illustrated in **Figure 4-5.** As shown in the figure, the more the ammonia concentration increases and the C:O ratio decreases, the more PHB is accumulated. In fact, PHB exceeded 45% when the ammonia concentration was higher than 7 mM and the C:O ratio was between 0.5 to 1. Whereas, decreasing the ammonia concentration to 1 mM and increasing C:O ratio to 3 resulted in PHB accumulation lower than 20%. The previous results are correlated to the previously described competitive inhibition exerted by ammonia at low methane concentrations.

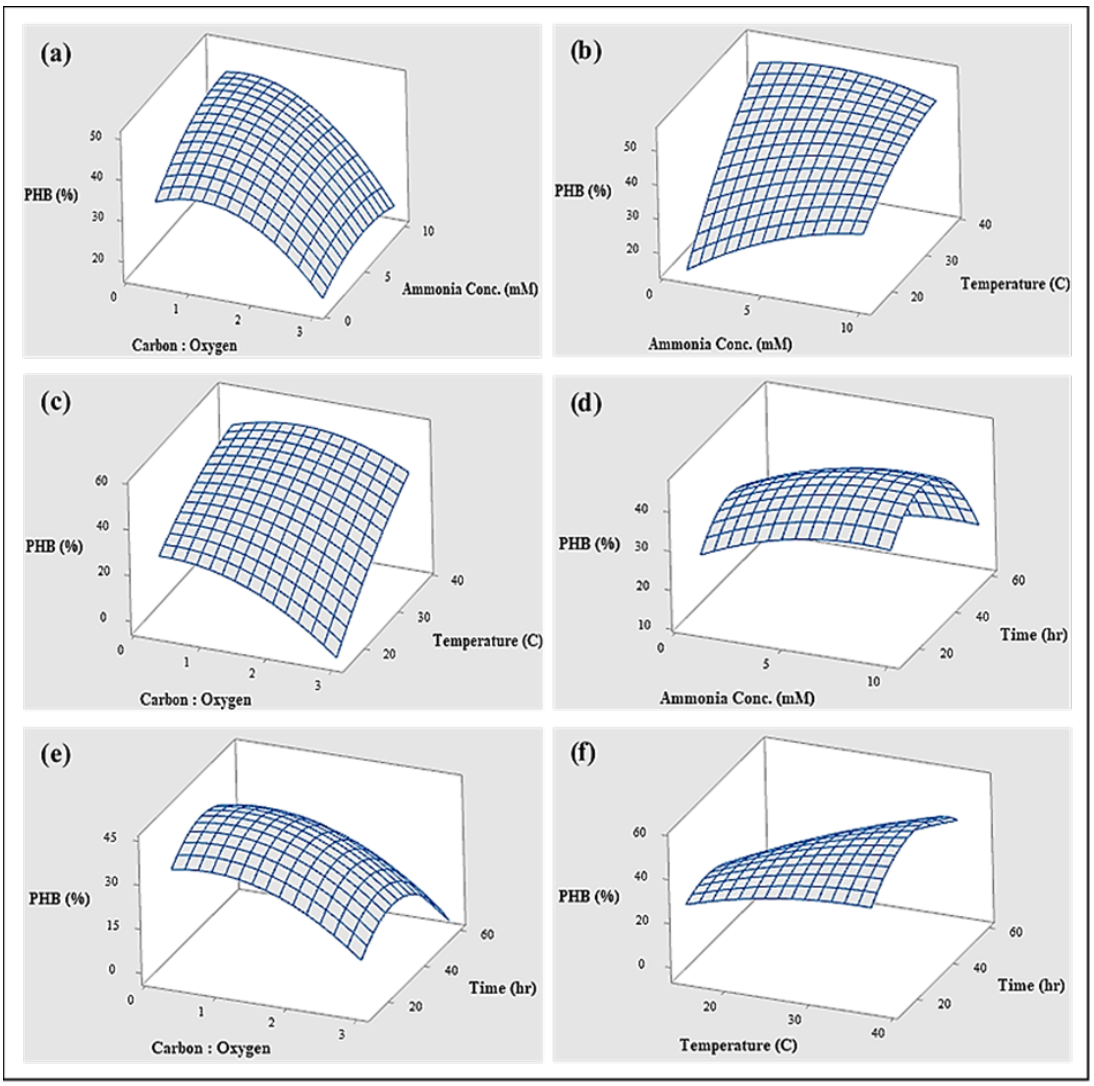

Figure 4-5: Effect of different variables interaction on PHB accumulation percentage

On the other hand, the growth rate of the culture media was greatly affected by the ammonia concentration having a growth rate between 0.14-0.15 hr^{-1} when the ammonia concentration was above 8 mM at all different C:O ratios. Whereas, C:O effect at different ammonia concentration did not have a significant effect on the growth rate. However, a growth rate that exceeds that 0.15 hr^{-1} was only observed at very high and low C:O and an ammonia concentration corresponding to 10mM. The previous observation can be correlated to the fact that two different groups of bacteria were enriched at high and low C:O ratio, type I and type II methanotrophs, respectively which was confirmed by looking at the PHB percentage graphs that show the highest PHB accumulation only at low C:O ratio.

