BIOLOGICAL CONVERSION PROCESS OF METHANE INTO METHANOL USING MIXED CULTURE METHANOTROPHIC BACTERIA ENRICHED FROM ACTIVATED SLUDGE SYSTEM

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Abstract

Wastewater treatment plants contribute to the global warming phenomena not only by GHG emissions, but also, by consuming enormous amount of fossil fuel based energy. Therefore, methane bio-hydroxylation has attracted the attention as methanol is an efficient substitute for methane (GHG) due to its transportability and higher energy yield.

This work is destined to investigate and optimize the factors affecting the microbial activity within methane bio-hydroxylation system using type I methanotrophs enriched from activated sludge system. The optimization resulted in a notable enhancement of the growth kinetics. The attained maximum specific growth rate (μ_{max}) (0.358 hr^{-1}) and maximum specific methane biodegradation rate (q_{max}) (0.605 g-$CH_{4,Total}$/g-DCW/hr^{-1}) were the highest reported in mixed cultures. Furthermore, the maximum methanol productivity achieved is comparable with pure cultures and equal to 2115±81 mg/L/day. Whereas, methanol concentration of 485±21 mg/L was attained which is two times higher than the reported using mixed culture.

Dedication

" Bountiful is your life, full and complete. Or so you think, until someone comes along and makes you realize what you have been missing all this time. Like a mirror that reflects what is absent rather than present, he shows you the void in your soul—the void you have resisted seeing. That person can be a lover, a friend, or a spiritual master. Sometimes it can be a child to look after. What matters is to find the soul that will complete yours. All the prophets have given the same advice: Find the one who will be your mirror! "

Thank you Issraa (my wife) and Yousr (my daughter) for being that person who complete my soul and make me feel the true meaning of love and happiness by just being in my life.

Acknowledgment

"And not you will except that wills Allah. Indeed, Allah is All-Knower, All-Wise."

Surah Al-Insan, Verse 30

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Chapter 1
Introduction and Justification

1.1 Background

1.1.1 Methane as Greenhouse Gas

Over the past few decades, the planet has witnessed the augmentation of greenhouse gases (GHG) emissions ensuing notable climate and environmental changes. If the same GHG emissions trend continuous, it is predicted that by 2050 the global warming would increase the earth's temperature by 2 Celsius degrees compared with the temperature level in the year of 1900 (Liao et al., 2016). Methane (CH_4) is considered the second major greenhouse gas at an atmospheric concentration of 1.75 ppm and is expected to reach 4 ppm by 2050. Compared to the major greenhouse gas (i.e. CO_2), methane molecule can absorb 30 times more heat with a lifetime span of approximately 12 years. Hence, the focus on CH_4 emissions mitigation has 20 to 60 times greater effect than the CO_2 emissions in reducing the global warming phenomena (Francisco José Fernández, 2005; Hanson and Hanson, 1996).

1.1.2 GHG Emissions from Wastewater Treatment Plants

In a typical municipal wastewater treatment plant (WWTP), biogas is generated throughout anaerobic biodegradation of the organic materials in the anaerobic reactors (Tchobanoglous et al., 2003). Therefore, WWTPs methane emissions count for 4% to the yearly global methane

budget (A. Ho et al., 2013). Therefore, WWTPs located in North America released methane for about 16 million metric tons of CO_2 equivalents in 2014 (Municipality of Toronto, 2016; US EPA, 2016). Additionally, WWTPs contribute to the global warming phenomena not only by GHG emissions including methane and carbon dioxide, but also, by consuming enormous amounts of fossil fuel based energy. For instance, WWTPs electricity consumption represented about ≈ 4% of the United states total electrical consumption which, results in about 45 million tons of GHG emissions annually (Y. Shen et al., 2015; US EPA, 2013). Nonetheless, WWTPs are required to treat higher wastewater volumes which is associated with higher energy requirements due to the ongoing population increase. Therefore, there is a great interest not only in GHG mitigation, but also, in resources recovery from waste streams to offset its treatment operational energy inputs.

1.1.3 Bio-Methane as a Resource in Wastewater Treatment Plants

Interestingly, it was reported in a study performed on WWTP located in Toronto, Canada, that the wastewaters have an energy content (in the form of organics) up to 9 times higher than energy consumed for its treatment (Logan, 2008). Combined together, the need to offset wastewater treatment operational cost and energy inputs and wastewater relatively high energy content led to the paradigm shift from considering the wastewater as waste that needs to be treated and disposed into an energy and value-added products resource (McCarty et al., 2011). Anaerobic digestion (AD) process has been widely adopted by WWTPs for biogas production as energy and resources recovery technology (Chen et al., 2008). It was estimated that WWTPs located in North America have the potential to produce about 3.90 billion cubic meters of biomethane per year. This could reduce the GHG emissions similar to taking 1.18 billion

passenger vehicles off the roads (Canadian Biogas Association, 2013; NREL, 2013; US EPA, 2011).

1.1.4 Methanol as Prominent Substitute for the Bio-Methane

Nevertheless, multiple challenges hinder the direct utilization of the produced AD derived biogas including the existence of impurities and moisture, its low handling and collecting capabilities, and lack of convenient infrastructure for gas distribution. Moreover, the combined heat and energy technologies show low electricity efficiency (η of 25-45%) (Bachmann et al., 2015; Park and Lee, 2013). Those obstacles, unfortunately, induce most of the WWTPs to either use the biogas only for facility internal heating (only in winter time) or to flare it. On the other hand, methanol, as a liquid fuel, consider to be more storable, and secured (Ge et al., 2014; Olah, 2005). Methanol as liquid fuel is much more compatible with the existing infrastructure (Liao et al., 2016). Additionally, more energy can be derived from methanol (15.8 MJ/L) compared to methane (38.1×10^{-3} MJ/L) (Hwang et al., 2014). Methanol can be used as a fuel either standalone or blended with gasoline (Olah, 2005; Taher and Chandran, 2013). In addition, methanol has lower NO_x and SO_2 emission than natural gas (Murray and Furlonge, 2009). Furthermore, methanol has been commonly used as an external carbon source to enhance biological nitrogen removal (BNR) processes (Ginige et al., 2008). Therefore, methanol is a multiple use commodity with a prominent role as an efficient and sustainable substitute for biomethane produced within WWTPs. Collectivity, two goals would be achieved by the conversion of methane into methanol; (1) the mitigation of GHG (biomethane) emitted from WWTPs instead of being flared to the atmosphere, and (2) the production of valuable liquid fuel to offset the WWTPs energy requirements.

1.1.5 Methanotrophs as Bio-Catalysts for Methane Hydroxylation

Unlike thermochemical techniques, methane can be oxidized biologically under ambient temperature and atmospheric pressure reducing the oxidation process energy requirements. Moreover, the employed biocatalysts are isolated from diverse environments what makes it adaptable to different operating conditions. Hence, biological methane hydroxylation can be considered more efficient, simpler, and cheaper in comparison to thermochemical conversion (Conrado and Gonzalez, 2014; Corder et al., 1986; Ge et al., 2014; Sheets et al., 2016). Biologically, two distinct clusters of microorganisms can utilize methane and activate the stable C-H bond in the methane and, subsequently, convert it into methanol; (1) ammonia oxidizing bacteria (AOBs) and (2) methane oxidizing bacteria (methanotrophs) (Fei et al., 2014; Hanson and Hanson, 1996; Yang et al., 2014). AOBs partially oxidize methane via the ammonia monooxygenase (AMO) and use ammonia as an energy source (Hanson and Hanson, 1996; Taher and Chandran, 2013). However, it was reported that AOBs yielded relatively low methanol productivities due to the competition between methane and ammonia on the AMO (Ge et al., 2014; Taher and Chandran, 2013; Wang et al., 2010). On the other hand, methanotrophs expose higher methane uptake and methanol yields as they rely on methane as their sole carbon and energy source. Furthermore, it can integrate methanol production with various biotechnological applications including BNR processes, biopolymers production (Myung et al., 2015; Semrau et al., 2010; Strong et al., 2015).

Descending from the Methylotrophic bacterial group, methanotrophs are gram-negative bacterial group that have the distinguish capability of exploiting methane as the cellular carbon and energy source (Hanson and Hanson, 1996). Methanotrophs are grouped into three types based on the are phylogenetic properties; type I, type II and type III (Semrau et al., 2010). Type III methanotrophs

are found in extreme environments such as volcanic mud and very acidic environments (Op den Camp et al., 2009; Teeseling et al., 2014), which limits its applications in WWTPs. In comparison with type II, type I methanotrophs were reported to have higher methane affinity, growth rates and more energy efficiency (Hanson and Hanson, 1996; Kalyuzhnaya et al., 2015). Furthermore, they can dominantly grow in mixed cultures under nitrate sufficient conditions (Pfluger et al., 2011). Thus, type I methanotrophs are more advantageous to be manipulated in methanol production. Hence, methane bio-hydroxylation using type I methanotrophs as the biocatalyst is an active, advantageous, and prominent research area, especially, in the area of methanotrophs productivity enhancement and overcoming the bioreactor engineering challenges.

1.2 Research Rationale

Even though intensive researches have been performed on methane bio-hydroxylation using methanotrophs. Several challenges still hinder the process upscaling. To the moment, no studies have successfully developed a feasible and stable methanol production process. Most of the reported studies were performed in batch scale and short term (from 8 to 52 hours). This is referred to the bacterial decay associated with methanol production results from the metabolic reactions inhibition. Additionally, the majority of these studies were using a pure culture bacteria, which is not economically feasible for WWTP (Ge et al., 2014). Thus, the development of a continuous methane bio-hydroxylation bioreactor requires a continuous supply of fresh biocatalysts using mixed-culture type I methanotrophs. The methane bio-hydroxylation system consists of two main bioreactors, as illustrated in **Figure (1-1)**. The first bioreactor is the biocatalysts (type I methanotrophs) cultivation bioreactor to simultaneously provide the second bioreactor with active biomass at the optimum density. The second bioreactor is the methanol production bioreactor, in which the biocatalysts are oxidizing methane into methanol. The

produced methanol can be used as a commodity (either as fuel or raw material for chemical industries) or as external carbon source for BNR process.

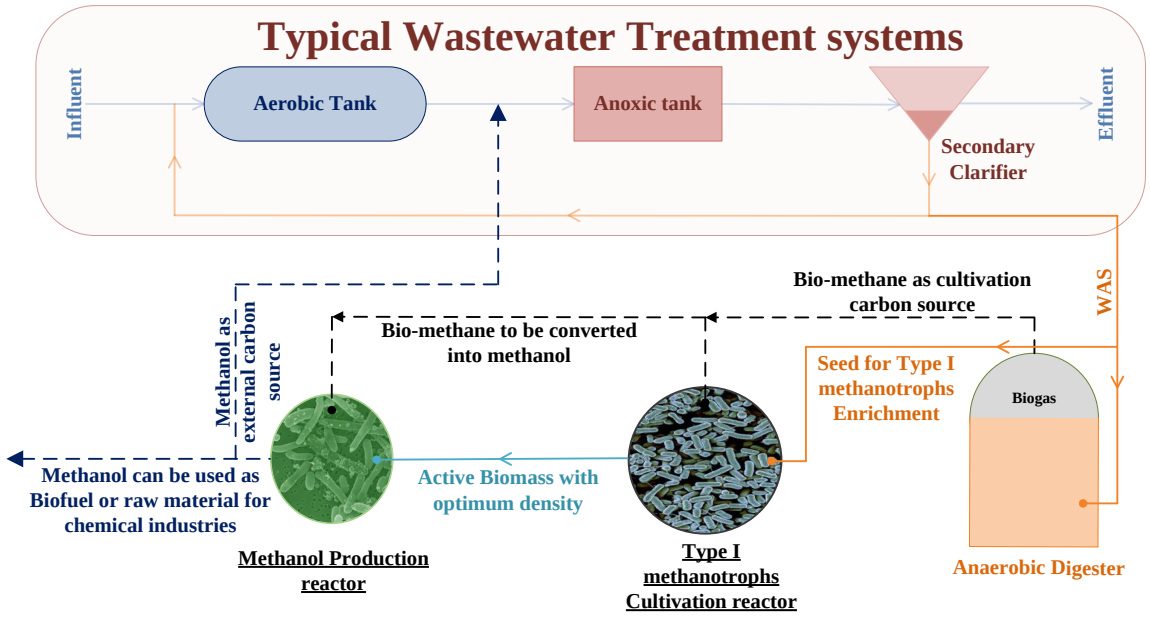

Figure (1-1): Proposed Methane Bio-Hydroxylation System in Wastewater Treatment Plant

On the other side, the microbial growth kinetics is crucial in order to design and scale-up methanotrophs cultivation bioreactor system. Those kinetic parameters varied significantly based on the operational conditions, however, few studies reported the microbial kinetics for methanotrophs. Those studies were performed using different operational conditions such as biomass density, nitrogen, methane and oxygen concentrations using pure cultures of type II methanotrophs (Ordaz et al., 2014; Rostkowski et al., 2013). Therefore, the optimization of the growth conditions is a prerequisite step to attain realistic growth kinetics in addition to determine the optimum values to be applied in the scaled-up process. Factors affecting methanotrophs microbial activity investigated throughout this study include nitrogen source and concentration, copper concentration, substrates concentrations, and biomass density under different food to microorganisms (F/M), carbon to nitrogen (C/N), and nitrogen to microorganisms (N/M) ratios.

Thereafter, Similar to methanotrophs growth, various factors were reported to affect methanol production such as -but not limited to- methanol dehydrogenase (MDH) inhibitors, headspace gaseous composition, and biomass density. However, the results reported in the literature are contradicted mainly due to the use of different strains at each study (Ge et al., 2014). Therefore, the optimization of such factors using mixed culture from waste activated sludge is a major breakthrough towards the scaling up of methane bio-hydroxylation process in WWTPs.

1.3 Research Objectives

The ultimate objective of this research is to find a commercially feasible engineering process for methane bio-hydroxylation. In particular, we aim to develop a stable and feasible bench-scale continuous process that integrate both biogas mitigation with methanol production. In order to do so, multiple research questions have been investigated throughout this study:

1- How to enrich type I methanotrophs dominant culture from waste activated sludge?

2- What are the factors affecting the growth of type I methanotrophs mixed culture enriched from waste activated sludge? How far those factors affect the microbial growth? What are the optimum values for those factors?

3- After growth optimization, what are the values of the microbial growth kinetics of the growth of type I methanotrophs mixed culture enriched from waste activated sludge to be able to design of methanotrophic cultivation bioreactor?

4- What are the factors affecting methanol production using type I methanotrophs mixed culture enriched from waste activated sludge? How far those factors affect the methanol productivity? What are the optimum values for those factors?

1.4 Research Approach and Thesis Layout

The first step to address the aforementioned research questions was to develop comprehensive literature review which has been done in **chapter 2**. Throughout this chapter, the differences between methanotrophs types and which of them is more advantageous to be employed in methane bio-hydroxylation were explored. In addition, methane assimilation and dissimilation pathways were identified. Most importantly, factors affecting methanotrophs microbial growth and selection were elucidated. Furthermore, methanol accumulation metabolism and the factors affecting the process were demonstrated. Thereafter, consecutive batch tests were conducted to explore each factor effect on type I methanotrophs type I methanotrophs mixed culture enriched from waste activated sludge microbial growth and identify the optimum values. Minutely, the inorganic nitrogen source, copper concentration, methane to oxygen ratio, biomass density, and methane concentrations influence were investigated in **chapter 3**. The biomass density effect including the influence of other parameters associated with biomass density change (i.e. F/M, C/N, and N/M ratios) on the microbial activity were detailed in **chapter 4**. Using the data attained from mixed culture enriched and biomass density, a complete set of microbial growth kinetics have been developed in **chapter 5**. Throughout **chapter 6**, the influence of different MDH inhibitors, formate concentration, headspace gaseous composition, copper concentration, and biomass density on methanol accumulation have been investigated and optimized.

Collectively, the thesis includes in total seven chapters; an introduction and justification chapter (**Chapter 1**), literature review chapter about methanotrophs growth and methanol accumulation (**Chapter 2**), four research chapters (**Chapter 3, Chapter 4, Chapter 5, and Chapter 6**), and a final concluding chapter (**Chapter 7**)

Chapter 2
Literature Review [1]

2.1 Methanotrophs

Methanotrophs are a unique cluster of microorganisms that have the ability to utilize methane as their sole carbon and energy source (Anthony, 1982; Chistoserdova et al., 2005; Hanson and Hanson, 1996). Naturally, methane oxidation is carried out by methane oxidizing bacteria and anaerobic methane oxidizing archaea (Cui et al., 2015; Ge et al., 2014). Archaea can anaerobically couple methane oxidation, via the reverse methanogensis pathway, with the reduction of multiple types of electron acceptors; i.e., sulfate, nitrate, and metal ion (Mn^{+4} and Fe^{+3}) (Beal et al., 2009; Boetius et al., 2000; Cui et al., 2015; Fei et al., 2014; Haroon et al., 2013; Scheller et al., 2010). On the other hand, the gram-negative methane oxidizing bacteria is a descending cluster from the methylotrophic bacteria (Anthony, 1982; Hanson and Hanson, 1996; Lieberman and Rosenzweig, 2004; Semrau et al., 2010). All methane oxidizing bacteria share the same methane utilization pathway. Methane oxidation is initiated through the methane monooxygenase (MMO) enzyme and converted into methanol (CH_3OH), which is converted subsequently into formaldehyde (CHOH). Formaldehyde is terminally oxidized into carbon

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dioxide (CO_2) with formate (CHOOH) as an intermediate (Anthony, 1982; Bowman, 2006; Kalyuzhnaya et al., 2015; Semrau et al., 2010). Furthermore, formaldehyde, formate, and carbon dioxide can be utilized for cell synthesis requirements via RuMP, Serine, and CBB cycles, respectively (Chistoserdova and Lidstrom, 2013). It is noteworthy that the nomenclature methanotrophs, in this study, is referring to the methane oxidizing bacteria not the archaea.

2.2 Methanotrophs Taxonomy

Aerobic methanotrophs are phylogenetically located in the verrucomicrobia phylum and the gamma and alpha subdivisions of proteobacteria phylum (Murrell, 2010; Semrau et al., 2010; Sun et al., 2013). Aerobic methanotrophs are clustered into three main types, **Figure (2-1)**, type I methanotrophs or gamma-proteobacteria methanotrophs forming the methylococcaceae and methylothermaceae families, type II methanotrophs or alpha-proteobacteria methanotrophs lying in the methylocystaceae and beijerinckiaceae families, and type III or verrucomicrobia methanotrophs descending from the methylacidiphilaceae family. Candidatus methylomirabilis oxyfera (M.oxyfera) is the only known anaerobic methanotrophic bacteria. It belongs to the NC10 phylum which all of its members haven't been isolated in pure culture yet (L. Shen et al., 2015).

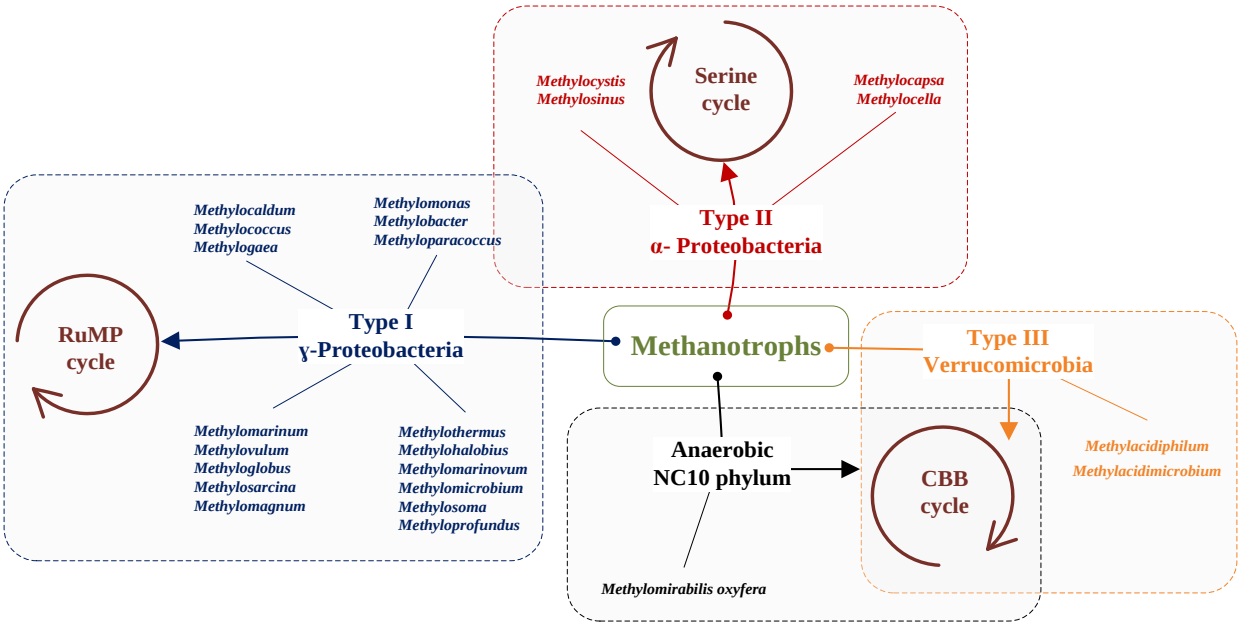


Figure (2-1): Methanotrophs Taxonomy and Each Type Carbon Assimilation Pathway

2.2.1 Type I Methanotrophs

Type I methanotrophs can be found in many environments including; freshwaters and sediments, marine environments, rice fields, hot springs, soils, landfills (Bowman, 2006, 2014; Bowman et al., 1993; Ge et al., 2014; Murrell, 2010; Semrau et al., 2010), sewage and activated sludge (Bowman, 2006; Bowman et al., 1993; Bowman, 2014; A. Ho et al., 2013; Kits et al., 2015), denitrification reactors, silage and manure wastes (A. Ho et al., 2013; Hoefman et al., 2014b; Trotsenko et al., 2009), anaerobic digesters (A. Ho et al., 2013; Sheets et al., 2016), coal-mine surface and drainage water (Bowman, 2006; Bowman et al., 1993). Type I methanotrophs possess a typical well developed intracytoplasmic membrane (ICM) throughout the cell, which appears as stacks of vesicular discs. Therefore, the expression of particulate methane monooxygenase (pMMO) is conferred due to its location inside the ICM (Bowman, 2014; Bowman et al., 1993; Semrau et al., 2010; Whittenbury et al., 1970). Some genus including methylomagnum, methaylovulum, and few strains within methylomonas and methylocaldum can express soluble methane monooxygenase (sMMO) as well as pMMO (Iguchi et al., 2011;

Kalyuzhnaya, 1999; Khalifa et al., 2015). Carbon, in the form of formaldehyde (CHOH), is assimilated via the ribulose monophosphate (RuMP) pathway (Anthony, 1982; Bowman, 2006). However, methylocaldum genus, methylomagnum ishizawai, and methylococcus capsulatus species possess Serine pathways enzymes (Bodrossy et al., 1997; Khalifa et al., 2015; Takeuchi et al., 2014; Trotsenko et al., 2009). Despite it cannot grow autotrophically, methylococcus strains can assimilate carbon dioxide (CO_2) in association with methane as a cellular carbon through a partially functional Calvin-Benson-Bassaham (CBB) cycle (Bowman et al., 1993).

2.2.2 Type II Methanotrophs

Type II methanotrophs widely exist in different environments especially at low oxygen and high methane concentrations (Amaral and Knowles, 1995; Graham et al., 1993; Hanson and Hanson, 1996; Semrau et al., 2010). These environments include soil and freshwater sediments, rice fields, coal-mine drainage water, landfills (Bowman, 2006; Bowman et al., 1993; Chen and Murrell, 2010; Knief, 2015; Marín and Arahal, 2014), groundwater aquifers (Lindner et al., 2007), sewage sludge (A. Ho et al., 2013; Knief, 2015), acidic wetlands and forest soils (Dunfield et al., 2003; Marín and Arahal, 2014). Methylocystis and methylosinus genus possess an ICM aligned to the cell periphery, while, the ICM for the methylocapsa genus appears as stacks of membrane vesicles packed in parallel on only one side of the cell membrane. Accordingly, the three of them express pMMO. Moreover, methylosinus and some methylocystis strains can possess sMMO (Bowman et al., 1993; Dedysh et al., 2002; Dunfield et al., 2010; Marín and Arahal, 2014; Semrau et al., 2010; Whittenbury et al., 1970). In contrast, methylocella and methyloferula genus miss this extensive ICM system and only express sMMO (Dedysh et al., 2004, 2000; Dunfield et al., 2003; Semrau et al., 2011, 2010; Vorobev et al., 2011). These two genera develop a vesicular membrane system composed of spherical or ovoid-shaped

membrane vesicles located on the periphery of the cytoplasm (Dedysh et al., 2004, 2000; Dunfield et al., 2003; Semrau et al., 2011, 2010; Vorobev et al., 2011). In order to increase their methane oxidation surface area, methanotrophs form more ICM in the presence of methane. It is noteworthy that a better ICM is developed while growing on methane rather than methanol (Bowman, 2006). All type II methanotrophs can accumulate poly-β-hydroxybutyrate (PHB) as a survival mechanism under nutrients unbalanced conditions (Bowman, 2006; Marín and Arahal, 2014). Carbon, in the form of formate (CHOOH), is assimilated via the serine pathway while all other methane assimilation pathways are not found (Bowman, 2006; Dedysh et al., 2015b; Marín and Arahal, 2014; Semrau et al., 2010).

2.2.3 Type III Methanotrophs

Type III methanotrophs were firstly isolated in 2007 from hot acidic habitats including acidic hot springs, volcanic mud, geothermal mud areas (Dunfield et al., 2007; Islam et al., 2008; Knief, 2015; Pol et al., 2007; Teeseling et al., 2014). Type III methanotrophs do not possess the typical proteobacterial ICM except for methylacidimicrobium fagopyrum which possess a type I-like ICM (Knief, 2015; Op den Camp et al., 2009). However, methylacidiphilum strains have an ICM of carboxysome-like structures or vesicular membranes (Op den Camp et al., 2009), while, no ICM system was observed in both methylacidimicrobium tartarophylax and methylacidimicrobium cyclopophantes (Teeseling et al., 2014). All methanotrophic verrucomicrobia possess pMMO only which raises the question about the location of pMMO in strains that do not have an ICM (Erikstad and Birkeland, 2015; Hou et al., 2008; Khadem et al., 2012; Op den Camp et al., 2009; Teeseling et al., 2014). Carbon, in the form of carbon dioxide (CO_2), is assimilated via the Calvin-Benson-Bassaham (CBB) cycle while the complete RuMP and serine cycle enzymes are not found (Erikstad and Birkeland, 2015; Teeseling et al., 2014).

Therefore, unlike proteobacterial methanotrophs, carbon dioxide presence notably stimulates the bacterial growth (Op den Camp et al., 2009; Teeseling et al., 2014), but, no growth was reported only on carbon dioxide.

2.2.4 Anaerobic Methanotrophs

Anaerobic methanotrophs were discovered in 2006 with the ability of coupling anaerobic methane oxidation (ANMO) with nitrite reduction (Ettwig et al., 2010; Raghoebarsing et al., 2006). They were enriched from freshwater environments, sewage sludge, and wetland soils. However, they have not been isolated in pure culture yet (L. Shen et al., 2015). M.oxyfera is the only bacterial type that can grow anaerobically on methane. No ICM system was observed in M.oxyfera, However, pMMO is the initiator of methane oxidation. The genomic analysis showed the existence of incomplete RuMP and serine pathways and the full CBB cycle (Wu et al., 2011). Recently, it was confirmed that carbon is fixed in the level of CO_2 via the CBB cycle (Rasigraf et al., 2014), while, it is suggested that the incomplete serine pathway is used to detoxify the formaldehyde (L. Shen et al., 2015; Wu et al., 2011).

2.3 Methane Metabolism in Methanotrophs

All methanotrophs oxidize methane terminally to carbon dioxide through a series of consecutive reactions to fulfill their energy and cell replication requirements with the aid of their secreted enzymes as shown in **Figure (2-2)** (Anthony, 1982; Hanson and Hanson, 1996; Kalyuzhnaya et al., 2015; Semrau et al., 2010).

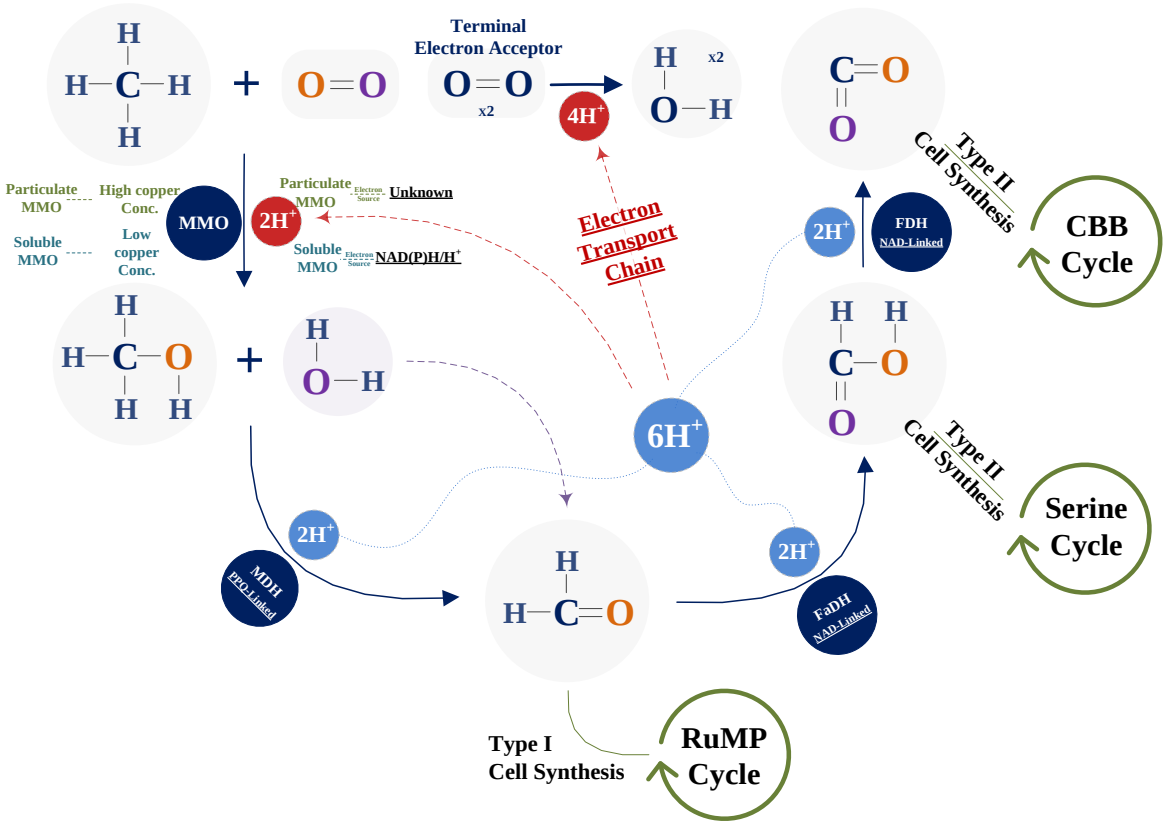


Figure (2-2): Methane Oxidation Pathway in Methanotrophs

MMO: Methane Monooxygenase enzyme, MDH: Methanol Dehydrogenase enzyme, FaDH: Formaldehyde Dehydrogenase enzyme, FDH: Formate Dehydrogenase enzyme, PQQ: Pyrroloquinoline Quinone, NAD(P)H: Nicotinamide Adenine Dinucleotide, RuMP: Ribulose Monophosphate pathway, CBB: Calvin-Benson-Bassaham.

Methane is oxidized to methanol, **equation (2-3)**, in a reaction catalyzed by methane monooxygenase (MMO) enzyme in which MMO splits O_2 bond into two atoms. Thereafter, one oxygen atom is incorporated in methane hydroxylation **equation (2-1),** while the other atom is reduced to H_2O, **equation (2)**. Moreover, MMO requires reducing equivalents as electrons donor in the latter reaction. Thus, methane oxidation is considered as an energy consuming reaction unlike the remaining reactions which are energy conserving (Chistoserdova and Lidstrom, 2013; Hanson and Hanson, 1996; Madigan et al., 2015).

$$CH_4 + ½\ O_2 \longrightarrow CH_3OH \quad \text{(Eq. 2-1)}$$

$$AH_2 + ½\ O_2 \longrightarrow H_2O + A^+ \quad \text{(Eq. 2-2)}$$

$$CH_4 + AH_2^* + O_2 \longrightarrow CH_3OH + H_2O + A^+ \quad \text{(Eq. 2-3)}$$

** A is a reducing equivalent providing $2e^-$ and $2H^+$; NAD(P)H in sMMO; not confirmed yet in pMMO*

The multi-function oxidase MMO is found in two forms; the cytoplasmic soluble form "sMMO" located in the cytoplasm and the copper containing particulate form "pMMO" located in the ICM (Semrau et al., 2010). sMMO utilizes nicotinamide adenine dinucleotide NAD(P)H generated from formaldehyde and formate oxidation as reducing equivalents (Hanson and Hanson, 1996; Karthikeyan et al., 2015). On the other hand, the electron donor for pMMO has not been identified yet (Karthikeyan et al., 2015). It was assumed that ubiquinol ($Q8H_2$) could be the most probable pMMO electron donor relying on its similarities with AMO. However, the mechanism of ubiquinone reduction to ubiquinol is still not clear (Kalyuzhnaya et al., 2015). One hypothesis is that electrons transfer between methanol oxidizing enzyme methanol dehydrogenase (MDH) and pMMO owing to their close locations (Culpepper and Rosenzweig, 2014). Alternatively, NAD(P)H from formaldehyde and formate oxidation may be the responsible for ubiquinone reduction based on the observed enhancement in methane oxidation and methanol production with external formate addition (Kalyuzhnaya et al., 2015; Trotsenko and Murrell, 2008). Hence, it can be concluded that pMMO might be utilizing various electron sources according to the growth conditions (Kalyuzhnaya et al., 2015). pMMO-expressing cells oxidize methane more efficiently than sMMO as they have higher methane affinity and growth yields (Kalyuzhnaya et al., 2015). On the other hand, sMMO have a broader substrate range than pMMO which makes it more attractive for several biotechnological applications (Chistoserdova and Lidstrom, 2013; Smith et al., 2010). The expression of both sMMO and pMMO enzymes is controlled by copper concentration in the growth medium. pMMO is expressed in copper concentrations above 1 µmol/gram (dry weight) of cells, whereas, sMMO is expressed in concentration below 1

μmol/gram (dry weight) of cells (Hanson and Hanson, 1996; Karthikeyan et al., 2015). Both enzymes properties are summarized in **Table (2-1)**.

Table (2-1): Particulate and Soluble Methane Monooxygenase (pMMO and sMMO) Enzymes Properties

	Particulate Methane Monooxygenase (pMMO)	**Soluble Methane Monooxygenase (sMMO)**
Location	Intercellular membrane (ICM)	Cytoplasm
Components	Copper containing and consist of three polypeptides	three subunits: hydroxylase (di-iron center), reductase, protein B
Copper Conc.	1 μmol/g (DW) or higher	below 1 μmol/g (DW)
Reducing equivalent	Not yet confirmed	NAD(P)H
Methane affinity	High	Low
Methanotrophs	All Methanotrophs except Methylocella and Methyloferula	Methylocystis, Methylosinus, Methylocapsa, Methylomagnum, Methaylovulum, and some strains within Methylomonas and Methylocaldum
Encoding genes	pmoB, A, and C	Six-gene operon mmoXYBZDC
Co-substrates	Narrow range of alkanes, alkenes, and alicyclic compounds	Wide range of alkanes, alkenes, Halogenated aliphatics, Monoaromatics, Diaromatics and alicyclic compounds

The produced methanol is oxidized further to formaldehyde via the quinoprotein methanol dehydrogenase (MDH) located in the periplasm, **equation (2-4)** (Chistoserdova and Lidstrom, 2013; Smith et al., 2010). Methanol oxidation to formaldehyde is accompanied with the reduction of pyrroloquinoline quinone (PQQ) to $PQQH_2$. Afterwards, $PPQH_2$ is oxidized and transfer electrons (2 electrons) either to the terminal oxidase with cytochromes-c and other carriers as intermediates or to reduce the pMMO electron donor as previously described (Chistoserdova and Lidstrom, 2013; Hanson and Hanson, 1996; Smith et al., 2010). Regardless the electrons destination, methanol oxidation is an energy-conserving step.

$$CH_3OH + PQQ \longrightarrow CH_2O + PQQH_2^{*} \quad \text{(Eq. 2-4)}$$

* $pQQH_2$ is further oxidized and transfer $2e^-$ and $2H^+$ to terminal oxidase or pMMO

Formaldehyde plays a pivotal role in methanotrophs metabolism as the central intermediate. Part of the formed formaldehyde is terminally oxidized to CO_2 for energy generation while the other part is incorporated in the carbon assimilation pathways for cell replication; RuMP pathway for type I and serine pathway for type II. Formaldehyde is rapidly directed to either cycles due to its toxic effect on methanotrophs (Hanson and Hanson, 1996; Karthikeyan et al., 2015).

Two systems have been suggested for formaldehyde oxidation into formate, **equation (2-5)**. Firstly, oxidation is catalyzed by formaldehyde dehydrogenase (FaDH) which is either NAD-linked or PQQ-containing and cytochrome-linked enzyme (Chistoserdova and Lidstrom, 2013; Smith et al., 2010). The quinoprotien FaDH was confirmed to be the major formaldehyde oxidizing enzyme in methylococcus capsulatus while expressing pMMO only (Zahn et al., 2001). This finding supports the hypothesis that the electron source for pMMO can be linked to the formaldehyde oxidation. The second suggestion is the tetrahydromethanopterin (H_4MPT)-linked formaldehyde oxidation pathway. Within the H_4MPT pathway, MtdB enzyme reduces $NAD(P)^+$ and produces NAD(P)H (Chistoserdova et al., 2009; Kalyuzhnaya et al., 2015). In addition, different formaldehyde oxidation systems were found in methylotrophs and might be existing in methanotrophs only as a formaldehyde detoxification strategy (Chistoserdova and Lidstrom, 2013). Finally, formate is oxidized to carbon dioxide, **equation (2-6)**. This last reaction is catalyzed by the NAD dependent enzyme formate dehydrogenase (FDH) which functions as sMMO electron source (Smith et al., 2010).

$$CH_2O + H_2O \longrightarrow CH_2O_2 + 2H^+ + 2e^- \quad \text{(Eq. 2-5)}$$

NAD(P)H or PQQH₂ is produced according to the pathway

$$CH_2O_2 + NAD^+ \longrightarrow CO_2 + NAD(P)H + H^+ \quad \text{(Eq. 2-6)}$$

Generally, the last three oxidation reactions produce 6 electrons ($2e^-$ each). Two of them travel back to methane oxidation in the form of reducing equivalent for simultaneous methane uptake. Whereas, the remaining electrons ($4e^-$) traverse the electron transport chain (ETC) generating energy by producing adenosine triphosphate (ATP) through the proton motive force, as shown in **Figure (2-2)**. Finally, the four electrons reduced the oxygen as the terminal electron acceptor to H_2O **equation (2-7)**. Accordingly, the overall reaction can be written as shown in **equation (2-8)**.

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O \quad \text{(Eq. 2-7)}$$

$$CH_4 + 2O_2 \longrightarrow CO_2 + 2H_2O \quad \text{(Eq. 2-8)}$$

The overall reaction

As mentioned before, the nitrite dependent anaerobic methane oxidizing bacteria M.oxyfera lacks the ICM. However, the same pathway took place with two preliminary reactions, expressed in **equations (2-9 and 2-10)**, in which nitrite (NO_2^-) is converted to NO via the periplasmic nitrite reductase (cd_1 Nir). Subsequently, two NO molecules are converted to nitrogen gas (N_2) and oxygen (O_2). The produced O_2 is furtherly utilized in the normal methane oxidation pathway and acts as the terminal electron acceptor (L. Shen et al., 2015; Wu et al., 2011). The overall anaerobic reaction is expressed in **equation (2-11)**.

$$8NO_2^- + 16H^+ \longrightarrow 4N_2 + \mathbf{6O_2} + 4H_2O \quad \text{(Eq. 2-9)}$$

$$3CH_4 + \mathbf{6O_2} \longrightarrow 3CO_2 + 6H_2O \quad \text{(Eq. 2-10)}$$

The oxygen produced is further involved in methane oxidation pathway

$$3CH_4 + 8NO_2^- + 8H^+ \longrightarrow 4N_2 + 3CO_2 + 10H_2O \quad \text{(Eq. 2-11)}$$

The overall reaction

For the methane assimilation pathways, as previously mentioned, type I undergoes the RuMP pathway and type II use the serine pathway while type III and the anaerobic methanotrophs assimilate carbon via CBB cycle. Each cycle has different reactions and enzymes involved in their assimilation pathway for energy production and cell replication.

The RuMP pathway is initiated by the reaction between formaldehyde and ribulose-5-phosphate yielding fructose-6-phosphate. Two specific enzymes mediate this reaction; hexulosephosphate synthase (HPS) and hexulosephosphate isomerase (HPI) found only in type I methanotrophs. Fructose-6-phosphate follows a series of reactions producing pyruvate and glyceraldehyde-3-phosphate. Glyceraldehyde is then utilized to regenerate ribulose-5-phosphate and complete the cycle for biomass synthesis, while the pyruvate is incorporated in an incomplete TCA cycle for CO_2 production. The main intermediates of the RuMP cycle are found in the form of sugar phosphates (Hanson and Hanson, 1996; Hwang et al., 2014; Kalyuzhnaya et al., 2015; Karthikeyan et al., 2015; Khmelenina et al., 2015).

In the serine cycle, formaldehyde is converted to methyl-H_4MPT then to methylene-H_4F mediating the reaction of formaldehyde with glycine to activate the serine cycle (Kalyuzhnaya et al., 2015; Karthikeyan et al., 2015). Furthermore, the cycle produce acetyl-CoA which is the key entry for the complete TCA cycle required for energy generation and biomass synthesis (Chidambarampadmavathy et al., 2015). The main intermediates in the serine cycle are found in the form of amino acids and CoA derivatives (Kalyuzhnaya et al., 2015).

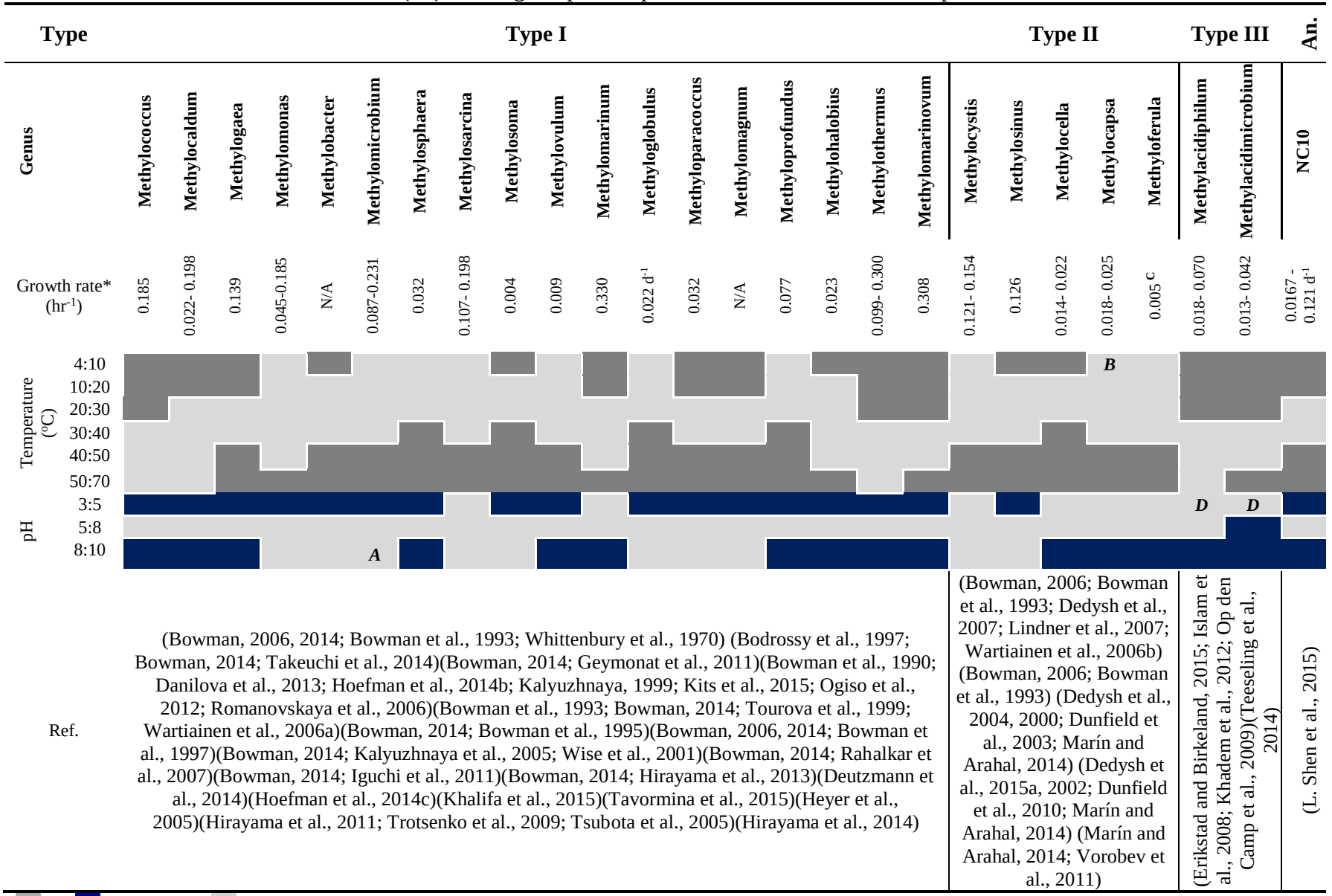
In general, type I methanotrophs have higher growth rate than type II (Kalyuzhnaya et al., 2015). Moreover, type I is more energy efficient as it requires 1 ATP for assimilation of 3 formaldehyde molecules compared to 3 ATP and 2 NAD(P)H to assimilate 2 formaldehyde molecules and 1

carbon dioxide molecule in type II (Karthikeyan et al., 2015). Up to 50% of the produced carbon dioxide by type II can be incorporated in cell synthesis compared to 15% in type I, what makes the biogas produced from anaerobic digesters consisting of about 40% CO_2 more suitable for type II (Kalyuzhnaya et al., 2015). One more advantage for type II is their ability to accumulate biopolymers under nutrients limited conditions.

2.4 Factors Affecting Methanotrophs Growth

The reported growth rates for methanotrophs using methane in pure cultures under optimum conditions is demonstrated in **Table (2-2)**. Type I methanotrophs have the highest growth rate ranging from 0.2 to 0.3 hr^{-1} in some strains (Hirayama et al., 2014, 2011; Whittenbury et al., 1970; Wise et al., 2001). Whereas, the highest growth rate reported in type II methanotrophs is from 0.15 to 0.18 hr^{-1} for methylocystis strains (Dedysh et al., 2007; Whittenbury et al., 1970). Type III methanotrophs are slower than both types with growth rate ranging from 0.013 to 0.07 hr^{-1} (Op den Camp et al., 2009; Teeseling et al., 2014). Anaerobic M.oxyfera is the slowest growing methanotroph with a doubling time that can reach up to 2 months (L. Shen et al., 2015). However, no specific type dominates in methane sufficient cultures, which can be referred to the fact that each type has different growth conditions rather than the methane presence such as the pH, temperature, nutrients, methane and oxygen ratio, etc. Furthermore, factors like methane solubility and bioreactor configuration can result in a better control on microbial yield and biomass density. Unfortunately, various factors affecting methanotrophs growth are still unclear and need to be reviewed and investigated either in natural habitats or in bioreactors.