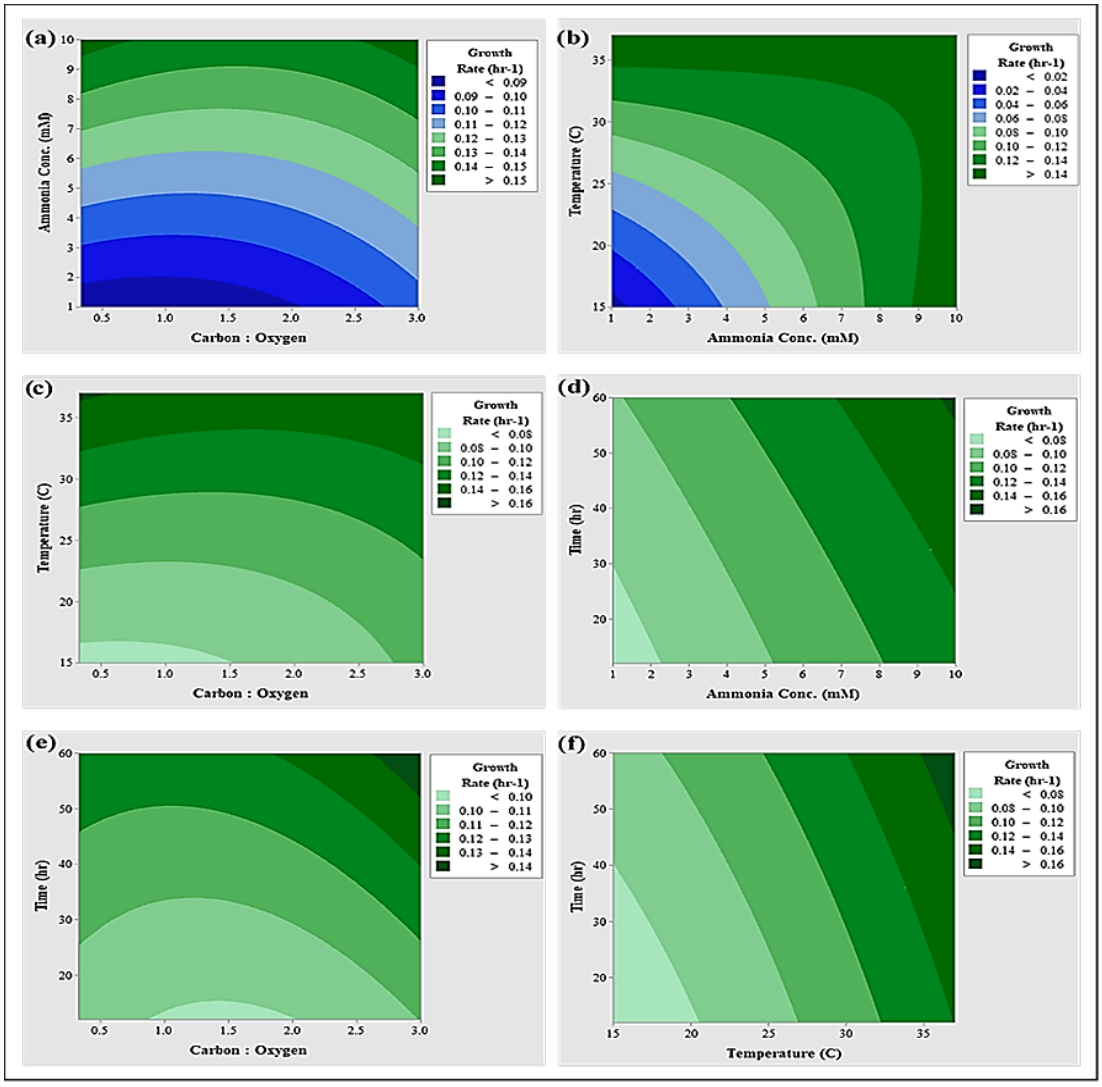

Figure 4-6: Effect of different variables interaction on PHB accumulation percentage

4.4.5.2. Interaction between Carbon to oxygen ratio and Temperature

Temperature of around 33ºC and a C:O ratio of 0.9 recorded an accumulation percentage of around 52%. While increasing C:O ration decreased the accumulation percentage the accumulated PHB was compensated by increasing temperature where higher temperature accompanied by higher C:O ratio recorded similar PHB accumulation percentage for example a C:O ratio of 2.3 incubated at 37 ºC also achieved accumulation percentage higher than 50%. However, further increase in the C:O ratio affected the production of PHB and caused severe reductions in the amount of PHB

accumulated, especially when accompanied with lower temperatures. Temperature variation exerted a stronger effect on the growth rate where it followed a similar pattern on the growth rate as Ammonia concentration when it interacted with C:O ratio where the highest Growth rate was observed at temperatures higher than 32 ºC achieving a growth rate higher than 0.16 hr^{-1} regardless of C:O ratio. This was also correlated to the PHB accumulation graphs where the highest PHB values were only found at lower C:O ratio. Time on the other hand was greatly influenced by the C:O ratio where the highest growth rates observed at high C:O and temperature of around 54 ºC of 0.13 to 0.14 hr^{-1}, lower growth rates negatively affected the growth rate when interacted with time which confirms that the abundance of carbon and increasing incubation achieve higher growth rate. However, increasing time slow the rate of growth rate increase, unlike in the PHB graph where increasing time and C:O affect the accumulation percentage negatively.

4.4.5.3. Interaction between Carbon to oxygen ratio and Time

The incubation time for the PHB phase had reduced significantly from an average of 33 h to an average of 22 h when the C:O ratio was less than 1.5 were the accumulation exceeds 40 %. Lower C:O ratio slow the growth rates where a C:O ratio of around 3 achieve a growth rate higher than 0.14 hr^{-1} after incubation period that is over 50 hours, unlike in the case of a C:O ratio of 1:1 where the growth rate drops to around 0.12 hr^{-1}.