Table (2-2): Growing Temperature, pH, and Growth rates for Methanotrophs



*or No growth, can grow, * growing on methane in pure and optimum culture,* ***A*** *methylomicrobium Kenyense can grow at pH up to 11,* ***B*** *methylocapsa aurea can grow at 2°C,* ***C*** *methyloferula stellate grow better on methanol with specific growth rate of 0.015 hr-1,* ***D*** *both Methylacidiphilum and Methylacidimicrobium can grow at pH lower than 3.*

2.4.2 Temperature

More than 70% of methanotrophs grow optimally in a temperature ranging from 20 to 35ºC, **Table (2-2)**. However, high temperatures (55 to 60ºC) is more preferable for the type I methylothermus genus and type III methylacidiphilum genus (Bowman, 2014; Hirayama et al., 2011; Op den Camp et al., 2009; Tsubota et al., 2005). Within type I, methylococcus and methylocaldum genus (which form type X a subset of type I methanotrophs (Bowman, 2006)) grow optimally at temperature range of 42-55ºC (Bodrossy et al., 1997; Bowman, 2006; Trotsenko et al., 2009). In contrast, methylosphaera genus grows at lower temperature ranging from 10 to 15ºC (Bowman, 2006; Bowman et al., 1997). Furthermore, all type II methanotrophs (except some strains within methylocystis) and some type I methanotrophs (methylosphaera, methyloprofundus, methyloglobulus, methylovulum, and some species within methylobacter, methylosarcina, methylomonas, methylomicrobium) are able to survive at temperatures as low as 4 to10ºC (Bowman, 2006, 2014; Bowman et al., 1993; Deutzmann et al., 2014; Iguchi et al., 2011; Marín and Arahal, 2014; Tavormina et al., 2015). Moreover, it was observed that type I methanotrophs predominated at lower temperature (3 to10ºC) in a sample enriched from landfill cover soils while both types grew normally at 20ºC (Börjesson et al., 2004).

2.4.3 pH and Salinity

More than 90% methanotrophs prefer to grow in pH ranges from 5.5 to 8, **Table (2-2)**. However, verrucomicrobial methanotrophs are more acidophilic with optimum growth at pH ranging from 1.5 to 3.5, while, methylacidiphilum fumariolicum (strain SoIV) and methylacidimicrobium tartarophylax sp. are the most acidophilic methanotrophs growing at pH between 0.5 and 0.8 (Op den Camp et al., 2009; Pol et al., 2007; Teeseling et al., 2014). In contrast, methylomicrobim species grow better in alkaline mediums (pH 8-10) (Bowman, 2006; Kaluzhnaya et al., 2001;

Kalyuzhnaya et al., 2008). For the salinity, most of methanotrophs don't require NaCl for their growth with wide tolerance range from 0.2 up to 10% NaCl (w/v) (Bowman, 2014; Bowman et al., 1993; Semrau et al., 2010). However, methylosoma genus is intolerant to NaCl (Rahalkar et al., 2007). Furthermore, a study on mixed culture showed that methane uptake and growth rate remained constant with salinity up to 7 mg/L, while, a noticeable decline occurred by increasing the salinity above this level (Ha et al., 2010).

2.4.4 Substrates

Generally, all methantrophs prefer methane as their substrate. However, methanotrophs except most of the methylocaldum species, methylobacter tundripaldum sp., and methylocystis rosea sp. can grow on methanol in the absence of methane (Bodrossy et al., 1997; Bowman, 2014; Lidstrom, 2006; Marín and Arahal, 2014; Wartiainen et al., 2006a, 2006b). Although, the growth is fully or partially inhibited at relatively high methanol concentrations. This was elucidated by the excessive accumulation of the toxic formaldehyde resulting from the methanol oxidation (Bowman, 2006; Graham et al., 1993; Tabata and Okura, 2008; Whittenbury et al., 1970). Despite its inhibitory effect, methylocella tundrae prefers methanol than methane (Dedysh et al., 2004; Dunfield et al., 2003). On the other hand, some strains within methylocapsa and methylocella genus have the ability to grow on other C_1 compounds like formate, and methylamines (Dedysh et al., 2004; Dunfield et al., 2010, 2003). For a long time, it was believed that all methanotrophs grow only on C_1 compounds (Anthony, 1982; Hanson and Hanson, 1996; Semrau et al., 2011, 2010; Theisen and Murrell, 2005). However, it was discovered that some type II strains can grow on multi-carbon substrates (Belova et al., 2013, 2011; Dedysh et al., 2005; Dunfield et al., 2010; Im et al., 2011; Semrau et al., 2011). Methylocella species can grow on acetate, ethanol, malate, succinate, and pyruvate without losing its vitality. Methylocella

silvestris BL2^T grew faster on acetate than on methane and interestingly the methane consumption rate increased after growing on acetate. Moreover, acetate was preferred when both substrates were available in the growing medium (Dedysh et al., 2005; Semrau et al., 2011). Contrarily, methylocapsa aurea can grow on acetate at the expense of the growth rate (Dunfield et al., 2010; Semrau et al., 2011). Moreover, many strains within methylocystis can grow on acetate, while Methylocystis bryophila can poorly grow on pyruvate and ethanol (Belova et al., 2013, 2011; Im et al., 2011).

2.4.5 Methane to Oxygen Ratio

In conditions where methane concentration is above 1%, type II methanotrophs form stable but slow growing communities in which the fluctuation in CH_4 and O_2 concentration does not affect the community structure or their abundance (Z. Chi et al., 2012; Henckel et al., 2000; Semrau et al., 2010). Moreover, type II dominates in very low methane concentration, below 0.06%, due to the possession of the unique pMMO2 which is found in most methylocystis spp., mehtylosinus sporium, some of methylosinus trichoporium strains (Baani and Liesack, 2008; Knief and Dunfield, 2005). Furthermore, it was reported in different studies that high methane to oxygen ratio, low oxygen concentration, is more preferable for type II growth (Amaral and Knowles, 1995; Graham et al., 1993; Knief, 2015; López et al., 2013; Semrau et al., 2010).

Unlike type II, type I methanotrophs grow faster with communities more sensitive to fluctuation methane and oxygen concentrations as the microbial structure changes with the variation of either one of them or both (Z. Chi et al., 2012; Henckel et al., 2000; López et al., 2014; Semrau et al., 2010). Thus, type I mostly is expected to dominate in the first stage of any enrichment process. Moreover, it was reported that type I outcompete at methane concentrations between 0.06 and 1% where type II can grow but with lower abundance (Cantera et al., 2016; Henckel et

al., 2000; Li et al., 2014; López et al., 2014; Semrau et al., 2010). This finding is supported by the fact that pMMO, unlike sMMO, has higher affinity to methane (Z. Chi et al., 2012; Kalyuzhnaya et al., 2015; López et al., 2013). It is noteworthy that methane and oxygen mixing ratio or concentrations are not reliable selection parameter between type I and type II. Unless, other parameters were manipulated, i.e., copper concentration or nitrogen source.

It was reported that microbial growth and methane uptake rate is remarkably increased with methane and oxygen concentrations increase as well as the existence of both types regardless their density (Z.-F. Chi et al., 2012; Henckel et al., 2000; Li et al., 2014; López et al., 2014). However, some studies claimed that a lack in methane oxidation occurred at high oxygen concentration. This hypothesis was based on two observations; (1) possessing oxygen sensitive nitrogenase, the enzyme responsible for fixing atmospheric nitrogen, which can be revealed by adding other nitrogen sources such as nitrate or ammonia, (2) the excess accumulation of the toxic intermediates such as formaldehyde (Amaral and Knowles, 1995; Costa et al., 2001; Henckel et al., 2000; Pfluger et al., 2011).

2.4.6 Methane Solubility

One of the major obstacles facing methanotrophs cultivation is the energy intensive agitation required to overcome methane low water solubility effects (dimensionless Henry's law constant equals 31.4 at 25ºC) (López et al., 2014). Whereas, attached growth systems showing very low yields and growth rates due to the very low methane transfer rate (Fennel et al., 1992; Pfluger, 2010).

In an approach to increase methane solubility and its delivery to the bacteria, a pressure bioreactor was tested and high biomass densities ranged from 18-65 g/l were achieved. However,

those types of bioreactors are considered to be cost intensive and less secured than reactors working under atmospheric pressure (Helm et al., 2008; Wendlandt et al., 2005, 2001). Another system was tested for methane solubility enhancement which is the two-phase partitioning bioreactor (TPPB) in which a non-aqueous phase (NAP) with higher affinity towards methane than water is employed. When 10% v/v of silicon oil was added, growth rate increased by 330% in the TPPB enriched with the methyosinus sporium (DSMZ 17706) strain (Ordaz et al., 2014). Using the same concept, the addition of 5% v/v paraffin oil to the growth medium including methylosinus trichosporium OB3b in a 5-l fermenter resulted in increasing the biomass density from 1 to 6 g/l after 160 hours. Furthermore, the biomass density reached 14 g/l in the medium supplemented with paraffin oil after 240 hours (Han et al., 2009). Loop bioreactors were also considered for increasing the methane delivery to methanotrophs. They have the advantage of their circular flow, which can be accomplished in fluidized systems by a propeller or a jet drive and optimum for gases having low solubility in water. Methylocystis hirsuta strain was cultivated in two loop bioreactors with different configurations; Bubble column bioreactor and forced-liquid vertical loop bioreactor and could reach a biomass density up to 2.9g/l (Rahnama et al., 2012).

2.4.7 Nitrogen Sources

All type II methanotrophs and few type I methanotrophs (methylococcus, methylosome, methyloglobulus, methyloprofundus, and some strains within methyomonas, and methylobacter) have the ability to fix atmospheric nitrogen via the oxygen sensitive nitrogenase. Whereas, methylosphaera has more oxygen tolerance to nitrogen fixation (Bowman, 2006; Bowman et al., 1997). Thus, type II methanotrophs dominates in N-limiting condition or high carbon to nitrogen (C/N) ratio, while type I demands high nitrogen content or lower C/N ratio for better growth (He

et al., 2011; López et al., 2013; Zhang et al., 2014). This distinguishing ability was manipulated as a selection tool in the enrichment of type II methanotrophs from mixed cultures in low dissolved oxygen concentrations (Graham et al., 1993; Pfluger et al., 2011; Pieja et al., 2011). However, methanotrophs grow better on inorganic nitrogen, specifically nitrate and ammonia, as the cellular nitrogen source (Bowman, 2006, 2014; Graham et al., 1993; López et al., 2013; Semrau et al., 2010). Ammonia presence can partially or fully inhibit the growth because of the competition between ammonia on MMO or the accumulation of excessive toxic hydroxylamine or nitrite (Hanson and Hanson, 1996; López et al., 2013; Semrau et al., 2010; Sundstrom and Criddle, 2015). Therefore, nitrate growing cultures exposes higher growth rates than ammonia enriched cultures for both type I and type II methanotrophs (Amaral and Knowles, 1995; Karthikeyan et al., 2016; Mohanty et al., 2006). Interestingly, it was found that higher moisture content and sufficient methane concentration in comparison with nitrogen load relieve ammonia toxicity and substrate competition effects, respectively (He et al., 2011; Wang et al., 2011; Zhang et al., 2014). Some batch tests showed that the addition of ammonia to different N-limiting soils was accompanied with an increase in methane oxidation rates and type I abundance (He et al., 2011; Lee et al., 2009; Mohanty et al., 2006; Visscher et al., 2001; Zhang et al., 2014). Afterwards, a notable decline in methane oxidation rate was noticed after reaching the peak (He et al., 2011; Zhang et al., 2014) which can be referred to the fast growth of type I and their sensitivity for nutrients sufficiency conditions (López et al., 2014; Pieja et al., 2011; Semrau et al., 2010; Wise et al., 1999). Supporting this hypothesis, nitrate was found to have greater stimulatory effect than ammonia which reflects that the previously reported increase in methane oxidation rate at higher ammonia concentrations was due to the increase in nitrogen source concentration generally not ammonia concentration specifically (Karthikeyan et al., 2016;

Mohanty et al., 2006; Zhang et al., 2017). On the other hand, type II methanotrophs can build a more stable community at the presence ammonia with minor inhibitory effects in methane sufficient conditions (He et al., 2011; Lee et al., 2009; Mohanty et al., 2006; Visscher et al., 2001; Zhang et al., 2014). Moreover, ammonia had a minor inhibitory effect on methylocystis sp. in comparison with methylomicrobium album, methylosinus sporium, and methylomonas methanica. Therefore, it was suggested that methylocystis sp. might possess multiple enzymes that can detoxify the produced hydroxylamine (Nyerges and Stein, 2009). Furthermore, methylocystis dominated an activated sludge culture after 24 days' incubation under continuous ammonia feeding conditions with slower growth rate (Myung et al., 2015). Interestingly, a recent study reported that sMMO expressing methylosinus trichosporium OB3b has a slightly higher growth yield and methane oxidation rate when growing on ammonia than nitrate (Zhang et al., 2017). Therefore, using nitrate as nitrogen source results in higher growth and methane oxidation rates with type I preferable cultures. Whereas, ammonia continues presence or N-limiting condition supports type II dominance with slower growth rates.

2.4.8 Copper

As mentioned before, copper concentration regulates the expression of MMO enzyme (Semrau et al., 2010). pMMO is well developed in concentrations above 1 µM while sMMO is expressed below this level (Ha et al., 2012; Hanson and Hanson, 1996; Adrian Ho et al., 2013). However, several studies discovered that copper concentration is not a reliable parameter in type I or type II selection as most of methanotrophs can express pMMO (Cantera et al., 2016; Pieja et al., 2011). Moreover, some sMMO lacking methanotrophs can survive and grow under very low copper concentration like methylomicrobium and methylobacter which can be referred to their possession of unique copper uptake mechanisms like the methanobactin one (Cantera et al.,

2016; Semrau et al., 2010). Also, some type I like Methylomonas possess both sMMO and pMMO and dominate in cultures having low copper concentrations (Cantera et al., 2016; Ha et al., 2012). In mixed cultures, some studies mentioned that copper addition significantly increase methane uptake and growth rate especially between 1 and 4.31 µM (Cantera et al., 2016; Graham et al., 1993; Ha et al., 2010; Adrian Ho et al., 2013; López et al., 2013). Conversely, others noticed either no or minor increase (Ha et al., 2012; Karthikeyan et al., 2016). These contradicted reports suggest that copper effect may depend on other factors: (1) initial community structure for instance, if the dominant genus was methylomonas, no or minor increase will be noticed, whereas in case of type II existence an increase will take place (Graham et al., 1993; Ha et al., 2012, 2010), (2) methane concentration and nitrogen load which may select a pMMO expressing cluster regardless copper concentration (Karthikeyan et al., 2016). It is noteworthy that excessive copper addition was reported to result in full or partially inhibitory effects due to its toxicity (Adrian Ho et al., 2013; Lee et al., 2009).

2.5 Bio-Methanol Production Metabolism

Bio-methanol can be produced either via utilizing the MMO enzyme or the whole methanotrophic cell as a biocatalyst. The former process has major drawbacks such as high cost and the enzymes instability. Hence, biological methanol production using methanotrophs can be more promising especially from the biogas produced from anaerobic digesters (Hwang et al., 2014; Park and Lee, 2013). As mentioned before, methanotrophs hydroxylate methane in an energy requiring step, **equations (2-1, 2-2, and 2-3),** which is catalyzed by the MMO. The produced methanol is rapidly oxidized to carbon dioxide with formaldehyde and formate as intermediates. Oxidation is catalyzed by methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FaDH), formate dehydrogenase (FDH), respectively. Accordingly, it is essential

to inhibit MDH activity for extracellular accumulation of methanol and subsequently, inhibiting the following oxidation steps (Duan et al., 2011; Ge et al., 2014; Mehta et al., 1987). Unfortunately, the last three steps are the electrons producing steps, **equations (2-4, 2-5, and 2-6)**. Therefore, MDH inhibition results in a shortage in the electrons needed for cellular energy and simultaneous methane hydroxylation. Also, a lack in carbon fixation can occurr as the cellular carbon is assimilated at the level of formaldehyde, formate and carbon dioxide (Hwang et al., 2014; Park and Lee, 2013). **Figure (2-3)** illustrates the methanol production pathway.

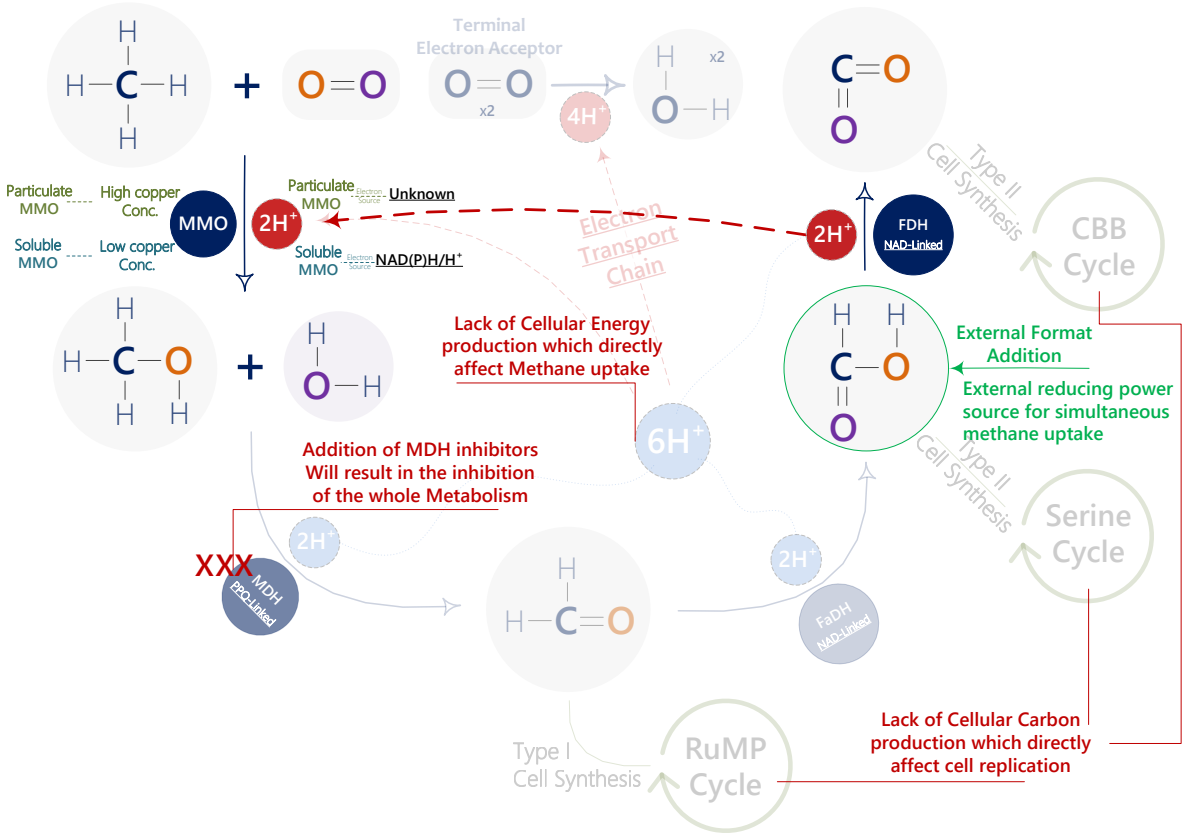


Figure (2-3)**: Methanol Production Metabolism.**

MMO: Methane Monooxygenase, MDH: Methanol Dehydrogenase, FaDH: Formaldehyde Dehydrogenase, FDH: Formate Dehydrogenase, PQQ: Pyrroloquinoline Quinone, NAD(P)H: Nicotinamide Adenine Dinucleotide, RuMP: Ribulose Monophosphate pathway, CBB: Calvin-Benson-Bassaham.

2.6 Factors Affecting Bio-Methanol Production

Several studies revealed various MDH inhibition strategies like, but not limited to, EDTA, high salts concentrations and cyclopropanol addition. However, methanol accumulation stopped within few hours due to the depletion of the reducing equivalents. This was resolved by sodium formate external addition as electron source. However, the addition of external electrons donors as expensive as formate limits the process upscaling. Furthermore, methanol yields have not reached the feasible range yet (Ge et al., 2014; Hwang et al., 2014; Park and Lee, 2013; Pen et al., 2014; Tabata and Okura, 2008). In order to make methanol production process industrially feasible, multiple factors, (i.e., pH, Temperature, cell densities, and gases mixing ratio) need be optimized as well as electrons supply and MDH inhibition strategies.

2.6.1 Bacterial Strains

Most of the studies were performed on pure cultures such as methylosinus trichosporium (Duan et al., 2011; Furuto et al., 1999; Hwang et al., 2015; Kim et al., 2010; Lee et al., 2004; Mehta et al., 1991, 1987; Pen et al., 2014; Takeguchi et al., 1997; Xin et al., 2007, 2004a, 2004b), Methylosinus sporium (Patel et al., 2016b; Yoo et al., 2015), methylocella tundrae (Mardina et al., 2016), methylocystis bryophila (Patel et al., 2016a), and strain 14B (Methyloculdum genus like) isolated from solid state anaerobic digester (Sheets et al., 2016). A consortium of strains (methylosinus trichosporium OB3b, Methylococcus capsulatus, and Methylosinus sporium) isolated from landfill soil were also studied (Han et al., 2013). Additionally, Patel et al. studied the potential of methylocella silvestris, methyloferula stellate, methylomonas methanica for methanol production (Patel et al., 2016a). It is noteworthy to mention that all of the studied strains except the strain 14B belong to type II despite type I has higher growth rate, exhibits

higher methane affinity, and require less energy for growth which make it more promising for methanol production.

2.6.2 pH and Temperature

All the studies were performed in pH and temperature ranging from 6.40 to 7 and 25 to 37ºC, respectively. Whereas, a notable decline in methanol was reported below or above those ranges. However, M. bryophila had the ability to produce methanol at higher pH of 8.5 (Patel et al., 2016a).

2.6.3 Nutrients

As mentioned before, copper is a crucial nutrient for pMMO and sMMO expression. Therefore, methanol productivity is expected to increase with copper addition due to the expression of the pMMO known for its higher methane uptake (Kalyuzhnaya et al., 2015). It was reported that concentrations ranging from 1 to 5 µM Cu^{+2} would notably increase methanol production. However, it was found that copper concentrations higher than 10 µM inhibited both the methanol production and bacterial growth. Also, addition of 10 µM iron in the culturing medium resulted in an enhanced methanol production due to its positive effect on MMO activity (Furuto et al., 1999; Hwang et al., 2015; Mardina et al., 2016; Patel et al., 2016b, 2016a; Pen et al., 2014; Sheets et al., 2016; Takeguchi et al., 1997). Interestingly, it was found that the addition of both 10 µM Fe and 5 µM Cu doubled methanol production of M.sporium (Patel et al., 2016b).

2.6.4 Liquid to Gas Ratio

The ratio between liquid and gas volumes directly affect methane and oxygen availability. Liquid to gas ratio used in the previous studies ranged from 1:1.5 to 1:9 (Duan et al., 2011; Furuto et al., 1999; Han et al., 2013; Hwang et al., 2015; Kim et al., 2010; Lee et al., 2004; Mardina et al.,

2016; Mehta et al., 1991, 1987, Patel et al., 2016b, 2016a; Pen et al., 2014; Sheets et al., 2016; Takeguchi et al., 1997; Xin et al., 2007, 2004a, 2004b; Yoo et al., 2015). Moreover, Pen et al. found that increasing the ratio from 1:1.67 to 1:9 significantly increased the methanol production, especially, at higher biomass concentration (Pen et al., 2014). Patel et al. reported that 1:5 is the optimum ratio, while, 1:7 and 1:9 are slightly lower (Patel et al., 2016b).

2.6.5 Gas Mixing Ratio and Composition

Theoretically, 1 mole of methane and 1 mole of oxygen are required to produce 1 mole of methanol, **equations (2-1, 2-2, and 2-3)**. However, methane to oxygen ratio in the headspace deviates from this ratio due to the limitation of oxygen and methane mass transfer and/or further methanol oxidation. The methane concentration used in the previous studies varied from 20% to 50% (Duan et al., 2011; Furuto et al., 1999; Han et al., 2013; Hwang et al., 2015; Kim et al., 2010; Lee et al., 2004; Mardina et al., 2016; Mehta et al., 1991, 1987, Patel et al., 2016b, 2016a; Pen et al., 2014; Sheets et al., 2016; Takeguchi et al., 1997; Xin et al., 2007, 2004a, 2004b; Yoo et al., 2015). Also, it was reported that increasing methane concentration leads to higher methanol production, especially, when the incubation time was 24 hours or higher (Mardina et al., 2016; Patel et al., 2016b, 2016a). However, the effect of methane concentration increase on the uptake rate and conversion ratio is not reported.

Biogas is approximately composed of 60% CH_4, CO_2, H_2S not pure methane. Interestingly, It was found that the presence of carbon dioxide increase methanol production (Patel et al., 2016b). This is can be referred to its inhibition effect on MDH activity (Xin et al., 2004a) or fixation of carbon dioxide as cellular carbon (Karthikeyan et al., 2015). Moreover, it was reported that 40% concentration of CO_2 in the headspace results in higher MDH inhibition (Xin et al., 2004a). On the other hand, Sheets et al. reported that up to 50 ppm of H_2S did not show any inhibitory effect

on methanol production (Sheets et al., 2016). Supportively, using artificial mix of CH_4, CO_2, and H_2 resulted in an increased methanol production (Patel et al., 2016b).

2.6.6 MDH Inhibition

Methanol oxidation is catalyzed by the PQQ linked MDH, **equation (2-4)**, in which cytochromes-c are utilized as the electron carriers to the terminal oxidase (Chistoserdova and Lidstrom, 2013; Hanson and Hanson, 1996; Smith et al., 2010). Only trace concentrations can be observed unless methanol oxidation is inhibited (Ge et al., 2014; Han et al., 2013; Hwang et al., 2014). The most common strategy used for MDH inhibition is the addition of chemical inhibitors including; phosphate buffer (Duan et al., 2011; Hwang et al., 2015; Mardina et al., 2016; Mehta et al., 1991, 1987, Patel et al., 2016b, 2016a; Sheets et al., 2016), NaCl (Han et al., 2013; Kim et al., 2010; Lee et al., 2004; Pen et al., 2014; Yoo et al., 2015), Cyclopropanol (Furuto et al., 1999; Takeguchi et al., 1997), EDTA (Hwang et al., 2015; Kim et al., 2010), $MgCl_2$ (Duan et al., 2011; Han et al., 2013; Mardina et al., 2016; Mehta et al., 1991, 1987, Patel et al., 2016b, 2016a; Yoo et al., 2015), and NH_4Cl (Han et al., 2013; Yoo et al., 2015). Such inhibitors were used either separately or combined in order to inhibit MDH activity.

Cyclopropanol with an optimum of 0.67 µM was reported as the most efficient MDH inhibitor through decreasing the MDH activity by 50% while decreasing the MMO activity by 5.2% only (Furuto et al., 1999; Ge et al., 2014; Han et al., 2013; Kim et al., 2010; Takeguchi et al., 1997). However, it is not used commonly due to the instability under aerobic conditions and difficulty in preparation. Moreover, cyclopropanol is considered as an irreversible inhibitor as it produces a stable compound from the interaction with the free and MDH-linked PQQ (Ge et al., 2014; Han et al., 2013; Kim et al., 2010). As shown in **Table (2-3)**, a high conversion efficiency was observed while using 40 mM NH_4Cl at the expense of methanol yields. These results can be

referred to the aforementioned inhibitory effect on MMO which decrease the methane uptake, thus, it is not preferred as inhibitor (Kim et al., 2010; Patel et al., 2016b, 2016b, 2016a; Yoo et al., 2015). $MgCl_2$ inhibitory mechanism is still unknown (Ge et al., 2014), however, various studies observed that addition of $MgCl_2$ with concentrations ranging from 5 – 20 mM to phosphate buffer significantly increased methanol production (Duan et al., 2011; Mardina et al., 2016; Mehta et al., 1987; Patel et al., 2016b, 2016a). Supportively, Mg ions support the sMMO activity and the methanotrophic cell growth (Duan et al., 2011). On the other hand, 50µM - 1mM of EDTA binds to the cytochrome-binding area in the MDH and decreases the MDH activity by blocking the electron transfer to the cytochrome-c (Ge et al., 2014; Han et al., 2013). Moreover, EDTA reduces -to lesser extent- MMO activity which might be due to EDTA chelation effect on the MMO metal ions. (Mardina et al., 2016; Patel et al., 2016b; Sheets et al., 2016). However, no studies relied on EDTA and $MgCl_2$ as the sole inhibitors and they were added with either phosphate or NaCl, **Table (2-3)** (Han et al., 2013; Kim et al., 2010; Yoo et al., 2015).

Furthermore, studies showed that high salt concentrations (i.e., NaCl and phosphate) disrupt electrons transport between PQQ and cytochromes-c which decrease the MDH activity (Ge et al., 2014). NaCl can be an advantageous inhibitor due to its stability, abundance and low cost. It was reported that 200 mM of NaCl is the optimum concentration for methanol production (Lee et al., 2004). However, it was observed that concentrations Higher than 100 mM NaCl distorts cell morphology. Thus, a combination of NaCl and other inhibitors such as EDTA were proposed to substitute the reduced NaCl concentration. Interestingly, EDTA addition resulted in approximately 1.8 times higher methanol production (Kim et al., 2010; Pen et al., 2014). On the other hand, phosphate was found to be the most commonly used methanol inhibitor used with concentrations ranging from 40 to 100 mM (Hwang et al., 2015; Mardina et al., 2016; Mehta et

al., 1991, 1987, Patel et al., 2016b, 2016a; Sheets et al., 2016). It is considered to be uncompetitive and reversible (Mehta et al., 1991, 1987).

Table (2-3): Methanol Dehydrogenase Inhibitors

Inhibitor	*Species*	*Optimum concentration*	*MDH Relative Activity (%)*	*MMO Relative Activity (%)*	*Conv. Efficiency (%)*	*References*
Cyclopropanol	M. Trichosporium	0.67 µM	51%	95%	61%	(Furuto et al., 1999; Takeguchi et al., 1997)
NH_4Cl	M.Sporium	40 mM	46%	70% [a]	90%	(Yoo et al., 2015) [c]
	Consortium	40 mM	55%	70% [a]	80%	(Han et al., 2013) [c]
EDTA	M. Trichosporium	0.5 mM	85%	ND	57% [b]	(Hwang et al., 2015)
	M.Sporium	50 µM	76%	95%	48%	(Yoo et al., 2015) [c]
	Consortium	50 µM	65%	95%	45%	(Han et al., 2013) [c]
NaCl	M. Trichosporium	100 mM	75%	80%	ND	(Kim et al., 2010; Lee et al., 2004)
	M. Trichosporium	200 mM	45%	70%	ND	(Kim et al., 2010; Lee et al., 2004)
	Consortium	100 mM	25%	40%	80%	(Han et al., 2013) [c]
NaCl + EDTA	M. Trichosporium	100 mM + 1 mM	ND	ND	ND	(Kim et al., 2010; Pen et al., 2014)
Phosphate	M. Trichosporium	100 mM	90%	ND	51%	(Hwang et al., 2015)
	M. Sporium, M. Tundrae	100 mM	83%	ND	ND	(Mardina et al., 2016; Patel et al., 2016b)
	M. Sporium	40 mM	72 %	95%	55%	(Yoo et al., 2015) [c]

	Consortium	40 mM	60%	80%	58%	(Han et al., 2013) [c]
	M.bryophila	100 mM	80%	ND	ND	(Patel et al., 2016a)
	Strain 14B	100 mM	ND	ND	ND	(Sheets et al., 2016)
Phosphate + $MgCl_2$	M. Trichosporium	80 mM + 5 µM	20%	84%	ND	(Mehta et al., 1987)
	M. Trichosporium	400 mM + 10 mM	ND	ND	64%	(Duan et al., 2011) [d]
	M.bryophila, M. Tundrae [e]	100 mM + 50 mM	66%	ND	ND	(Mardina et al., 2016; Patel et al., 2016a)
	M. Sporium [e]	100 mM + 20 mM	65%	ND	ND	(Patel et al., 2016b)
Phosphate + NaCl	M.bryophila, M. Tundrae [e]	100 mM + 50 mM	68%	ND	ND	(Mardina et al., 2016; Patel et al., 2016a)
	M. Sporium [e]	100 mM + 80 mM	70%	ND	ND	(Patel et al., 2016b)
Phosphate + EDTA	M.bryophila, M. Tundrae [e]	100 mM + 10 mM	52%	ND	ND	(Mardina et al., 2016; Patel et al., 2016a)
	M. Sporium [e]	100 mM + 1 mM	60%	ND	ND	(Patel et al., 2016b)
Phosphate + NH_4Cl	M.bryophila, M. Tundrae [e]	100 mM + 0.1 mM	74%	ND	ND	(Mardina et al., 2016; Patel et al., 2016a)
	M. Sporium [e]	100 mM + 15 mM	73%	ND	ND	(Patel et al., 2016b)

***a**: sMMO activity, **b**: higher results achieved in optimized conditions, **c**: without formate addition, **d**: high cell density, **e**: Phosphate and $MgCl_2$ was the highest in methanol production*

Phosphate is used in combination with other inhibitors such as NaCl, EDTA, $MgCl_2$ (Mehta et al., 1987; Patel et al., 2016b) which can be referred to the notable decline in MMO activity at phosphate concentration above 100 mM (Mardina et al., 2016; Patel et al., 2016a; Takeguchi et al., 1997). However, 400 mM phosphate concentration and 10 mM $MgCl_2$ were used while the cell density was 17.3 g /L in a study that achieved the highest methanol concentration 1.13 g/L. This result showed that a higher methanol production can be achieved by increasing the inhibitors concentration as well as the cell density (Duan et al., 2011). Interestingly, it was reported that using phosphate with $MgCl_2$ was more productive than EDTA, although EDTA had higher inhibitory effect on the MDH activity (Mardina et al., 2016; Patel et al., 2016a). Additionally, NH_4Cl had higher MDH inhibition but low methanol production (Kim et al., 2010; Patel et al., 2016b, 2016b, 2016a; Yoo et al., 2015). These results showed that the methanol production not only depends on MDH inhibition but also MMO activity. Moreover, it was suggested that MDH relative activity should be in the range of 50% to achieve the highest methanol production (Takeguchi et al., 1997) which was supported by the other studies, **Table (2-3)**. This hypothesis can be referred to the suggestion that MDH supplies MMO with electrons and the fact that 100% MDH inhibition means no carbon would be assimilated and no electrons would be produced. Thus, methane to methanol conversion ratio should be considered with methane uptake rate. For example, NH_4Cl and NaCl have high methane to methanol conversion ratio, however, a significant decline in methane uptake rate was observed (Han et al., 2013; Yoo et al., 2015) due to the reduction in MMO activity.

Another MDH inhibition strategy, Xin et al. reported that the presence of high carbon dioxide concentration may partially inhibit MDH activity while the other electrons producing steps still occurring. The maximum methanol production rate was too low (0.1 mg/L/hr.) in comparison

with other results (Xin et al., 2004a). Nevertheless, this strategy has the advantage of not adding either expensive external electron donor or chemical MDH inhibitors.

2.6.7 Electron Donor

Theoretically, 2 electrons (1 mole of NADH) are needed for production of 1 mole methanol. Both formaldehyde and formate can be added as reducing power source. However, formaldehyde can be toxic and has inhibitory effects. Hence, most of studies were adding formate for simultaneous methanol production (Duan et al., 2011; Furuto et al., 1999; Hwang et al., 2015; Kim et al., 2010; Lee et al., 2004; Mehta et al., 1991, 1987; Pen et al., 2014; Takeguchi et al., 1997; Xin et al., 2007, 2004a, 2004b). Different formate concentrations for different strains were reported as the optimum for methanol production 14.3, 20, and 40 mM for M.trichosporium (Duan et al., 2011; Furuto et al., 1999; Hwang et al., 2015; Kim et al., 2010; Lee et al., 2004; Mehta et al., 1991; Pen et al., 2014; Takeguchi et al., 1997), 40 mM for both M.sporium, and Strain 14B (Patel et al., 2016b; Sheets et al., 2016) and 100 mM for M.tundrae and M.bryophila (Mardina et al., 2016; Patel et al., 2016a). Interestingly, Sheets et al mentioned that excessive formate addition resulted in a methanol accumulation reaching in concentrations up to (500 to 1000 mg/L/day) without adding any MDH inhibitors. It was referred to the higher activity of FDH than MDH resulting in a higher production rate of reducing power and methanol (Sheets et al., 2016). While methanol production was inhibited after 3-4 hours in other studies (Hwang et al., 2015; Mehta et al., 1987) it was successfully accumulated for about 24 hours without formate addition (Han et al., 2013; Yoo et al., 2015). However, the production rate was low and a sharp decline was noticed after 24 hours (Han et al., 2013; Yoo et al., 2015) suggesting that methanotrophs can utilize other electron donors such as lipids.

Unfortunately, formate is not economically feasible to be used at the commercial scale. Therefore, alternative strategies should be invistigated such as generating electrons electrochemically or using facultative methanotrophs. Interestingly, Xin et al used Poly-β-Hydroxybutyrate (PHB) as a reducing power reserve while converting carbon dioxide (not methane) into methanol. After 144 hours, Cells with 38.6% PHB produced 3 mg/L methanol without losing its vitality (Xin et al., 2007). Despite the low methanol yield, employing PHB as an electron donor is shown to be viable. The use of methane with MDH inhibitors can overcome the low methanol yields problem.

2.6.8 Cell Density

Biomass density have contradictive results on its effect on methanol accumulation (Duan et al., 2011; Furuto et al., 1999; Han et al., 2013; Hwang et al., 2015; Kim et al., 2010; Lee et al., 2004; Mardina et al., 2016; Mehta et al., 1991, 1987, Patel et al., 2016b, 2016a; Pen et al., 2014; Sheets et al., 2016; Takeguchi et al., 1997; Xin et al., 2007, 2004a, 2004b; Yoo et al., 2015). It was reported that the optimum cell density of M.trichosporium was 3000 mg/L and a decline in methanol accumulation was noticed with further increase (Mehta et al., 1991, 1987, Xin et al., 2007, 2004a, 2004b). However, Takeguchi et al. found that the maximum methanol accumulation occurred at a low cell density of 35 mg/L (Furuto et al., 1999; Takeguchi et al., 1997), while, Lee et al. reported the peak accumulation at a cell density of 600 mg/L (Kim et al., 2010; Lee et al., 2004). However, it was found that increasing the cell density of M.sporium from 1 mg/L to 50 mg/L resulted in higher methanol accumulation (Lee et al., 2004). Other studies using M.tundrae (Mardina et al., 2016), M.bryophila (Patel et al., 2016a) reported an increase by 1.35 and 1.5 times in methanol concentration while increasing the cell density from 1.5 to 9 and 3 to 18 g/L, respectively. These contradicted results can be referred to limitation of

oxygen and methane mass transfer at higher cell densities or the increase in the rate of methanol oxidation (Lee et al., 2004; Mehta et al., 1987; Takeguchi et al., 1997). Duan et al. used 5% paraffin oil to eliminate the mass transfer effect, and examined the methanol accumulation with higher concentration of MDH inhibitors in order to inhibit any further methanol concentration. Interestingly, high Methanol concentration (1130 mg/L) was achieved significantly by increasing the M.trichosporium cell density up to 17000 mg/L accompanied with notable increase in MDH inhibitors (Duan et al., 2011). Furthermore, another study noticed that at low methane concentration (in the terms of low gas to liquid ratio) the culture with lower cell density had a slightly higher methanol accumulation. at high methane concentration, methanol was 3.2 times higher than in cultures with higher biomass (Pen et al., 2014). These data suggest that increasing cell density should be accompanied with an increase in methane concentration, MDH inhibitors, and overcoming mass transfer limitation. However, it's noteworthy that the methanol productivity decreases with the biomass increase (Duan et al., 2011; Mardina et al., 2016; Patel et al., 2016a) which means that more optimized conditions such as bioreactor configuration, optimized concentrations are still needed to minimize the limitation factors.

Chapter 3

Effect of Growth Medium Composition and Methane to Oxygen (M/O) Ratio on Mixed Culture Proteobacteria Phylum (Type I Methanotrophs) Enriched from Waste Activated Sludge [2]

3.1 Introduction

In a typical municipal WWTP, biogas is generated from the anaerobic biodegradation of the organic materials in the anaerobic digesters (Tchobanoglous et al., 2003). The produced biogas mainly consists of methane (CH_4) and carbon dioxide (CO_2) with concentrations of 50:70% and 30:50%, respectively (Yang et al., 2014). Having a global warming potential of 25 for a 100-year time horizon makes the mitigation of CH_4 emissions pivotal for the global warming phenomena control (Hanson and Hanson, 1996). On the other hand, WWTPs are considered extensively energy demanding facilities (Y. Shen et al., 2015) which is expected to be increased due to the ongoing population increase. Therefore, it is crucial to develop sustainable technologies not only for biogas (as consortium of greenhouse gases) mitigation but also for value-added resources recovery.

Methanotrophs are the major biological methane sink due to their unique ability to utilize methane as cellular carbon and energy source (Hanson and Hanson, 1996). Aerobic methanotrophs are

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phylogenetically clustered into three types; type I (gamma subdivision of proteobacteria phylum), type II (Alpha subdivision of proteobacteria phylum), type III (verrucomicrobia phylum) (Semrau et al., 2010). Type I has higher growth rates, and is more energy efficient in comparison with type II (Bowman, 2006; Dedysh and Dunfield, 2011; Henckel et al., 2000; Karthikeyan et al., 2015; Whittenbury et al., 1970). Furthermore, type I methanotrophs can be easily enriched in mixed cultures under nutrients sufficient conditions (Henckel et al., 2000; López et al., 2014). On the other hand, type III prefers to grow in extreme conditions such as acidic (optimal pH ranges from 1.5 to 3.5) and hot (optimal temperature ranges from 40 to 55^{o}C) environments (Op den Camp et al., 2009; Teeseling et al., 2014), which limits its applications in wastewater treatment plants (WWTPs). Therefore, type I is more advantageous to be manipulated in methane mitigation coupled with any other biotechnological application.

Diversified factors affect methanotrophs microbial activity such as nitrogen source and concentration, copper concentration, substrates concentrations, and biomass density. As comprehensively reviewed throughout chapter 2, contradicted reports are found in the literature about how the aforementioned factors influence the methanotrophic microbial activity.

For the nitrogen source, some batch tests showed that the addition of ammonia to different N-limiting soils was accompanied with an increase in methane oxidation rates and type I abundance (He et al., 2011; Lee et al., 2009; Mohanty et al., 2006; Visscher et al., 2001; Zhang et al., 2014). Whereas, methylocystis (type II genus) dominated an activated sludge culture after 24 days' incubation relying on continuous ammonia addition (Myung et al., 2015).

Considering the copper influence, some studies mentioned that copper addition significantly increase methane uptake and growth rate especially between 1 and 4.31 µM (Cantera et al.,

2016; Graham et al., 1993; Ha et al., 2010; Adrian Ho et al., 2013; López et al., 2013). Conversely, others noticed either no or minor increase (Ha et al., 2012; Karthikeyan et al., 2016). These contradicted reports suggest that copper effect may depend on the initial community structure (Graham et al., 1993; Ha et al., 2012, 2010) and methane concertation and nitrogen load which may select a pMMO expressing cluster regardless of the copper concentration (Karthikeyan et al., 2016). It is noteworthy that excessive copper addition was reported to result in full or partially inhibitory effects due to its toxicity (Adrian Ho et al., 2013; Lee et al., 2009).

Furthermore, too limited studies investigated the aforementioned conditions influence on type I methanotrophs even in pure or mixed cultures. Therefore, the investigation of those factors effect on methanotrophs type I enriched from mixed culture of waste activated sludge, as well as the determination of the optimum values are a pivotal milestone on the way of developing an efficient methanotrophs cultivation bioreactor.

3.2 Materials and Methods

3.2.1 Operational Conditions

Batch experiments were performed using copper and nitrogen free mineral salts medium (MSM) containing the following concentrations (in mg/L): $MgSO_4.7H_2O$, 1000; $CaCl_2.6H_2O$, 200; KH_2PO_4, 272; K_2HPO_4, 610; Ferric EDTA, 4. 1 mL/L; and 1 mL of trace elements solution (Disodium EDTA, 500; $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $Na_2MoO_4.2H_2O$, 3; $FeSO_4.7H_2O$, 200; $NiCl_2.6H_2O$, 2; $CoCl_2.6H_2O$, 20). Copper sulfate ($CuSO_4.5H_2O$) was used to prepare copper stock solution to control copper concentration throughout the experiments. Nitrate (in the form of $NaNO_3$) and ammonium (in the form of NH_4Cl) concentrations were varied based on the targeted concentration for each experiment.

Unless otherwise specified, cells were suspended in 50 mL of MSM with specific copper and nitrogen concentrations in 250-mL sealed serum bottles capped with rubber stoppers. Methane and oxygen with purity above 99% (Praxair Technology, Inc., Danbury, CT, USA) were added to the headspace with 1:1 volumetric ratio. The serum bottles were incubated at room temperature (ranges from 25 to 28^{o}C) and continuously mixed at 165 rpm. Gas samples were withdrawn periodically throughout the experiments. While, liquid measurements were carried out at the start and end of each incubation.

3.2.2 Enrichment Experiment

Fresh waste activated sludge from Humber wastewater treatment plant (Toronto, Canada) were used as a seed for type I methanotrophs enrichment. The sludge was filtered through 100-µm cell strainer and the filtered sludge was centrifuged and re-suspended in the MSM with 5 µM copper sulfate and 10 mM of sodium nitrate. Four bottles were running in fed-batch mode with initial optical density (OD_{600}) of 0.3 ± 0.1. Every two days, cultures were centrifuged (4200 g) for 20 mins and re-suspended in fresh medium and 200 mL of methane and oxygen were added. After two weeks, cultures totally shifted to the pinkish color known for type I methanotrophs. Samples were taken and microbial analysis were performed for the confirmation of the type I methanotrophs existence/dominance.

3.2.3 Growth Culture Optimization Conditions

Three different concatenations of sodium nitrate (10, 20, and 40 mM) and ammonium chloride (5, 10, 20 mM) were added to 50 mL MSM with 5 µM of copper sulfate. The previously enriched biomass obtained from enrichment phase was inoculated in the reaction medium with initial OD_{600} of 0.575 ± 0.75.

As mentioned before, copper concentration regulates the MMO enzyme expression, which is directly affect the competition between methane and ammonium. Thus, the effect of copper addition to the optimum nitrate and ammonium concentrations was investigated. Cultures with 40 mM nitrate and 5 mM ammonium with no copper were compared to the same cultures with copper of 10 µM. Biomass harvested from the previous phase was inoculated to the reaction medium with initial OD_{600} of 0.433 ± 0.2 after being centrifuged.

Thereafter, higher nitrate and copper concentrations were employed to identify the inhibitory and optimum concentrations. The biomass obtained from previous phases were collected and centrifuged. The nitrate enriched biomass was inoculated with initial cell density (OD_{600}) of 0.170 ± 0.039 for nitrate optimization. The used nitrate concentrations were as ranging from 10 to 160 mM added $NaNO_3$. Using the optimum nitrate concentration as the nitrogen source, the copper experiments were performed using copper concentration of 0, 20, 40, and 80 mM with initial cell density (OD_{600}) of 0.185 ± 0.038.

For the biomass density, the initial biomass densities of 0.148 ± 0.01, 0.595 ± 0.04, 1.263 ± 0.03, 2.063 ± 0.14, and 2.743 ± 0.14 measured as OD_{600} were used to investigate biomass density effect on type I methanotrophs. The same methane and oxygen concentrations were used in this phase. The reaction medium consisted of 40 mM of sodium nitrate and 20 µM of copper added to the MSM.

Oxygen to methane with various molar ratios (1, 2, 3, and 4) were added to the bottles headspace using biomass harvested from previous phases. Volume of methane added was fixed in all bottles to maintain equal food to microorganisms (F/M) ratio effect. Moreover, Helium was added to the headspace to fix the partial pressure in all bottles. Experiments kept running until most of the

methane was consumed from one of the bottles. The initial biomass densities (OD_{600}) were equal to 1.330 ± 0.12.

3.2.4 Analytical Methods

Samples withdrawn using gas tight syringe from bottles headspace were injected to SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD), methanizer and 6' molecular sieve column (Restek, Bellefonte, PA.) to measure methane and oxygen concentrations. The temperature program was as following: injector, 80°C; Oven, 80°C; FID, 300°C; TCD, 155°C and helium gas was used as carrier gas with flowrate of 20 mL/min. For cell density measurements, optical density (OD_{600}) at 600 nm were obtained using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). To determine the dry cell weight (DCW), a correlation equation between OD_{600} and DCW was developed. DCW was measured after the filtered samples (TSS Glass Fiber Filter, Pore Size 1.5 µm, Diameter 47 mm, 100/pk) dried overnight in the oven at 105°C. Liquid samples were collected from the supernatant after the cultures centrifuge (4200 g) for 20 min. Then, HACH methods and testing kits were used to measure inorganic nitrogen (NH_3-N, NO_2-N, and NO_3-N).