4.4.5.4. Interaction between Ammonia concentration and Temperature

On the other hand, the interaction between was more influential as the more temperature rise and the more ammonia concentration increased the more PHB was accumulated, where a maximum accumulation was recorded at ammonia concentration between 7-8 and a temperature bet 32- to 35ºC where the accumulation in this range was higher than 50%. When the incubation period is

between 40 to 50 h or 10-20 h and the C:O ratio is in the range of 0.5 to 2 the accumulation percentage is dropped to 30%. The relationship between temperature and ammonia concentration is almost linear, where increasing temperature ranging from 34 to 37 ºC accompanied with increasing ammonia concentration ranging from 9-10 mM achieve the highest growth rate of more than 0.14 hr^{-1}

4.4.5.5. Interaction between Ammonia concentration and Time

Ammonia concentration which was higher than 7 mM achieved the highest accumulation percentage of more than 45%, only when it is coupled with an incubation period that is between 25 to 32 hours. Excessive and insufficient incubation time results in very low accumulation percentage regardless of the ammonia concentration since time was a significant factor. Ammonia has higher influence on the growth rate than C:O ratio, whereas growth rate reaches up to 0.16 hr^{-1} at temperatures higher than 50 hours while C:O ratio achieve lower growth rate at the same duration.

4.4.5.6. Interaction between Temperature and Time

Both time and Temperature are significant factors where the increase in temperature above 33 ºC coupled with a an incubation time ranging from 24 to 42 hours had an accumulation percentage of more than 50%, however increasing the incubation time to more than 45 hours drop the accumulation severely to less than 20% at temperature less than 25 ºC. Time and temperature have a more influential linear relationship on the growth. Their directly proportional relationship reaches over 0.16 hr^{-1} above 37 ºC and time over 50 hours incubation, unlike the PHB accumulation curve where it peaks at around 30 hours of incubation.

4.5. Conclusion

A mixed culture methanotrophic community was successfully enriched using waste activated sludge. Moreover, this methanotrophic community successfully optimized and produced high PHB accumulation percentage in different operational conditions using CCD. The optimization of C:O ratio, ammonia concentration, temperature and incubation duration were found to contain three significant factors affecting PHB accumulation; temperature and incubation duration and C:O ratio respectively. The optimization of both the growth rate and PHB accumulation achieved high values of PHB accumulation that reached more than 55% low C:O ratio and temperature higher than 35 ºC at 24 hours incubation in PHB phase and ammonia concentration of around 7 mM. On the other hand, the growth rate that exceeded 0.16 hr^{-1} in the case of high ammonia concentration of 10 mM and temperature higher than 35 and after 48 hours of incubation and low methane to oxygen ration. These values were obtained after five operational cycles with expectations that the PHB accumulation value and growth rate increase with increasing the cycles number as witnessed in the previous experiment. The value reached was one time and a half higher than the control sets which indicates the success of the optimization in achieving higher accumulation percentages while giving valuable information about influential factors and interactions.

Chapter 5: Conclusions and Future Work

5.1 Conclusions

Methanotrophs have the ability to perform multiple roles, ranging from methane mitigation to many product recoveries, one of which is biopolymers production. This capability has made methanotrophic bacteria one of the top candidates for industrial applications. Moreover, PHB production has been demonstrated to be crucial for overcoming all environmental concerns associated with petroleum-based plastic since it is biodegradable and possess the right chemical and physical qualities needed in industry. the abundance of methanotrophs in WWTPs due to the emission of methane gas in anaerobic digestion process will not only help mitigate this harmful greenhouse gas, but it can also be used to recover these products at the same time. This can be achieved through the incorporation of a sequential batch bioreactor after the anaerobic digestion process to cultivate type II methanotrophs (PHB producers) since WWTPs possess all the necessities for maintaining a methane-based system for PHB production.

This chapter summarizes the major findings of this thesis along with the direction of future work. The main objective of this thesis is to develop a robust, efficient and sustainable system for achieving high PHB accumulation percentages in mixed culture methanotrophic communities, this was delivered through the proposal of a new intermittent cycling of nitrogen availability and nitrogen starvation over a long period of time with both nitrate and ammonia. Moreover, with the help of the literature, it was possible to specify the most influential factors that can affect the PHB accumulation rate to perform the optimization for the nitrogen source with the highest PHB accumulation. Operational parameters were optimized to achieve maximum yield using response surface methodology fitted to central composite design where methane gas was mainly used as the