The bacterial behavior throughout the experiments were evaluated using main three parameters. First is specific growth rate (µ) which was determined using **equation (3-1)**.

$$\mu = \frac{(\mathrm{DCW}_{initial} - \mathrm{DCW}_{final})/t}{(\mathrm{DCW}_{initial} - \mathrm{DCW}_{final})/2} \qquad \text{(Eq. 3-1)}$$

Where $DCW_{initial}$ is the initial dry cell weight (mg), DCW_{final} is the final dry cell weight (mg), t is the experiment duration (hrs), µ is the specific growth rate (g $DCW_{increase}$/ g $DCW_{average}$ / hr). Secondly, the methane uptake rate (mg-CH_4 consumed/hr) was calculated by dividing the

consumed methane by the incubation time. In some cases, methane uptake rate was normalized by the initial biomass density in order to eliminate the biomass density effect, especially in the case of comparing the results from two different experiments. The last parameter is cells observed growth yield (Y_{CH4}) which used mainly to demonstrate how efficient was the cells in converting the methane uptake into cell increase. Biomass increase was divided by methane consumed to obtain the growth yield.

3.3 Results and Discussions

3.3.1 Growth Culture Optimization

3.3.1.1 Type I Methanotrophs Behavior under Nitrate and Ammonium as Nitrogen Sources

Nitrogen is one of the macronutrients needed for any living organism to build their nucleic acids and proteins (Madigan et al., 2015). However, some studies reported that nitrate increase has inhibitory effect on methanotrophs growth, even though, its metabolism and inhibition mechanism in methanotrophs are still unclear (Karthikeyan et al., 2016). As shown in **Table (3-1)**, the increase in nitrate concentration from 10 to 20 mM resulted in minor increase in bacterial activity. Whereas, growth rate and methane uptake increased by 14% and 25%, respectively, by increasing nitrate concentration to 40 mM. The highest growth rate and methane uptake were equal to 0.574 ± 0.022 day^{-1} and 1.054 ± 0.084 mg_{CH4}/hr. In agreement, the tolerance to nitrate of some type I strains in pure cultures were previously investigated and all the tested strains were tolerant to 40 mM of nitrate (Hoefman et al., 2014a). Hence, it can be deduced that the presence of nitrate with concentrations up to 40 mM as a macronutrient has a positive effect on the performance of type I methanotrophs.

It was reported that the increase in methane uptake may result in excessive concentration of formaldehyde known for its toxicity (Amaral and Knowles, 1995; Pfluger et al., 2011). This can

elucidate a notable decline in the yield was noticed at higher nitrate concentration, **Table (3-1)**. Another hypothesis is that the cells pushed its metabolism towards the energy production pathway at the expense of the cell synthesis to produce more electrons to maintain this higher methane uptake rate. Supportively, oxygen uptake observed at 40 mM nitrate was 20% higher than the uptake at 10 mM, **Table (3-1)**. Interestingly, this observed decline took place only throughout this phase and higher growth rates were observed in the next phases. Therefore, it can be deduced that the cells successfully adapted with the new conditions by either developing formaldehyde detoxification mechanism and/or increasing formaldehyde oxidation rate or the cells regulating both the methane assimilation and dissimilation pathways.

Table (3-1): The Influence of Nitrogen Source & Concentration on Type I Methanotrophs Growth Rate and Yield, and Methane and Oxygen Uptake Rates

	Specific growth rate (day^{-1})	Observed growth yield ($gDCW_{increase}/gCH_{4\ uptake}$)	Methane uptake rate (mg_{CH4}/hr)	Oxygen uptake rate (mg_{O2}/hr)
NIT-10*	0.510 ± 0.003	0.68 ± 0.07	0.839 ± 0.084	2.659 ± 0.048
NIT-20	0.526 ± 0.085	0.70 ± 0.06	0.840 ± 0.059	2.676 ± 0.077
NIT-40	0.574 ± 0.022	0.52 ± 0.03	1.054 ± 0.084	3.194 ± 0.093
AMM-5**	0.536 ± 0.092	0.65 ± 0.05	0.857 ± 0.015	2.771 ± 0.191
AMM-10	0.460 ± 0.049	0.62 ± 0.03	0.773 ± 0.022	2.679 ± 0.063
AMM-20	0.443 ± 0.065	0.57 ± 0.09	0.721 ± 0.021	2.475 ± 0.069

**NIT-10*: 10mM of Nitrate, **AMM-5*: 5mM of Ammonium*

As illustrated in **Table (3-2)**, The amount of nitrogen utilized per average biomass (nitrogen utilization ratio) increased from 0.13 ± 0.08 for 10 mM nitrate to 0.40 ± 0.07 for 40 mM which supposed to be around 0.13 (the cellular nitrogen) (Madigan et al., 2015). Moreover, the consumed C/N ratio (g Carbon as CH_4 consumed/g Nitrogen consumed) dropped by 50% when nitrate concentration increased from 10 to 40 mM. This drop indicates that less carbon is needed to remove the same amount of nitrogen in form of nitrate. In addition, nitrite with relatively high

concentrations were observed at the end of the experiments, for instance, 18.76 ± 0.18 mgN-NO_2/L was noticed at 40 mM of nitrate, **Table (3-2)**. Combined together, those findings raise the potential of the integration of methane mitigation and denitrification processes. Further investigations and enhancements are needed to confirm such potential.

Table (3-2): Type I Methanotrophs Nitrogen Consumption under Different Nitrogen Sources and Concentrations

	Final nitrogen concentration (mg-N/L)	Nitrate conc. (mg-N/L)	Nitrite conc. (mg-N/L)	N-utilization ratio ($g\text{-}N_{uptake}/gDCW_{increase}$)	Consumed C/N ratio (g $C\text{-}CH_4$ /g-N)
NIT-10*	100 ± 6.81	--	2.80 ± 0.09	0.13 ± 0.08	10.05 ± 0.88
NIT-20	214 ± 5.52	--	6.82 ± 0.24	0.22 ± 0.03	5.85 ± 0.09
NIT-40	466 ± 4.92	--	18.76 ± 0.18	0.40 ± 0.07	4.71 ± 0.01
AMM-5**	18 ± 1.54	16.00 ± 0.93	0.18 ± 0.08	0.14 ± 0.02	9.87 ± 0.90
AMM-10	84 ± 4.80	12.00 ± 0.87	0.32 ± 0.02	0.16 ± 0.01	8.27 ± 0.28
AMM-20	220 ± 6.5	10.00 ± 0.27	0.34 ± 0.09	0.20 ± 0.05	7.19 ± 0.70

**NIT-10*: 10mM of Nitrate, **AMM-5*: 5mM of Ammonium*

As shown in **Table (3-1)**, type I has a relatively higher growth rate, growth yield, and methane uptake rate at 5 mM of ammonium. It was equal to 0.536 ± 0.092 day^{-1}, 0.65 ± 0.05 $gDCW_{increase}$ /$gCH_{4uptake}$, and 0.857 ± 0.015 mg-CH_4/hr, respectively. Moreover, increasing ammonium concentrations above 5 mM caused a decline of 17% and 11% in the growth rate and methane uptake rate. This decline was accompanied with an increase in ammonium uptake, **Table (3-2)**. A lot of similarities have been reported between MMO and ammonia monooxygenase (AMO) enzymes (Kalyuzhnaya et al., 2015). Therefore, it can be concluded that the competition between ammonium and methane on the MMO enzyme is the main reason for the lower methane uptake and by consequence the lower growth rate and growth yield. The nitrogen consumption ratio was about 75 ± 3% at ammonium concentration of 5 mM. However, the nitrogen utilization ratio was equal 0.14 ± 0.02 which indicates that the amount consumed was mainly for the cellular

nitrogen, **Table (3-2)**. In addition, the consumed C/N ratio at the optimum ammonium concentration was equal to 9.87 ± 0.9 gC-CH_4/g-N which relatively high in comparison with nitrate.

In terms of comparing ammonium and nitrate as a nitrogen source for type I methanotrophs, the growth rate at 40 mM nitrate was 0.574 ± 0.022 day^{-1}, while at 5 mM ammonium was 0.536 ± 0.092 day^{-1}. The methane uptake was equal to 42.14 ± 3.37 mgCH_4 and 34.28 ± 0.62 mgCH_4 for 40 mM nitrate and 5 mM ammonium, respectively. In addition, the amount of methane consumed to remove 1 mg of nitrogen as ammonium was 2 times higher than nitrate, **Table (3-2)**. These results show that the type I bacterial activity is better while utilizing nitrate which can be referred to the ammonium inhibitory effect discussed before.

3.3.1.2 Copper Effect on Type I Methanotrophs Behavior under Different Nitrogen Sources

Genetically, it was reported pMMO and AMO are more identical than the sMMO (Zhang et al., 2017). Therefore, ammonium inhibitory effect would be higher in pMMO expressing cultures. Therefore, the effect of copper was evaluated for the optimum concentrations for both ammonium and nitrate cultures to make the ammonium and nitrate comparison more reliable. Moreover, 10 µM of copper was tested instead of 5 µM used in the previous phase as an indication of the influnce of increasing copper concentration.

In the presence of ammonium, the methane uptake rate was higher by 5 % at the copper free cultures which confirms the effect of sMMO expression on the competition between methane and ammonium, **Figure (3-1)**. sMMO is known for its lower methane uptake ability which can elucidate this minor increase (Kalyuzhnaya et al., 2015). Furthermore, Ammonium consumption ratio increased from 80 ± 0.5% to 86 ± 1.7% by copper elimination. This observation is an evidence on the substrate competition release. Moreover, the growth rate and yield was increased

from 0.576 ± 0.085 to 0.606 ± .022 day^{-1} and 0.79 ± 0.07 to 0.86 ± 0.08 $gCH_{4consumed}/gDCW_{increase}$, respectively, by removing the copper from the ammonium growing cultures, **Figure (3-1)**. The increased growth rate and yield confirms the positive effect of the removing the copper from the medium.

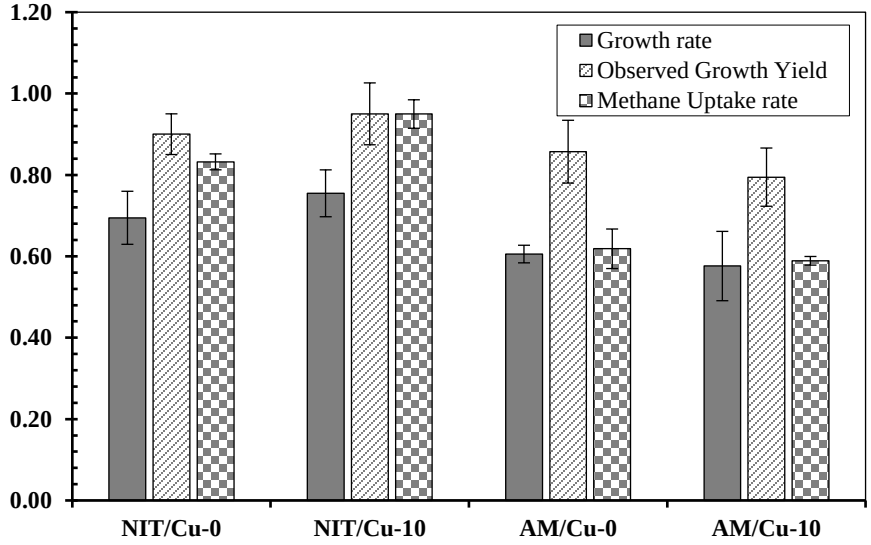

Figure (3-1): The Effect of Copper with Ammonium and Nitrate as Nitrogen Source on Type I Methanotrophs

Specific growth rate (day-1), Observed growth yield (gDCW increase/gCH4 consumed), Methane uptake rate (mg-CH4/hr), NIT/Cu-0: Nitrate 40 mM and Copper 0 µM, NIT/Cu-10, Nitrate 40 mM and Copper 10 µM, AM/Cu-0: Ammonium 5 mM and Copper 0 µM, AM/Cu-10, Ammonium 5 mM and Copper 10 µM.

As demonstrated in **Figure (3-1),** the methane uptake rate of the nitrate cultures with 0 and10 µM copper was equal to 0.832 ± 0.020 and 0.950 ± 0.035 $mgCH_4$/hr, respectively. Moreover, the growth rate and growth yield was increased by 8% and 5%, respectively. **Figure (3-1)**. These results can be explained by the higher methane uptake of the pMMO. Hence, copper addition has a positive effect on the bacterial activity of the nitrate growing cultures.

As shown in **Figure (3-1)**, type I methanotrophs showed better performance in cultures using nitrate as their nitrogen source regardless the expression of sMMO or pMMO. In this phase, the difference was even more obvious than the previous one due to the adaptation effect discussed

before. The growth rate was almost 2 times higher at nitrate growing cultures. Moreover, the growth yield and uptake was higher by 10% and 40%, respectively. All these observation lead to that it is more advantageous to use nitrate as nitrogen source for type I methanotrophs. Furthermore, increasing copper concentration from 5 to 10 µM had a positive effect on the bacterial activity in both cultures. Specific growth rate increased by 30% due to copper concentration to 10 µM. The methane uptake rate in this phase was lower than the previous phase which is due to the lower initial biomass density. In agreement, the methane uptake/initial biomass density was equal to 3.533 ± 0.197 and 2.499 ± 0.106 $gCH_{4consumed}/gDCW_{initial}$ at 40 mM nitrate and 5 mM ammonium, respectively, in both phases. Therefore, 10 µM of copper sulfate was added to the upcoming phases.

Finally, it is noteworthy that the bacterial activity (uptake and growth) in the previous two phases was lower than the upcoming batch tests due to the cultures storage at 4°C in limited methane conditions. It was reported that bacterial activity especially the MMO activity is negatively affected by the storage in limited methane conditions (Jensen et al., 1998; Karthikeyan et al., 2016).

3.3.1.3 Nitrate and copper concentration optimization

Consistently, the addition of 40 mM nitrate resulted in the best bacterial activity. As shown in **Figure (3-2)**, It led to specific growth rate of 0.613 ± 0.035 day^{-1} and growth yield of 0.92 ± 0.06 $gCH_{4consumed}/gDCW_{increase}$. In comparison with cultures growing using 10 mM of nitrate, the consumed C/N ratio was 50% less which was equal to 5.05 ± 0.03. Moreover, the methane uptake/initial biomass was significantly equal to 13.707 ± 1.09 $gCH_{4consumed}/gDCW_{initial}$ which is 45% higher than uptake observed at 10 mM. It was concluded from the previous results that

increasing nitrate concentration enhance the overall bacterial performance. However, a severe decline in the bacterial activity occurred when the nitrate concentration increased above 40 mM.

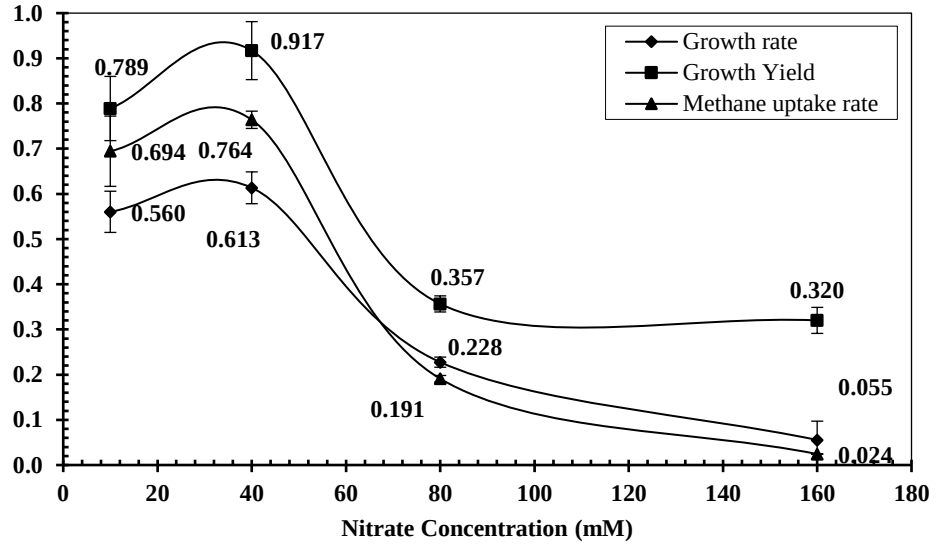

Figure (3-2): Type I Methanotrophs Behavior under Different Nitrate Concentrations

Specific growth rate (day^{-1}), Observed growth yield ($gDCW_{increase}/gCH_{4\,consumed}$), Methane uptake rate (mg-CH_4/hr).

As shown in **Figure (3-2)**, increasing nitrate concentration from 40 to 80 mM results in a decrease in growth rate and methane uptake by 90% and 75%, respectively. Moreover, almost no activity was observed while adding 160 mM of nitrate. As previously mentioned, nitrate inhibitory mechanism is still not clear, however, some studies reported that the inhibition might be due to the increased salinity (Sundstrom and Criddle, 2015). Also, it can be referred to the low initial carbon to nitrogen (C/N) ratio which was less than 1 for 80 and 160 mM. Collectively, it was deduced that the optimum sodium nitrate is equal to 40 mM which was used in the further experiments.

Increasing copper from 0 to 20 µM resulted in slight enhancement in the growth rate and methane uptake, **Table (3-3)**. At copper concentration of 20 µM, growth rate reached 0.622 ±

0.050 day^{-1} while maintain high growth yield of 0.94 ± 0.03. The further increase in the copper concentration to 40 µM resulted in minor inhibitory effect, while, a notable inhibition in the bacterial activity was observed at copper concentration of 80 µM, **Table (3-3)**. Most of the studies reported an inhibitory effect at high copper concentration, but, the inhibitory concentration varied from study to another (Ha et al., 2010; Adrian Ho et al., 2013; López et al., 2013). However, the cultures in this phase showed higher tolerance to copper than the previous studies. This can be referred to the effect of the associated bacterial community as most of the previous studies was hold on pure culture. Hence, it can be deduced that type I enriched from waste activated sludge optimally grow at copper concentration of 20 µM and tolerate copper concentration up to 40 µM. Thereafter, copper sulfate was added with concertation of 20 µM in the further experiments based on these findings.

Table (3-3): Type I Methanotrophs Behavior under Different Copper Concentrations

Initial Copper Concentration (µM)	Specific growth rate (day^{-1})	Growth yield ($gDCW_{increase}/gCH_{4\ uptake}$)	Methane uptake ($gCH_4/gDCW_{initial}$)
0	0.612 ± 0.019	0.94 ± 0.05	13.111 ± 0.156
10	0.613 ± 0.026	0.92 ± 0.09	13.707 ± 0.371
20	0.622 ± 0.050	0.94 ± 0.03	14.829 ± 0.186
40	0.611 ± 0.033	0.87 ± 0.08	14.062 ± 0.266
80	0.326 ± 0.060	0.54 ± 0.05	3.140 ± 0.183

3.3.2 Biomass Density Influence

Throughout the previous phases, it was noticed that decreasing the initial biomass density has resulted in a notable enhancement in the performance of type I methanotrophs. For instance, the highest growth rate in the study was obtained at the copper optimization experiment (0.622 ±

0.050 hr^{-1}). This growth rate was achieved at the lowest initial biomass density (OD_{600}) equal to 0.148 ± 0.01. Thus, those observations drove us to run an experiment to assess the effect of biomass density on the bacterial activity. The results showed that the culture with the lowest optical density ratio which was equal to 0.595 ± 0.04 had the best performance. Growth rate was equal to 0.617 ± 0.077 hr^{-1} while the growth yield was 0.66 ± 0.03 $gCH_{4consumed}/gDCW_{increase}$. Those values are still lower than the obtained values at lower biomass density in copper optimization phase as demonstrated in **Figure (3-3)**. It can be concluded that type I methanotrophs growth rate and yield was positively affected by the decrease in the biomass density. On the other hand, it was observed that the methane removal ratio was the same in all bottles and equal to 83 ± 3% by the end of the incubation time. Correspondingly, the total methane uptake was almost the same with only 6% higher at optical density of 2.743 ± 0.14 in comparison with 0.595 ± 0.04. However, the methane uptake rate was not the same over the incubation time.

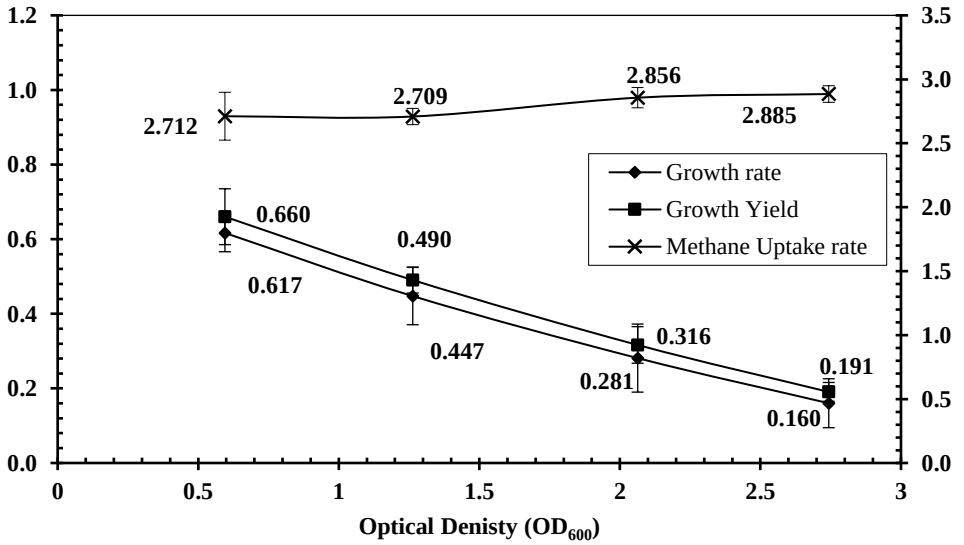

Figure (3-3): Biomass Density Influence on Type I Methanotrophs

Specific growth rate (day^{-1}), Observed growth yield ($gDCW_{increase}/gCH_{4\ consumed}$), Methane uptake rate ($mg\text{-}CH_4/hr$).

As shown in **Figure (3-4)**, It was 1.6 times higher at biomass densities of 2.743 ± 0.14 and 2.063 ± 0.09 measured as OD_{600} than the lower biomass densities after 8 hours. Whereas, the uptake rate for both cultures were declined by 2 ± 0.1 folds when measured after 21 hours and remained the same at the lower biomass densities. Thus, it can be concluded that higher biomass densities (above 2 OD_{600}) are more effective in methane removal only at higher methane loading rate.

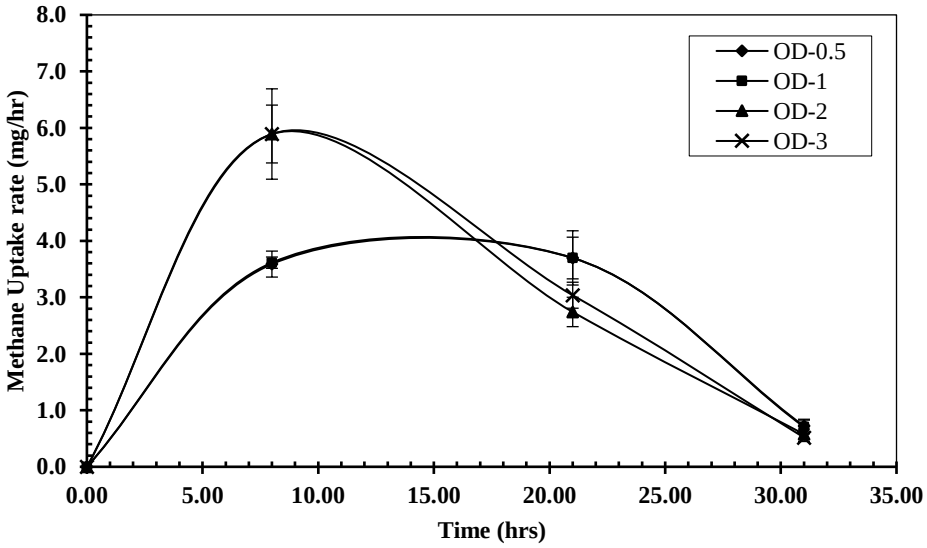

Figure (3-4): Time Course Methane Uptake Rate over Incubation Time.

3.3.3 Methane to Oxygen Ratio Effect

Running the bottles at different oxygen to methane molar ratios resulted almost in the same growth rate which were equal to 0.522 ± 0.013 day^{-1}. As illustrated in **Figure (3-5)**, methane and oxygen uptake was increased by 20 and 81%, respectively. In contrast, the growth yield declined by 24% because of the increase in oxygen to methane molar ratio from 1 to 4. The increase in gases uptake can be referred the abundance of the terminal electron acceptor which stimulate the cells to uptake more methane. However, the decline growth yield indicate that the increased uptake has to some extent negative effect. As an elucidation, the increased existence of the electron acceptor stimulates the bacteria to push its metabolism more towards the energy

generation stream on the expenses of the cell syntheses resulting in lower yields and the same growth rate. Thus, it is not favorable to apply oxygen to methane molar ratio higher than 1. Even though, increasing oxygen concentration result in higher methane uptake by 20%. This is because of (c) the lower observed yields, and (b) the observation that it was achieved with 80% higher aeration requirements and 4 times less methane loading (assuming fixed bioreactor volume) what makes it not economically feasible.

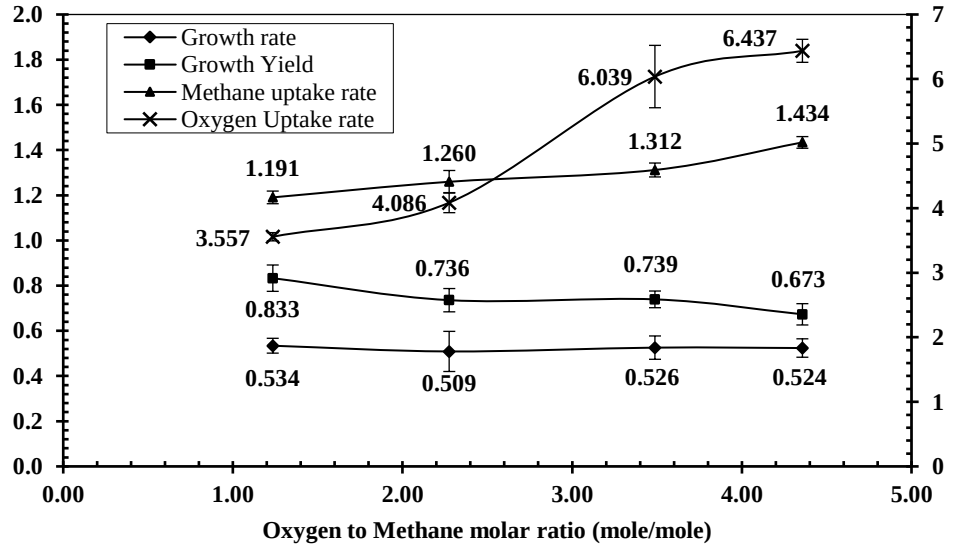

Figure (3-5): Type I Methanotrophs Behavior under Different Methane to Oxygen Ratios

Specific growth rate (day^{-1}), Observed growth yield ($gDCW_{increase}/gCH_{4\ consumed}$), Methane uptake rate (mg-CH_4/hr), Oxygen uptake rate (mg-O_2/hr).

3.3.4 Nitrogen Uptake Rate

In order to estimate the nitrogen (in form of sodium nitrate) uptake rate, the optimized conditions were applied on five different biomass densities and Liquid samples were withdrawn every 3-5 hours and filtered to be used for nitrogen measurements. At all the biomass densities, nitrogen uptake rate was higher during the first 6 hours and sufficient nitrogen, methane and oxygen conditions, **Figure (3-6)**. The maximum nitrogen uptake rate was equal to 5.83 ± 0.83, 6.67 ± 1.33, 7.00 ± 0.33, 17.67 ± 3.00, 15.67 ± 0.33 mgN-NO_3/L/hr for the cultures with optical

densities of 0.20 ± 0.02, 0.52 ± 0.01, 0.91 ± 0.01, 2.11 ± 0.11, and 2.98 ± 0.03, respectively. Thereafter, nitrogen uptake rate declined which can be referred to the effect of biomass increase on gas diffusion, methane and oxygen decrease. The overall specific nitrate uptake rate was equal to 0.62 ± 0.03, 0.23 ± 0.01, 0.19 ± 0.00, 0.06 ± 0.01, 0.05 ± 0.01 mgN-NO_3/mg-TSS/day for cultures from 0.20 to 3, respectively. Those results are comparable to the specific denitrification rates in the literature. However, biomass densities in this study is lower than in the mixed liquor volatile suspended solids (MLVSS) of activated sludge processes by 10 times at least which would significantly affect the results (Tchobanoglous et al., 2003). Further investigations are needed to enhance nitrogen removal and methane mitigation integration.

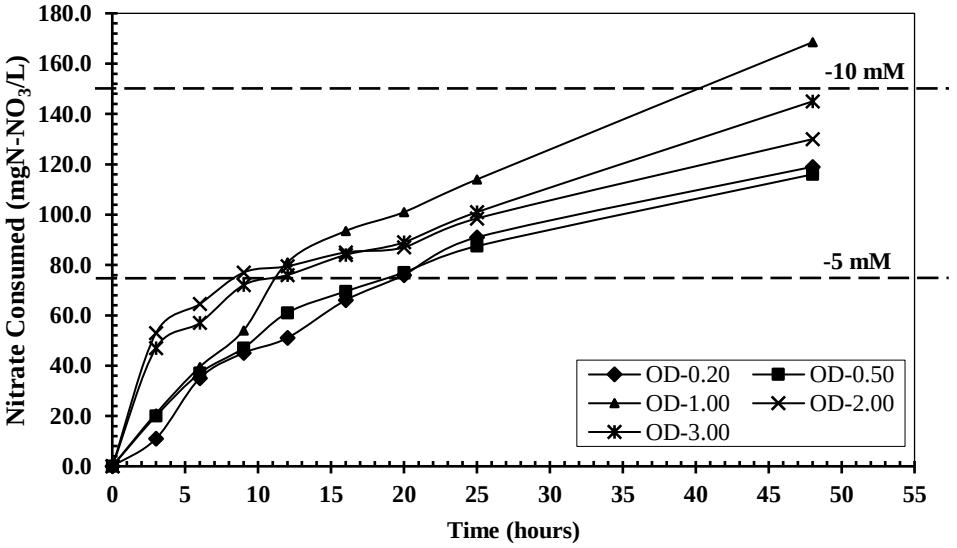

Figure (3-6): Nitrogen Uptake at Different Initial Biomass Densities.

Representing 13% of the cells weight, nitrogen as a macronutrient is the most important macronutrient after carbon (Madigan et al., 2015). In addition, nitrogen source and concentration has a notable effect on methanotrophs community structure (Semrau et al., 2010). For instance, all type II and few strains of type I methanotrophs -with slower growth rates- can fix the atmospheric nitrogen (Pfluger et al., 2011; Pieja et al., 2011). Therefore, the deterioration of the

nitrate from the growing cultures may lead to a notable shift in type I cultures shifting to type II and, by consequence, a totally different behavior. Hence, nitrogen uptake rate is a decisive parameter in methanotrophic bioreactor design.

As demonstrated in **Figure (3-6)**, nitrate concentrations were declined by 3.4 to 5.4 mM after 12 hours, which have a minor effect on the bacterial growth rate ranging from 1.5 to 2.5% only. Whereas, only cultures with optical density of 0.91 ± 0.01 have consumed more than 10mM after 48 hours which may decrease the methanotrophic activity by 5%. Having the same trend, nitrate concentration will reach 10mM after 128 hours which would decrease the bacterial activity by 12%. The decline estimation is based on the data obtained throughout nitrogen optimization phase which can be obtained under non-limiting methane and oxygen conditions. Therefore, less methanotrophic activity would be observed by increasing in the hydraulic retention time (HRT). However, the corresponding decline in the bacterial activity is accepted (≈ 12%) after up to 128 hours.

Having gaseous electron donor and acceptor makes HRT determined based on the nitrogen uptake rate. Typically, Lower HRTs are more desirable to maintain higher flow rates with lower bioreactor volumes. In agreement, lower HRT would be beneficial for methanotrophs in which higher nitrogen (nitrate) concentrations. The maximum HRT can be a decisive factor in bioreactors operated hydraulically in fed-batch mode such as fluidized bed reactor (FBR). Such hydraulic mode can be beneficial for methantrophic bioreactor because of the gaseous substrates, while, minimizing the water and pumping requirements. On the other hand, the minimum HRT can be determined based on the growth rate in order to prevent the bacterial washout. As discussed previously, methanotrophs growth rate is function of biomass density in addition to the

substrate concentration. Further investigation to identify the biomass density effect and the bacterial optimum growth rates is currently under development.

3.4 Conclusion

Throughout the literature, numerous studies have been held to investigate the methanotrophs behavior under multiple conditions. However, most of the studies were performed using pure cultures of type II methanotrophs. In this chapter, diversified batch tests were performed to assess the behavior of type I methanotrophs enriched from waste activated sludge under different environmental and operational conditions. Also, optimized values were identified for those conditions. Based on the targeted process, two different perspective can be adopted to assess methontrophs behavior. The first perspective is based on methane mitigation as the target in which methane uptake and removal ratio is the decisive parameter. The other one is targeting methanotrophs cultivation as a first step for further biotechnological application. From the latter prospective, the growth rate and growth yield are more critical because the target is to have higher growth with the minimum methane uptake and the lowest possible retention time to achieve less pumping requirement while maintaining the solids retention time (SRT).

In this chapter, it was found that higher methane uptake and growth rate was observed while using nitrate as nitrogen source. It was concluded that the optimum nitrate concentration for type I methanotrophs is 40 mM of sodium nitrate. In addition, using 20 µM of copper sulfate resulted in the highest growth rate, growth yield and methane uptake. Those findings identify the optimum composition for growth culture for any envirmontal application. Moreover, the addition ratio of methane to oxygen was found to be more feasible at 1:1 ratio due to the higher methane loading and lower aeration requirements. Regarding the biomass density, it was found that initial biomass density of 0.148 measured as OD_{600} have the higher growth rate and growth yield. On

the other hand, higher methane uptake was observed in the higher biomass densities up to 2.743 ± 0.14. Furthermore, the maximum overall nitrate uptake rate was obtained at 0.91 ± 0.01 and was equal to 3.51 ± 0.07 mgN-NO_3/L/hr. Therefore, it was determined that HRT up to 128 hours can be applied to Type I methanotrophs with limited effect on the bacterial growth. Whereas, further investigations are underdevelopment to determine the minimum HRT and optimum growth rate for Type I methanotrophs mixed culture enriched from waste activated sludge.

Chapter 4

Influence of Biomass Density and Food to Microorganisms (F/M) Ratio on the Mixed Culture Proteobacteria Phylum (Type I Methanotrophs) Enriched from Waste Activated Sludge [3]

4.1 Introduction

Due to the ongoing population increase, WWTPs are required to treat higher wastewater volumes, which is associated with higher energy requirements. There is a great interest in resources recovery from waste streams to offset its treatment operational cost and energy inputs. Methane in the form of biogas is commonly produced throughout the anaerobic digestion of the wastewater collected sludge (Tchobanoglous et al., 2003). However, enormous energy input is required to store and transport methane to be used as a commodity. Therefore, most of the WWTPs are interested in converting methane into more transportable and storable commodities (Ge et al., 2014). Methane can be utilized biologically under ambient temperature and atmospheric pressure using methanotrophs (Conrado and Gonzalez, 2014).

As previously discussed, type I methanotrophs are more beneficial to be employed in biotechnological application in comparison with other types. Operating in a pollutant control facility such as WWTP, methanotrophic bioreactor cannot be operated using methanotrophic pure

[3] This chapter has been submitted to "**Environmental Research Journal**".

cultures. Even though, methane is an efficient selective carbon source. Other types of heterotrophic bacteria can rely on the methanotrophic intermediates and/or any organics contamination to invade the growing cultures (Strong et al., 2016). Whereas, autotrophic bacteria can grow on the carbon dioxide released from methanotrophs and/or existing in the feeding biogas (Tchobanoglous et al., 2003). In addition, the use of mixed cultures enriched from wastewater sludge is more economically feasible.

The challenge to maintain stable microbial growth at higher cell densities is one of the major obstacles facing the process upscaling. It was reported that high cell densities are associated with poor microbial growth (Han et al., 2009; López et al., 2014). This was referred to the limited gas diffusion from the gas phase to the aqueous phase (Strong et al., 2016). In order to overcome such obstacle, the addition of methane vectors (5% paraffin oil) to enhance methane solubility was proposed (Han et al., 2009). Furthermore, different bioreactor configurations were developed and relatively higher microbial activity was achieved (Helm et al., 2008; Wendlandt et al., 2005, 2001). However, the previous studies were performed under different operational conditions such as methane loading rate and initial biomass density. Moreover, biomass density change is typically associated with the change in other conditions such as the methane to microorganisms ratio. Throughout this chapter, the biomass density effect has been explored independently. Furthermore, the influence of other parameters associated with biomass density change including food to microorganisms (F/M), carbon to nitrogen (C/N), and nitrogen to microorganisms (N/M) ratios on the microbial activity were assessed.

4.2 Materials and Methods

4.2.1 Chemicals and Operational Conditions

Throughout all the incubation, modified nitrate mineral salts medium (Mod-NMS) was used as the growing medium. Mod-NMS has been modified from the NMS described in (Bowman, 2006) and its composition is as following (in g/m^3): $NaNO_3$, 3400; $MgSO_4.7H_2O$, 1000; $CaCl_2.6H_2O$, 200; KH_2PO_4, 272; K_2HPO_4, 610; Ferric EDTA, 4. 1 mL/L; $CuSO_4.5H_2O$, 5. In addition, trace elements solution is used with concentration of 1 mL/L. it contains the following (in ppm): Disodium EDTA, 500; $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $Na_2MoO_4.2H_2O$, 3; $FeSO_4.7H_2O$, 200; $NiCl_2.6H_2O$, 2; $CoCl_2.6H_2O$, 20. Nitrate and copper concentration was modified based on growth optimization performed in the previous section. gases (oxygen, methane, and helium) of more than 99% pureness was used (Praxair Technology, Inc., Danbury, CT, USA).

Unless otherwise stated, all incubations were performed in 250-mL sealed serum bottles capped with rubber stoppers. Specific biomass densities were suspended in 50 mL of Mod-NMS as the growth medium. The headspace was filled with methane and oxygen with 1:1 as molar ratio after being evacuated for 5 minutes. The mixing speed were controlled using MaxQ™ 4000 Benchtop Orbital Shakers (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 165 rpm. Whereas, the bottles were running at room temperature ranging from 23 to 27ºC. Incubations time ranged from 31 to 34 hours.

4.2.2 Inoculum and Methanotrophs Type I Enrichment

Filtrated waste activated sludge were suspended, after being centrifuged, in the Mod-NMS. The sludge was obtained from Humber wastewater treatment plant (Toronto, Canada). The initial biomass density was equal to 0.5 ± 0.07. The gaseous headspace was replenished on daily basis.

Whereas, the biomass was transferred into fresh medium three times a week in which cultures were centrifuged (4200 g) for 20 mins and re-suspended into the fresh Mod-NMS medium. After the first transfer, the methane consumption was observed. After 12 to 15 days, gaseous uptake and bacterial growth became stable in the four seeded bottles and the biomass turned into the pinkish color. Thereafter, the cultures were transferred to 2 Liters bottle with liquid volume of 500 mL as biomass source. Methane and oxygen were added daily, while, the biomass was transferred into fresh media twice a week.

4.2.3 The Biomass Density and F/M Ratio Experiments

A series of consecutive batch tests were performed to evaluate the biomass density effect on type I methanotrophs bacterial growth. In the first phase, Biomass was inoculated with five different densities equal to 0.201 ± 0.01, 0.595 ± 0.04, 1.263 ± 0.03, 2.063 ± 0.09, 2.743 ± 0.14. The same methane and oxygen volume (400 mL as total volume) were added to the headspace. The fixed methane concentration resulted in different food to microorganisms (F/M) and nitrogen to microorganisms (N/M) ratios.

In the second phase, the objective was to investigate the F/M ratio effect while eliminating the biomass density influence on the bacterial activity. The biomass was suspended with the same biomass density which was equal to 1.318 ± 0.013. The change was in the growth medium volume to obtain different F/M ratios. Medium volumes of 10, 15, 25, 35, 50 mL were corresponding to 17.33 ± 0.59, 11.92 ± 0.43, 7.15 ± 0.36, 4.98 ± 0.35, 3.50 ± 0.31 g-CH_4/g-$DCW_{initial}$.

In the third phase, incubations were performed to elucidate the N/M ratios influence. Different nitrogen concentrations were applied to three different initial biomass densities. The biomass

densities were equal to 1.062 ± 0.042, 0.503 ± 0.004, and 0.201 ± 0.004 measured as OD_{600}. At each biomass density, nitrate concentrations of 10 mM, 40 mM, 80 mM, and 160 mM were added growth culture. The same methane volume (200 mL) was added to maintain the same carbon to nitrogen (C/N) ratio effect. In order to assess the C/N ratio effect, lower methane volume (75 mL) were added to four bottles with initial OD_{600} of 0.188 ± 0.026 to be compared with the similar biomass density but under 200 mL of methane. The remaining headspace was filled with helium to maintain the same partial pressure in all the bottles.

In the fourth phase, the biomass density effect was evaluated after identifying of the influence of F/M, N/M, and C/N ratios on the bacterial growth. Therefore, F/M was maintained constant to eliminate its effect on cells activity. biomass was suspended in 50 mL of the Mod-NMS with five different densities 0.265 ± 0.01, 0.567 ± 0.04, 0.991 ± 0.09, 2.192 ± 0.05, and 2.927 ± 0.20 measured as $OD_{600.}$ Added methane volume was adjusted to maintain the same F/M which was equal to 1.51 ± 0.14 g-CH_4/g-$DCW_{initial}$.

It is noteworthy that in all incubations the oxygen to methane molar ratio was maintained 1:1 to eliminate any effect to the oxygen. Throughout the previous three phases the cell density was measured at the beginning and the end of the incubations, whereas, the headspace composition was measured periodically over the incubation time.

In the final phase, time course type I methanotrophs growth and methane consumption was monitored. Different initial biomass densities were employed in the test 0.20 ± 0.02, 0.52 ± 0.01, 0.91 ± 0.01, 2.11 ± 0.11, and 2.98 ± 0.03. Achieving similar conditions to the first phase, the maximum methane volume was added to headspace which was equal to 200 mL. Liquid and

gaseous samples were withdrawn every 3-5 hours to monitor both the microbial activity as well as the methane uptake.

4.2.4 Analytical Methods

Cell density was measured using DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). Optical density (OD_{600}) at 600 nm were obtained and correlated using developed equation to calculate the dry cell weight (DCW). Liquid samples were harvested from the supernatant after being centrifuged. Thereafter, HACH methods and testing kits were used to measure inorganic nitrogen (NH_3-N, NO_2-N, and NO_3-N). SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) was used for gas composition (methane and oxygen concentrations) measurements in which thermal conductivity detector (TCD), methanizer and 6' molecular sieve column (Restek, Bellefonte, PA.) was used. The temperature program was as following: injector, 80ºC; Oven, 80ºC; FID, 300ºC; TCD, 155ºC and helium gas was used as carrier gas with flowrate of 20 mL/min.

The specific growth rate (μ) was determined using **equation (4-1)**.

$$\mu = \frac{(\mathrm{DCW}_{initial} - \mathrm{DCW}_{final})/t}{(\mathrm{DCW}_{initial} - \mathrm{DCW}_{final})/2} \qquad \text{(Eq. 4-1)}$$

Where $\mathrm{DCW}_{initial}$ is the initial dry cell weight (mg), DCW_{final} is the final dry cell weight (mg), t is the experiment duration (hrs), μ is the specific growth rate (g $\mathrm{DCW}_{increase}$/ g $\mathrm{DCW}_{average}$ / hr). Biomass increase was divided by methane consumed from the headspace to obtain the growth yield to determine Cells observed growth yield (Y_{CH4}). The F/M ratio was calculated through dividing the initial methane mass in the headspace by the initial dry cell weight.

4.3 Results and Discussions

Low growth rates and yields have been widely reported at high cell densities of methanotrophs. Such slow growth has hindered the upscaling of several methanotrophic biotechnological application (Han et al., 2009). It was suggested that higher cell densities directly affect the substrates diffusion from the gas phase to the aqueous phase (Strong et al., 2016). Therefore, the effect of biomass increase was evaluated in the first phase by using multiple initial biomass density while adding the same methane and oxygen volume. As shown in **Figure (4-1)**, Increasing the biomass density from 0.201 ± 0.01 to 0.595 ± 0.04 OD_{600} resulted in only 5% decline in the growth. Whereas, it resulted in 70% decline by increasing the biomass density to 1.263 ± 0.03 OD_{600}. At cell density of 0.201 ± 0.01, the highest bacterial activity was obtained in which growth rate was equal to 1.075 ± 0.085 day^{-1}, whereas, the growth yield was 0.98 ± 0.10 $gCH_{4consumed}/gDCW_{increase}$. The methane uptake rate was equal to 0.713 ± 0.021 which is lower by 3.8 times than the uptake at cell density of 0.595 ± 0.04 OD_{600}. This can be referred to the low final cell density obtained at cultures with initial optical density of 0.201 ± 0.01 which was 2.6 times lower than those obtained at 0.595 ± 0.04 OD_{600}. Therefore, it can be concluding that decreasing the cell density has a positive effect on the microbial growth of methanotrophs type I. The effect was more obvious at cell densities above optical density of 0.595 OD_{600}. Initially, this increase in the microbial activity can be referred to release of the limited gases diffusion into the liquid medium by decreasing the biomass density. In agreement, it was reported that preliminary biomass density optimization by decreasing it to 51.7 ± 14.7 mg/L resulted in enhanced and more realistic microbial kinetics. It was obtained at very low biomass density of 51.7 ± 14.7 mg/L (López et al., 2014).

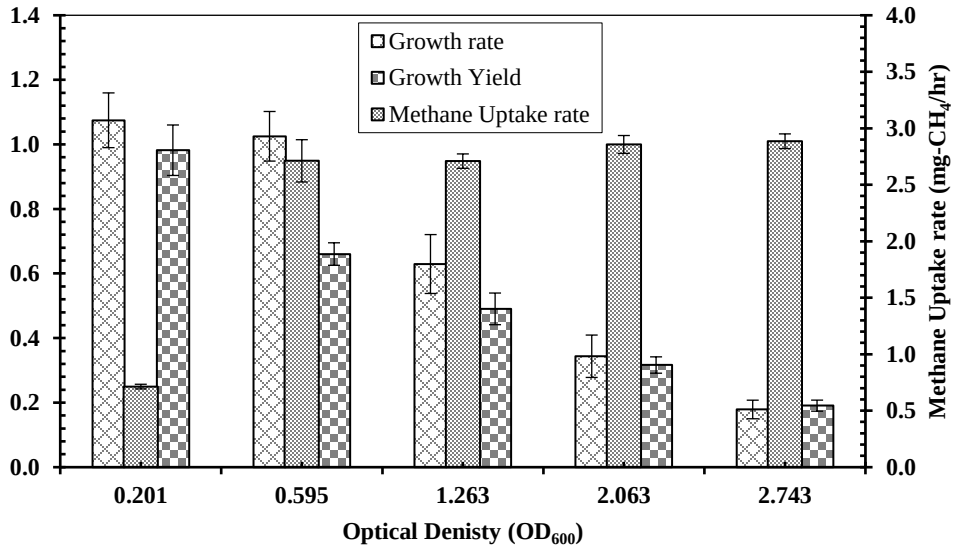

Figure (4-1): Biomass Density Effect on Type I Methanotrophs at the Same Methane Concentrations

Specific growth rate (day^{-1}), Observed growth yield ($gDCW_{increase}/gCH_{4\ consumed}$), Methane uptake rate ($mg\text{-}CH_4/hr$).

However, the change in the biomass density from 0.201 ± 0.01 to 2.743 ± 0.14 OD_{600} throughout this phase was associated with the change of F/M ratios from 19.85 ± 1.02 to 1.69 ± 0.13 $g\text{-}CH_{4\ initial}/g\text{-}DCW_{initial}$. Whereas, the N/M ratio decreased from 6.27 ± 0.12 to 0.46 ± 0.07 $g\ N\text{-}NO_{3\ initial}$ /g-DCW, **Table (4-1)**. Four dependent variables (biomass density, F/M ratio, C/N ratio, and N/M ratio) are varying in accordance with each other. Hence, the observed behavior cannot be clearly elucidated unless their effect on the microbial activity have been assessed independently.