only carbon substrate for methanotrophic bacteria. In **Chapter 3**, A novel recycling method has been successfully developed to eliminate the limitation imposed by the nitrogen source. Furthermore, high PHB accumulation percentage of 59.4% and 54.3%, was achieved in the new method for both AMS and NMS, respectively, compared to 37.8% and 9.1% for the conventional method. Finally, a microbial shift towards PHB producers was observed in the microbial analysis after 9 weeks of operation which confirmed the success of the new setup in creating an environment where methanotrophic bacteria can co-exist with other types of PHB producers. the experiment was conducted to compare the conventional set-up and the new setup (design-based selection) based on two different recycling techniques while also comparing the ammonia and nitrate. High accumulation percentage was reached using ammonia (ammonia-based selection). The new setup achieved high methane consumption 2.69 mg CH_4/ hr rates and high PHB accumulation percentages using both ammonia and nitrate which proves the success of the proposed setup where the newly proposed recycling technique could save money by using only one reactor instead of two (one for the growth phase and one for the PHB accumulation phase). Afterwards, after sustaining a stable type II methanotrophic community, the screening of the polymer was achieved through staining with Sudan Black dye and characterization of the extracted polymer was confirmed using both H^1 and C^{13}-NMR. The success of retaining methane for producing PHB, will mostly rely on ensuring the consistency of the selection of type II methanotrophs consortium. Hence, employing selective pressures should reflect on eliminating any inhibition that would hinder the PHB accumulation potential of PHB producers. On the other hand, in **Chapter 4**, The effect of the different operational parameters on PHB accumulation and microbial growth rate have been successfully studied using CCD which is a crucial step towards maximizing PHB accumulation and minimizing its production cost. After selecting CCD as the

most suitable optimization tool, the five levels of the four previously selected factors (time, temperature, Duration, and C:O ratio) were determined and the model generated 30 experimental trials for determining the most significant factors and assess variables interaction. The 30 experiments consisted of premediated factors interaction levels and PHB accumulation was estimated in all of the 30 runs along with the growth rate. The optimization confirmed the role of temperature and time in PHB accumulation of PHB as they were most influential factors and the maximum optimizable response can be estimated using the generated empirical equation. Overall, Both the growth rate and the PHB accumulation percentage are crucial in estimating the amount of PHB that can be extracted from a specific HB accumulating community since both cell storage capacity and the amount of biomass in the reactors can boost the accumulation threshold.

5.2 Direction of Future work

The main direction of future work for batch scaled experiments, is applying the process on a larger scale and afterwards an industrial scale. Furthermore, Running the experiment in a sequential reactor mode would give a clearer vision how the system will perform in an industrial scale. With the possibility of adjusting the cultivation process in order to recover multiple product simultaneously which can increase economic value. Moreover, an optimization with other nitrate and applying the CCD design with nitrate as well to find the most influential factors to compare between the two nitrogen sources in order to develop a more sustainable and viable design. Moreover, studying the effect of phosphorus deprivation and developing an anerobic stage that can induce the PHB cycle the study of different enzyme activity molecular level reactions in order to grasp a deeper understanding of different interactions that are key in PHB formation and unique to type II methanotrophs. Furthermore, analysis of the consortium composition within the mixed culture and the interactions among them can be exploited it to shift the internal metabolic activities

in the direction that could maximize PHB accumulation. Additionally, investigating the effect of citrate addition on initiating the PHB cycle can have a key effect on shifting key metabolic activities towards polymer production. Additionally, assessing the chemical and physical properties of the extracted polymer, would give an idea about the sort of industrial applications it is most suited for. For instance, the formation of co-polymers to introduce a material that is characterized with enhanced physicochemical properties. Finally, exploring the molecular aspects to try and understand the PHB accumulation process through assessing the enzyme activity, exploring the PHB associated protein and enhancing their role in maximizing PHB. As well as studying the possibility of creating a recombinant strain that could have higher PHB capability.

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