Table (4-1): Corresponding F/M, C/N, and N/M Ratios at Different Cell Densities

Cell Density (OD_{600})	F/M ($g\text{-}CH_{4\ initial}/g\text{-}DCW_{\ initial}$)	C/N ($g\ C\text{-}CH_{4\ initial}/g\ N\text{-}NO_{3\ initial}$)	N/M ($g\ N\text{-}NO_{3\ initial}$ /g-DCW)	Specific Growth rate (day^{-1})
0.201 ± 0.01	19.85 ± 1.02	2.37 ± 0.09	6.27 ± 0.12	1.075 ± 0.085
0.595 ± 0.04	6.87 ± 0.54	2.43 ± 0.02	2.12 ± 0.07	1.025 ± 0.077
1.263 ± 0.03	3.47 ± 0.23	2.60 ± 0.04	1.00 ± 0.03	0.629 ± 0.091
2.063 ± 0.09	2.11 ± 0.19	2.58 ± 0.05	0.61 ± 0.05	0.344 ± 0.066
2.743 ± 0.14	1.69 ± 0.13	2.75 ± 0.03	0.46 ± 0.07	0.179 ± 0.029

4.3.1 Food to Microorganisms(F/M) Ratio Effect

In this phase, the initial biomass density (as OD_{600}) was fixed to be equal to 1.318 ± 0.013 OD_{600} to explore the F/M ratio on the microbial activity. As shown in **Figure (4-2)**, the same trend as observed throughout the previous phase was obtained in which increasing the F/M ratio resulted in the increase in the growth rate. Growth rate at F/M 17.33 ± 0.59 g-$CH_{4\ initial}$/g-$DCW_{initial}$ was higher by 1.7 times than culture with 3.50 ± 0.31 g-$CH_{4\ initial}$/g-$DCW_{initial}$. The highest growth rate achieved at F/M of 17.33 ± 0.59 g-$CH_{4\ initial}$/g-$DCW_{initial}$ was equal to 0.983 ± 0.052. Interestingly, this value is only 9% and 4% less than the growth rate obtained at lower cell densities which are 0.201 and 0.595 OD_{600}, respectively. Thus, it can be hypothesized that F/M ratio increase has higher influence on the microbial rather than the cell density. In contrast, the growth yields slightly decreased by the increase of the F/M, **Figure (4-2)**. Further investigations are required to clarify such observation. However, it can be initially explained by the significant increase in the final biomass density, which agreed with the F/M ratio increase. In general, the growth yields were lower than the values obtained at lower biomass densities in the previous phase.

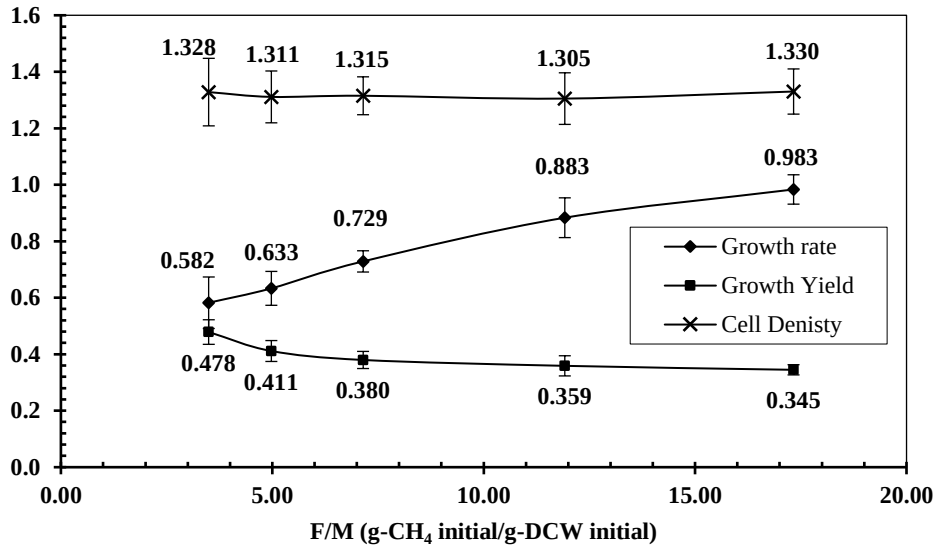

Figure (4-2): Type I Methanotrophs Behavior under Different Food to Microorganisms Ratios

Specific growth rate (day^{-1}), Observed growth yield ($gDCW_{increase}/gCH_{4\,consumed}$), Methane uptake rate (mg-CH_4/hr).

The highest achieved methane uptake rate was equal to 2.728 ± 0.104 mg-CH_4/hr obtained at the lowest F/M ratio (≈3.5 g-$CH_{4initial}$/g-$DCW_{initial}$). This was only 10% higher than the uptake observed at the highest F/M ratio (≈17.5 g-$CH_{4initial}$/g-$DCW_{initial}$). In contrast, the amount of methane consumed at F/M ratio ≈3.5 g-$CH_{4initial}$/g-$DCW_{initial}$ was 3.3 times higher after 8 hours than F/M ratio ≈17.5 g-$CH_{4initial}$/g-$DCW_{initial}$, as demonstrated in **Figure (4-3a)**.

After 16 hours, the methane consumed at the lowest F/M was equal to 86% of the total methane consumed in this culture, while, 63% only was consumed at the highest F/M. Accordingly, the maximum methane uptake rate was achieved at F/M ratio ≈3.5 g-$CH_{4\ initial}$/g-$DCW_{initial}$ after 8 hours and was equal to 4.805 ± 0.18 mg-CH_4/hr which the highest methane uptake rate in this phase. While by the end of the experiment, the methane uptake rate for the same bottle declined to 2.728 ± 0.104 mg-CH_4/hr, **Figure (4-3a)**. At F/M ratio ≈17.5 g-$CH_{4\ initial}$/g-$DCW_{initial}$, the removal ratio was increased from 32% at the start to final removal ratio of 83% which was almost the same as other cultures. **Figure (4-3b)** demonstrate that the cultures with F/M ratios

≈3.5 and 5.0 have the same pattern of consumption, while, different behavior took place at cultures with F/M ratios ≈7.0, 12.0, and 17.5. Therefore, it can be deduced that a methane limited environment has been occurred at F/M ratio equal to or below 5 due to the low methane loading. These findings provide another explanation for the lower growth rates at lower F/M ratios. Moreover, it highlights the significant of the methane loading rate which is determined based on the methane uptake rate to prevent any methane limited conditions.

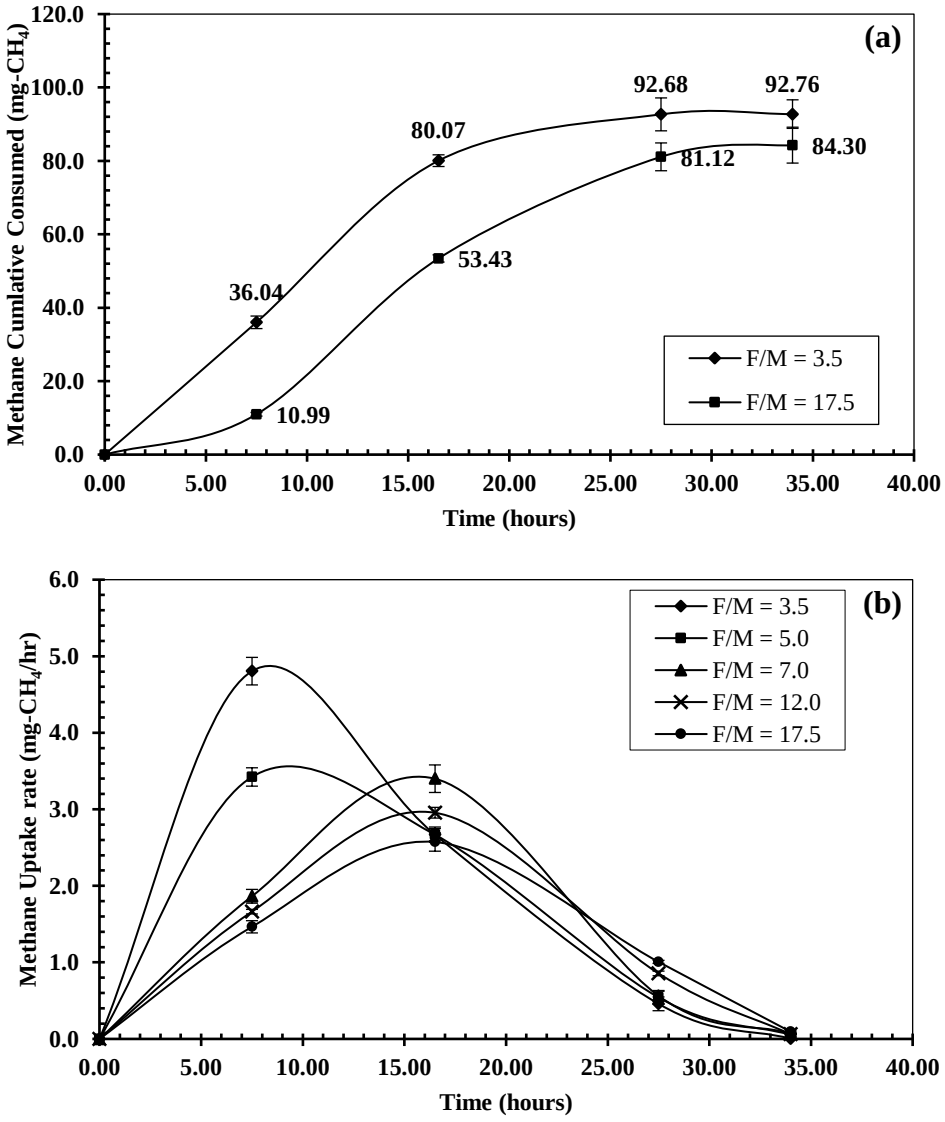

Figure (4-3): Methane Consumption Pattern under Different F/M Ratios

(a) cumulative methane consumed in milligrams over the experiment time, (b) methane uptake rate (mg-CH_4/hr) over the experiment time.

It is noteworthy that F/M ratio of ≈17.5 at biomass density of 1.318 ± 0.013 measured as OD_{600} was obtained by using a ratio between the headspace and liquid volumes of 20:1 which is not feasible in the industrial scales. Such obstacle can be addressed by using different methane delivery technology and bioreactor configuration (Strong et al., 2016) or the addition of methane vectors to increase the methane aqueous solubility (Han et al., 2009).

4.3.2 Nitrogen to Microorganisms (N/M) and Carbon to Nitrogen (C/N) Effect

Throughout the previous phases, both C/N and N/M ratios were changed according to the change in the biomass density and the reaction volume. Therefore, their influence on the microbial activity must be identified. Different nitrogen concentrations were added to cultures with the same biomass density. For those cultures, two different parameters have changed the nitrogen concentration and the N/M ratio. Thereafter, the test would be repeated using different biomass density. As a result, the same N/M ratios would be achieved at different nitrogen concentrations which can be used to identify the influencing factor on the microbial growth. Therefore, nitrogen was added in the form of sodium nitrate in for different concentrations ranging from 10 to 160 mM. It was added to three different biomass densities equal to 0.202 ± 0.005, 0.501 ± 0.003, and 1.058 ± 0.047 OD_{600}. If the same results were obtained at the same N/M regardless the nitrogen concentration. Then, the ratio between the nitrogen to microorganisms is the decisive parameter not the nitrogen concentration.

At 40 mM nitrate, the optimum growth rates and growth yields were achieved for all the biomass densities. Whereas, The N/M ratios were equal to 1.21 ± 0.08, 2.50 ± 0.09, and 6.27 ± 0.12 g $N\text{-}NO_{3\ initial}$/g-$DCW_{\ initial}$ at biomass densities measured as OD_{600} of 0.202 ± 0.005, 0.501 ± 0.003, and 1.058 ± 0.047, respectively, **Figure (4-4)**. The same trend was followed for the methane uptake rate; the highest uptake rates were achieved at 40 mM nitrate in all biomass densities with

less than 8% difference from the uptake rate achieved in the previous phases. Supportively, it was observed that over the three biomass densities both the growth rate and growth yield were positively affected by the increase in F/M ratio and the decrease of the biomass density in agreement with the previous phases. Moreover, the difference between cultures with biomass density of 0.202 and 0.501 was much lower than the difference between 0.202 and 1.058, **Figure (4-4)**. confirms that the decline in the microbial at the three biomass densities did not occur because of the N/M decline. The nitrogen removal ratio in at the optimum cultures was ranging from 52% to 43% which confer the nitrogen availability regardless the biomass density which provide an explanation to the limited effect of the N/M ratio. Hence, it can be confirmed that N/M is not a decisive parameter regarding the bacterial activity of type I methantrophs.

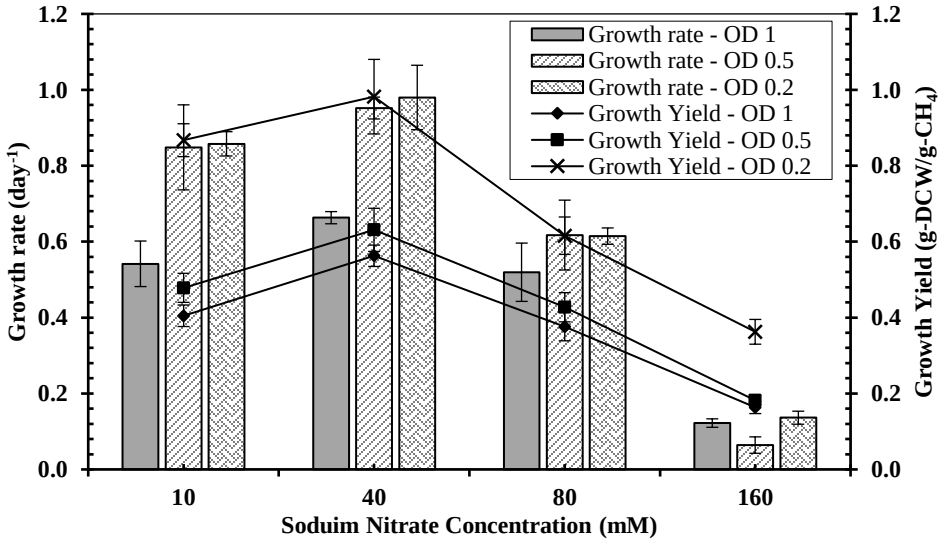

Figure (4-4): Type I Methanotrophs Behavior under Different N/M Ratios

Following the same concept, C/N ratio influence on type I methanotrophs was evaluated. The methane was added with two different volumes 200 mL and 75 mL to cultures with the same biomass density which was equal to 0.202 ± 0.005 and 0.188 ± 0.026 OD_{600}. Whereas, the nitrate concentration varied from 10 to 160 mM. For instance, C/N ratio of ≈1.25 g C-CH_4/g N-NO_3

was achieved at 80 mM nitrate for cultures with 200 mL of methane, while, the same ratio was obtained at 40 mM at the cultures with 75 mL of methane. The same N/M ratios were achieved at the same nitrogen concentrations because of using the same initial biomass densities to eliminate its effect. The cultures were again following the same trend regardless the C/N ratio. As demonstrated in **Figure (4-5)**, the highest growth rate and growth yield were achieved at 40 mM of nitrate in which the C/N ratio was equal to 2.37 ± 0.06 and 1.14 ± 0.02 g C-CH_4/g N-NO_3. The decline in the growth rate and yield between the two incubations was due to the decline in F/M ratio from 20 ± 1.25 to 11 ± 2.17 g-$CH_{4\ initial}$/g-$DCW_{initial}$. Hence, the change in the C/N ratio do not affect methantrophs type I bacterial activity.

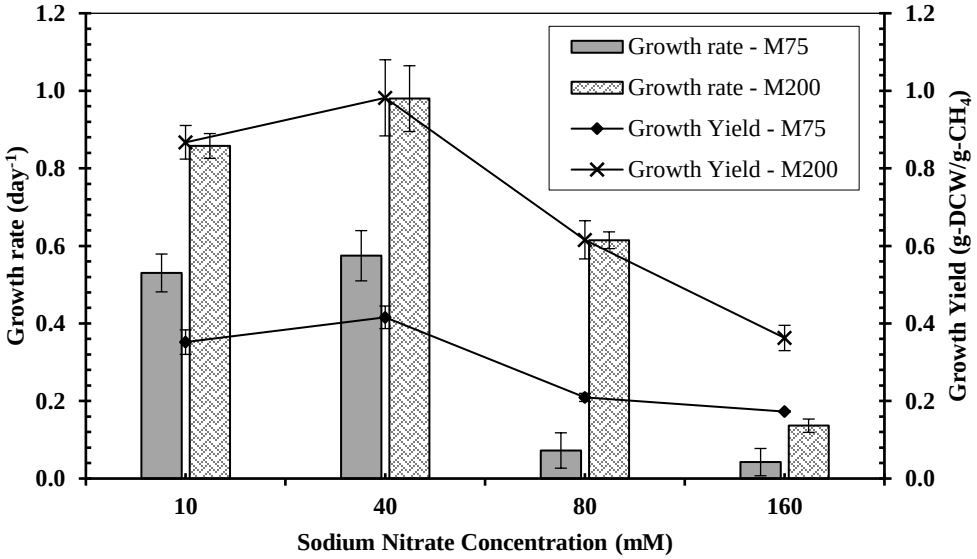

Figure (4-5): Type I Methanotrophs Behavior under Different C/N Ratios

M200: 200 mL methane added, M75: 75 mL methane added

4.3.3 Biomass Density Effect

In this phase, the biomass density influence after elucidating the F/M, C/N, and N/M ratios effect on the microbial activity of type I methanotrophs. The F/M ratio was maintained the same and was equal to 1.51 ± 0.14 g-$CH_{4\ initial}$/g-$DCW_{initial}$. The C/N and N/M ratios were varying from

biomass density to another which was neglected based on the results obtained in previous phases. As shown in **Figure (4-6)**, the highest growth rate and yield were obtained at biomass density (OD_{600}) of 0.99 ± 0.09 which were equal to 0.436 ± 0.012 day^{-1} and 0.41 ± 0.04 g-$CH_{4consumed}$/g-$DCW_{increase}$, respectively. The low growth yields observed can be referred to the low F/M ratio used in this experiment. On the other hand, the methane uptake rate (4.177 ± 0.075 mgCH_4/hr) was significantly higher at the highest optical density (2.927 ± 0.20 OD_{600}) but with the lowest growth rate (0.192 ± 0.067 day^{-1}).

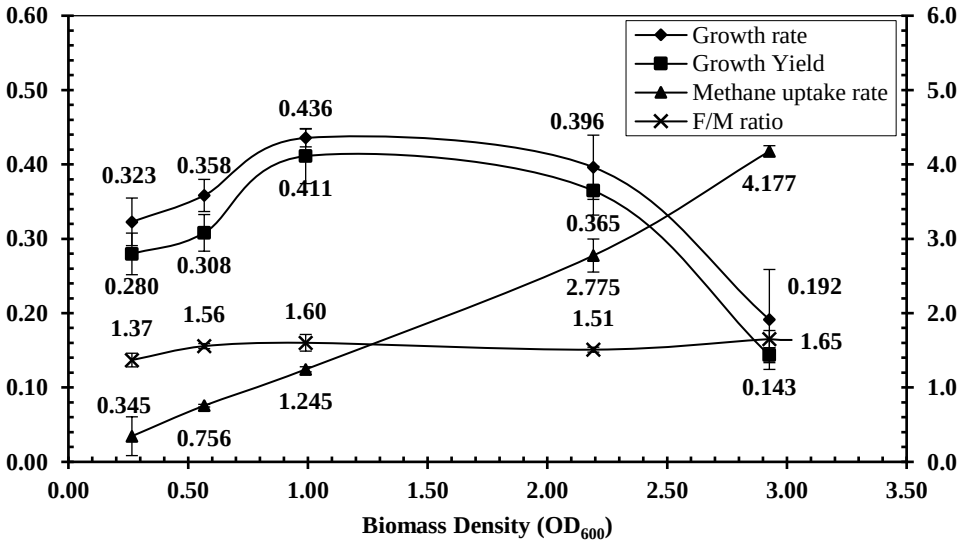

Figure (4-6): Type I Methanotrophs Behavior under Different Biomass Densities

Specific growth rate (day^{-1}), Observed growth yield ($gDCW_{increase}/gCH_{4\,consumed}$), Methane uptake rate (mg-CH_4/hr), F/M ratio (g-$CH_{4\,initial}$/g-$DCW_{initial}$).

As deduced from **Figure (4-6)**, the increase in the biomass density from 0.265 ± 0.01 to 0.991 ± 0.09 was associated with 35% and 46% increase in growth rate and yield respectively. In comparison with the first experiment (in which F/M ratio and biomass density effect was combined), the similar increase in the biomass densities resulted in 70% and 100% decrease in in growth rate and yield respectively. Such comparison shows that the most dominant factor that affect the bacterial growth is the F/M ratio not the biomass density. In addition, a relatively high

growth rate (0.983 ± 0.052 day^{-1}) was observed at optical density of 1.330 ± 0.08 OD_{600} and 17.33 ± 0.59 g-$CH_{4\ initial}$/g-$DCW_{initial}$ as the F/M ratio. This growth rate was only 9% less than the achieved at 0.201 ± 0.01 and F/M ratio of 19.85 ± 1.02 g-$CH_{4\ initial}$/g-$DCW_{initial}$. Combined together, it can be confirmed that F/M ratio has higher influence on the microbial growth than the biomass density. Furthermore, higher growth rates and yields can be achieved by maintaining high F/M ratios regardless the biomass density. On the other hand, the biomass density effect is related to the gases delivery technique. Hence, it can be hypothesized that different methane delivery technique (such as diffusers) resulted in different optimum biomass density which can also be enhanced. Such factor was neglected in most of the studies performed either using pure or mixed cultures. This study shows that F/M ratio and cell density in association with methane delivery technique must be clearly identified at any further bacterial activity assessment due to their significant effect. Furthermore, those findings prove that methanotrophic bioreactors with its diverse biotechnological application have the potential to be scaled up to the commercial results. further investigations on developing bioreactors that maintain high F/M ratios and better gas delivery should be performed.

4.3.4 Time Course Bacterial Growth and Methane Uptake

Methane to oxygen ratio and growth medium composition have been optimized in previous study. Furthermore, F/M ratio and initial cell density influence on the microbial activity have been identified. As shown in **Figure (4-7)**, methanotrophs bacterial growth passes through exponential, stationary, and decay phases which vary based on the F/M ratio and the biomass density. The previously reported values were calculated over the incubation time regardless the exponential phase duration. Thus, a time course observation was performed to report more realistic kinetics.

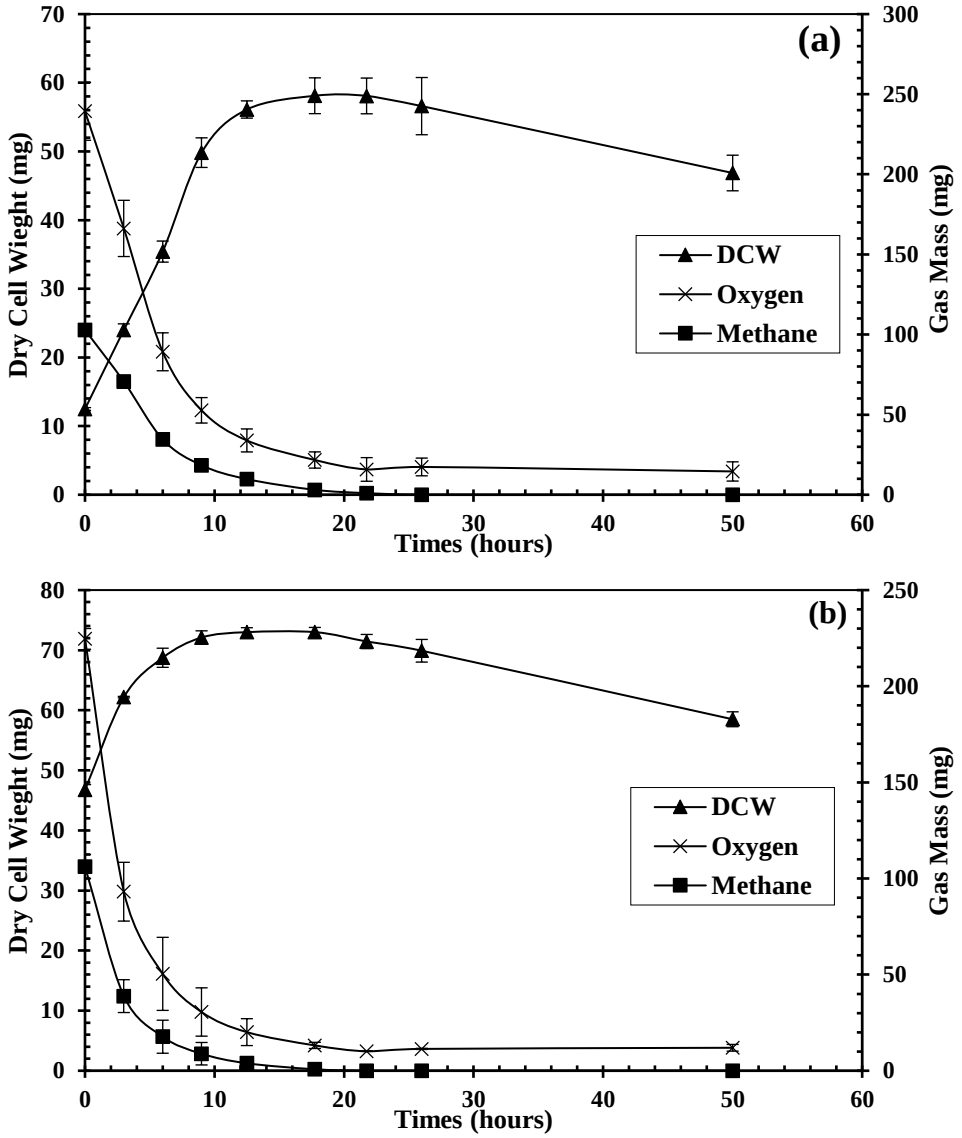

Figure (4-7): Type I Methanotrophs Time Course Growth and Methane and Oxygen Uptake

(a) Initial optical density ≈ 0.50, (b) Initial optical density ≈ 2.00.

Throughout this phase, F/M ratio and biomass density were changing similar to the first phase. The reason is that our objective from this phase is to report the growth kinetics based on feasible operational conditions that can be scaled up. The ratio between the gas phase volume and liquid phase volume applied in this phase was equal to 9:1. Higher gas to liquid ratios are needed to achieve higher F/M at higher biomass densities which is not feasible in larger scales. Therefore, we preferred to report the kinetics based on similar conditions. However, it should be highlighted

that by achieving higher F/M ratios using different gas delivery techniques higher microbial activity is expected. **Table (4-2)** shows the growth kinetics that can used to obtain different design parameters relying on the same bioreactor configuration and gas delivery technique.

Table (4-2): Growth Kinetics at Different Biomass Densities & F/M Ratios

Cell Density **(OD_{600})**	F/M ratio ($gCH_{4\ initial}$/g-$DCW_{initial}$)	μ (day^{-1})	$Y_{obs.}$ ($gCH_{4\ consumed}$/g-$DCW_{increase}$)	Methane uptake rate (mg-CH_4/hr)	q ($gCH_{4\ consumed}$/g-$DCW_{average}$/day^{-1})
0.20 ± 0.02	16.65 ± 1.92	3.014 ± 0.092	0.531 ± 0.011	5.318 ± 0.096	5.672 ± 0.054
0.52 ± 0.01	8.24 ± 0.18	2.442 ± 0.007	0.469 ± 0.003	7.449 ± 0.229	5.211 ± 0.050
0.91 ± 0.01	4.20 ± 0.23	1.987 ± 0.025	0.324 ± 0.003	10.302 ± 0.086	6.130 ± 0.139
2.11 ± 0.11	2.27 ± 0.18	1.523 ± 0.018	0.248 ± 0.002	14.746 ± 0.389	6.128 ± 0.031
2.98 ± 0.03	1.49 ± 0.06	0.911 ± 0.007	0.147 ± 0.012	19.553 ± 1.238	6.247 ± 0.458

μ: Specific growth rate, Y_{obs}: Observed growth yield, q: Methane utilization rate

4.4 Conclusion

Methanotrophs are prominent biological agents that can be employed in greenhouse gases mitigation integrated with multiple biotechnological applications such as methanol and PHB productions. Methanotrophs slow growth rates at higher biomass densities have limited the biotechnological application. In this chapter, the biomass density influence on the microbial activity have been investigated. It was concluded that the microbial is affected mainly by the decline in the F/M ratio associated with the biomass density increase. Furthermore, it was confirmed that both N/M and C/N ratios have no effect on the microbial activity of type I methanotrophs. Based on these findings, higher growth rates can be obtained at higher biomass densities by either maintaining higher F/M ratios or enhancing the gas delivery techniques.

Finally, growth kinetics have been determined under different biomass densities and F/M ratios as the most feasible conditions.

Chapter 5

Kinetics and Bioreactor Design Parameters of Type I Methanotrophs mixed culture enriched from waste activated sludge [4]

5.1 Introduction

Two contrary perspectives can be adopted while dealing with methane generated within WWTPs. First is the methane as the second major GHG with 20 to 60 times higher effect than carbon dioxide (Francisco José Fernández, 2005). In 2012, The methane released from WWTPs around the world was about 4% of the global methane budget (A. Ho et al., 2013). Whereas, the second perspective is the methane as a resource. The collected methane can be converted into multiple prominent commodities such as biopolymers, methanol, and single cell proteins. It was estimated that WWTPs located in North America have the potential to produce about 3.90 billion cubic meters of biomethane per year. The utilization of such amount prevents the GHG emissions similar to taking 1.18 billion passenger vehicles off the roads (Canadian Biogas Association, 2013; NREL, 2013; US EPA, 2011). Those facts highlight how significant and reliable is the methane generated within the WWTPs. Therefore, the development of a methanotrophic bioreactor is essential either for methane mitigation due the increasing global warming phenomena or as the first step in methane-based resource recovery system to offset part

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of the WWTPs increasing energy and cost requirements. The first step to design methanotrophs cultivation bioreactor is to identify its growth kinetics. Throughout the literature, those kinetic parameters varied significantly based on the operational conditions such as biomass density, nitrogen, methane and oxygen concentrations.

Therefore, all the reported operational conditions have been investigated and optimized throughout the previous two chapters. Few studies reported the microbial kinetics for methanotrophs. However, most of those studies were performed using pure cultures and using type II methanotrophs. Even though, type I methanotrophs are more energy efficient and have higher growth rates and methane uptake rates (Hanson and Hanson, 1996; Kalyuzhnaya et al., 2015). In general, few studies were performed using type I methanotrophs mixed culture from wastewater sludge and activated sludge in particular. Those studies were mainly focusing on the treatment of chlorinated organic wastewater (Chang and Alvarez-Cohen, 1997; Fennel et al., 1992) or the resulted microbial community structure targeting the cultivation of type II due to their unique capability to accumulate biopolymers (López et al., 2014; Myung et al., 2015; Pieja et al., 2011).

The reported kinetics, regardless the type of the growing culture, have a very wide range as mentioned before what makes it unreliable (Rostkowski et al., 2013). For instance, maximum specific growth rate (μ_{max}) ranged from 0.018 to 0.34 hr^{-1} (Boiesen et al., 1993; Delhoménie et al., 2008; Heijnen and Roels, 1981; Ménard et al., 2014; Ordaz et al., 2014; Rostkowski et al., 2013). Furthermore, Ordaz et al. reported Monod half saturation constant (K_s) up to 100 times lower than other values within the literature (Delhoménie et al., 2008; Ménard et al., 2014; Ordaz et al., 2014).

Therefore, it is pivotal to determine the growth kinetics after optimizing all the conditions affecting the process due to the contradicted data in the literature either in pure or mixed cultures. Furthermore, the protocol and calculations followed in this study should be attentively elucidated to be avoid any wrong comparison. Throughout this chapter, all Monod kinetic parameters including maximum specific growth rate (μ_{max}), Monod half saturation constant (K_s), maximum specific substrate (methane) utilization rate (q_{max}) have been determined. Moreover, the cellular growth yield (Y_x) and the endogenous decay rate (K_d) have been calculated. Thereafter, it was demonstrated how the kinetics parameters can be employed to obtain the main bioreactor design parameters such as the solids retention time (SRT) and the methane loading rate (MLR).

5.2 Materials and Methods

5.2.1 Type I Methanotrophs Mixed Culture Enrichment

In order to enrich type I methanotrophs, the inoculum was suspended in modified nitrate mineral salt (Mod-NMS) solution after being filtered. The inoculum was obtained from Humber wastewater treatment plant (Toronto, Canada). The Mod-NMS composition was as following (mg/L): $NaNO_3$, 3400; $MgSO_4.7H_2O$, 1000; $CaCl_2.6H_2O$, 200; KH_2PO_4, 272; K_2HPO_4, 610; Ferric EDTA, 4. 1 mL/L; and 1 mL of trace elements solution (Disodium EDTA, 500; $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $Na_2MoO_4.2H_2O$, 3; $FeSO_4.7H_2O$, 200; $NiCl_2.6H_2O$, 2; $CoCl_2.6H_2O$, 20); and 20 µM of copper sulfate. The enrichments were performed in 250-mL serum bottles with 50 mL as liquid volume. The cells were initially suspended at 0.5 ± 0.1 measured as optical density (OD_{600}) was used for 3 bottles. Methane and oxygen were added on daily bases, while, cultures were transferred every two to three days into fresh media. After the third transfer, cultures started to shift into the pinkish color recognized for type I

methanotrophs. After two weeks, cultures showed stable growth and methane consumption confirming methanotrophs existence. Thereafter, the cells were harvested and re-suspended in 800 mL of modified NMS solution running in 2L Bottle at 165 rpm and room temperature. On daily basis, methane and oxygen were added to the headspace, after being evacuated, with volumetric ratio of 1:1. cells were transferred to fresh media every three days with initial OD_{600} of 0.818 ± 0.107 to ensure that any used biomass are in the exponential phase.

5.2.2 Time Course Methanotrophs Growth

The cells were suspended in 50 mL of the Mod-NMS medium with initial biomass density of 0.20 ± 0.02 OD_{600}. The bottles headspace was evacuated for 30 mins. Thereafter, it was filled with methane and oxygen with molar ratio of 1:1. The bottles were incubated at MaxQ™ 4000 Benchtop Orbital Shakers (Thermo Fisher Scientific Inc., Waltham, MA, USA) at mixing speed of 165 rpm and room temperature (25 to 28^{o}C). the pH was adjusted to be 7 ± 0.3. Liquid and gaseous samples were withdrawn every 3-5 hours to monitor both the microbial growth as well as the gaseous uptake.

5.2.3 Methanotrophs Growth Kinetics

In order to determine the maximum specific growth rate (μ_{max}) and the Monod half-saturation constant (K_s), different incubations with different initial methane concentrations were used. The added methane volumes were equal to 10, 25, 50, 100, 150, and 200 mL of methane to the headspace resulting in initial aqueous methane concentration of 0.26 ± 0.01, 0.96 ± 0.07, 1.79 ± 0.07, 5.29 ± 0.07, 7.53 ± 0.04, 8.33 ± 0.11 mg-CH_4/L, respectively. The initial cell density was maintained the same at the six incubation which was equal to 118.57 ± 2.86 mg cells/L. Low initial biomass was used to eliminate any effect to the high biomass density on the methane diffusion from the gas phase to the aqueous phase. The incubation time for the cultures was three

hours which was decided based on the previous phase. The molar ratio between the added methane and oxygen was equal to 1:1. Furthermore, the cells were suspended in the previously described Mod-NMS. Methane, oxygen, and the cell density were measured before and after the incubation for all cultures.

Another incubation was performed to estimate the endogenous decay rate (K_d). The headspace was evacuated for 30 minutes. Thereafter, no methane or oxygen were added to the headspace. It was filled with helium to ensure methane and oxygen removal. The cells were suspended with initial optical density of 0.280 ± 0.05 OD_{600}. After twenty hours, the biomass density was measured to determine the endogenous decay rate (K_d). All the incubations were performed in 25^{o}C and at shaking speed of 165 rpm.

5.2.4 Analytical Methods

Samples from the bottles gaseous headspace were withdrawn by gas tight syringe. Thereafter, it was injected to the gas chromatography SRI 8610C GC (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD), methanizer and 6' molecular sieve column (Restek, Bellefonte, PA.) to obtain methane and oxygen peaks. The resulted peaks were compared with previously calibrated values using pure gases to be converted into gaseous concentrations. The temperature program was as following: injector, 80˚C; Oven, 80˚C; FID, 300˚C; TCD, 155˚C and helium gas was used as carrier gas with flowrate of 20 mL/min. For biomass density was measured as optical density at 600 nm using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). To determine the dry cell weight (DCW), a correlation equation between OD_{600} and DCW was developed. DCW was measured after the filtered samples (TSS Glass Fiber Filter, Pore Size 1.5 µm, Diameter 47 mm, 100/pk) dried overnight in the oven at 105˚C.

5.3 Calculations

5.3.1 Stoichiometric Calculations

Rostkowski et al. have developed the stoichiometric model for methanotrophic growth (Rostkowski et al., 2013). Three half reactions were developed to describe methanotrophs overall growth using methane. The first half reaction is for the methane as electron donor (R_d) taking into consideration that one mole of oxygen and two electrons are incorporated throughout this half reaction, as shown in **equation (5-1)**. The second half reaction described oxygen (as the terminal electron acceptor) reduction into water (R_a), **equation (5-2)**. Finally, the electrons derived from the methane used for cell synthesis are described in the third half reaction (R_c), as expressed in **equation (5-3)**. The cell synthesis half reaction varies according to the nitrogen source. As demonstrated in chapter 3, methanotrophs type I exposed better microbial activity while relying on nitrate as the nitrogen source. Thus, the cell synthesis half reaction is described using nitrate. The overall reaction ($R_{overall}$) was determined as following $R_{overall} = f_e\ R_e + f_s\ R_c - R_d$, where the f_e is the electrons fraction used for energy generation; f_s the electrons fraction used for cell synthesis. The overall methanotrophic equation is as shown in **equation (5-4)**.

$$CO_2 + 4H^+ + 4e^- \longrightarrow CH_4 + O_2 \qquad \text{(Eq. 5-1)}$$

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O \qquad \text{(Eq. 5-2)}$$

$$5CO_2 + NO_3^- + 29H^+ + 28e^- \longrightarrow C_5H_7O_2N + 11H_2 \qquad \text{(Eq. 5-3)}$$

$$\frac{1}{4}\,CH_4 + \left(\frac{1}{4} + \frac{f_e}{4}\right) O_2 + \left(\frac{f_s}{28}\right) NO_3^- + \left(\frac{29\,f_s}{28} + f_e - 1\right) H^+ \longrightarrow \left(\frac{1}{4} - \frac{5\,f_s}{28}\right) CO_2 + \left(\frac{f_e}{2} - \frac{11\,f_s}{28}\right) H_2O + \left(\frac{f_s}{28}\right) C_5H_7O_2N \qquad \text{(Eq. 5-4)}$$

The f_e and f_s can be calculated using the overall equation along with the gas consumption and cellular growth measurements. The molar ratio between the oxygen and methane consumed is

equal to $(1 + f_e)$. Alternatively, the growth yield is can be used to determine the f_s as it is equal to $(\frac{113fs}{28/4})$.

5.3.2 Growth Kinetics Calculations

The experiments were performed in batch bioreactors in which no inflow or outflow. Throughout the experiments, the substrate, which is the methane, was added to the gas phase serving as sole carbon source and electron donor. The total mass of the substrate (methane) is equal to the summation of the mass in the gas phase and in the aqueous phase, as described in **equation (5-5)**.

$$dM_{T,CH4} = M_{gas} + M_{aq.} = C_{gas}V_{gas} + C_{aq.}V_{aq.} \quad \text{(Eq. 5-5)}$$

Where $M_{T,\,CH_4}$ is the total methane mass in the system (mg), M_{gas} is the methane mass in the gas phase (mg), $M_{aq.}$ is the methane mass in the aqueous phase (mg), C_{gas} is the methane concentration in the gas phase (mg/L), V_{gas} is the gas volume (L), $C_{aq.}$ is the methane concentration in the aqueous phase (mg/L), and $V_{aq.}$ is the liquid volume (L). The methane concentration in the aqueous phase can be estimated by using the dimensionless Henry's law constant (H_c) at 25°C and 1 atmospheric pressure which is equal to 31.40. Thus, the total methane mass can be expressed using the methane concentration in the gas phase. Furthermore, the rate substrate (methane) mass accumulation can be calculated as shown in **equation (5-6)**.

$$\frac{\Delta M_{T,CH4}}{\Delta t} = \frac{\Delta C_{gas}}{\Delta t}\left(V_{gas} + \frac{V_{aq.}}{H_c}\right) \quad \text{(Eq. 5-6)}$$

The relation between the substrate (methane) uptake rate and biomass increase rate can be described using **equation (5-7)**. Whereas, the specific substrate (methane) utilization rate (q) can be calculated as shown in **equation (5-8)** and the specific growth rate (μ) can be calculated as

expressed in **equation (5-9)**. Thus, the equation can be re-written as expressed in **equation (5-10)**.

$$\frac{\Delta X}{\Delta t} = Y_x \frac{\Delta C_{T,CH4}}{\Delta t} - K_d X \quad \text{(Eq. 5-7)}$$

$$q = \frac{(\frac{\Delta C_{T,CH4}}{\Delta t})}{X_{average}} = \frac{(\frac{\Delta M_{T,CH4}}{\Delta t})}{DCW_{average}} = (\frac{\frac{\Delta C_{gas}}{\Delta t}\ (V_{gas} + \frac{V_{aq.}}{H_c})}{\frac{DCW_{t-1} + DCW_t}{2}}) \quad \text{(Eq. 5-8)}$$

$$\mu = \frac{(\frac{\Delta X}{\Delta t})}{X_{average}} = \frac{(\frac{\Delta DCW}{\Delta t})}{DCW_{average}} \quad \text{(Eq. 5-9)}$$

$$\mu = Y_x\, q - K_d \quad \text{(Eq. 5-10)}$$

Where, X is the biomass concentration (mg cells/L), Y_x is the cellular growth yield (g-$DCW_{increase}$/g-$CH_{4\ consumed}$), $C_{T,\ CH_4}$ is the methane (substrate) concentration (mg-CH_4/L), K_d is the endogenous decay rate ($hour^{-1}$), t is the step time or the incubation time (hours), q is the specific substrate utilization rate (g-CH_4/g-DCW/hr^{-1}), $DCW_{average}$ is the average dry cellular weight (mg) over specific time step or the incubation time, and μ is the specific growth rate (hr^{-1}). Therefore, the growth yield and decay rate can be determined by plotting the relation between the specific growth rate and specific substrate utilization rate. The slope of the regression line is the growth yield (Y_x) and the Y-intercept is the decay rate (K_d). Furthermore, the decay rate (K_d) was re-calculated using equation (9), while, the substrate (methane) and the electron acceptor (oxygen) was eliminated from the culture.

Monod equation is used to describe the methanotrophic microbial growth using the specific growth rate and the methane aqueous concentration, as expressed in **equation (5-11)**. Hence, the maximum specific growth rate (μ_{max}) (hr^{-1}) and the Monod half-saturation constant (K_s) (mg-CH_4/L) were determined using lineweaver-Burk correlation derived from Mondo equation, as

shown in **equation (5-12)**. It can be assumed that the decay rate tends to zero when the specific growth rate is maximum. Therefore, the maximum specific substrate (methane) utilization rate (q_{max}) can be calculated using the maximum specific growth rate (μ_{max}) and the growth yield (Y_x) using **equation (5-13)** derived from **equation (5-10)**.

$$\mu = \mu_{max} \frac{C_{aq.,CH4}}{K_s + C_{aq,CH4}} \quad \text{(Eq. 5-11)}$$

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}} \times \frac{1}{C_{aq.,CH4}} + \frac{1}{\mu_{max}} \quad \text{(Eq. 5-12)}$$

$$q_{max} = \frac{\mu_{max}}{Y_x} \quad \text{(Eq. 5-13)}$$

5.4 Results and Discussion

5.4.1 Type I Methanotrophs Growth Kinetics

Incubations throughout the previous two chapters were performed to evaluate the initial biomass density effect. It showed that initial biomass density of 0.225 ± 0.025 measured as OD_{600} yielded the best microbial activity. At lower biomass density, higher food to microorganisms (F/M) ratios were achieved as discussed in chapter 4. In agreement, previous kinetic studies stressed on the significance of using low biomass densities to eliminate any mass transfer limitation (Cantera et al., 2016; López et al., 2014; Ordaz et al., 2014).

The biomass density and methane and oxygen concentration variation over incubation time is shown in **Figure (5-1)**. It followed the typical bacterial growth and substrate removal patterns in batch reactors (Tchobanoglous et al., 2003). Interestingly, the incubated cultures did not expose any lag phase based on three hours' time step. The biomass density continued to increase until 17.75 hours when the stationary phase started, **Figure (5-1)**. The specific growth rate (μ) attained over the exponential phase was equal to 0.092 ± 0.02 hr^{-1}. At 17.75 hours, the percentage of the

methane removal was equal 94% equivalent to specific methane utilization rate of 0.164 ± 0.004 g-CH_4/g-DCW/hr^{-1}.

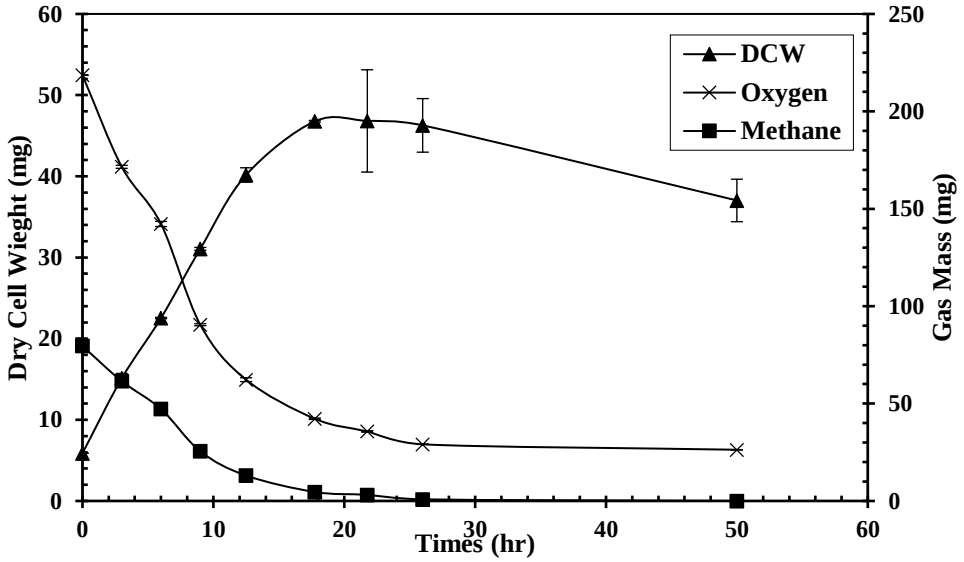

Figure (5-1): Type I Methanotrophs Time Course Growth and Methane and Oxygen Uptake

The specific growth rates (μ) along with the corresponding specific substrate utilization rates (q) over the exponential phase were plotted to obtain the growth yield. The resulted coefficient of determination (R^2) was equal to 0.9491. The cellular growth yield (Y_x) was computed based on **equation (5-10)**, and was equal to 0.592 g-DCW/g-CH_4. Whereas, the endogenous decay rate (k_d) was equal to 0.0109 hr^{-1}. Growth yields of 0.585 ± 0.085 g-cells/g-CH_4 was obtained using type I methanotrophs pure culture of methylococcus capsulatus (Leak and Dalton, 1986). Whereas, a new type I methanotrophs isolate from solid state anaerobic digesters expressed growth yield of 0.19 ± 0.02 g-cells/g-CH_4 (Sheets et al., 2016). Moreover, the growth yield of methanotrophic consortium from a packed bed column was equal to 0.36 g-cells/g-CH_4 (Delhoménie et al., 2008). Mixed culture enriched from landfill soil resulted in growth yield of 0.33 g-cells/g-CH_4 (Chang and Alvarez-Cohen, 1997). Additionally, (Heijnen and Roels, 1981) reported growth yields of 0.46 - 0.59 for mixed cultures and 0.35 - 0.65 g-cells/g-CH_4.

Therefore, the growth yield value attained in this study using waste activated sludge are comparable with the values obtained throughout the literature using either pure or mixed cultures. Using type II pure cultures, Rostokowski et al. reported growth yield of 0.63 and 0.73 g-cells/g-CH_4 for methylosinus trichosporium OB3b and methylocystis parvus OBBP, respectively (Rostkowski et al., 2013). Whereas, methylosinus sporium (DSMZ 17706) exposed growth yield of 0.78 ± 0.04 g-cells/g-CH_4. Hence, it can be deduced that type I methanotrophs growth yield is slightly lower growth yield than type II methanotrophs.

As demonstrated in **Figure (5-2)**, the specific growth rate (μ) notably declined over time. It can be referred to the associated increase in the biomass density and the decline in the in the F/M ratio. Hence, the further growth kinetics experiments were performed for three hours only to eliminate any effect for the cell density increase or F/M ratio decrease.

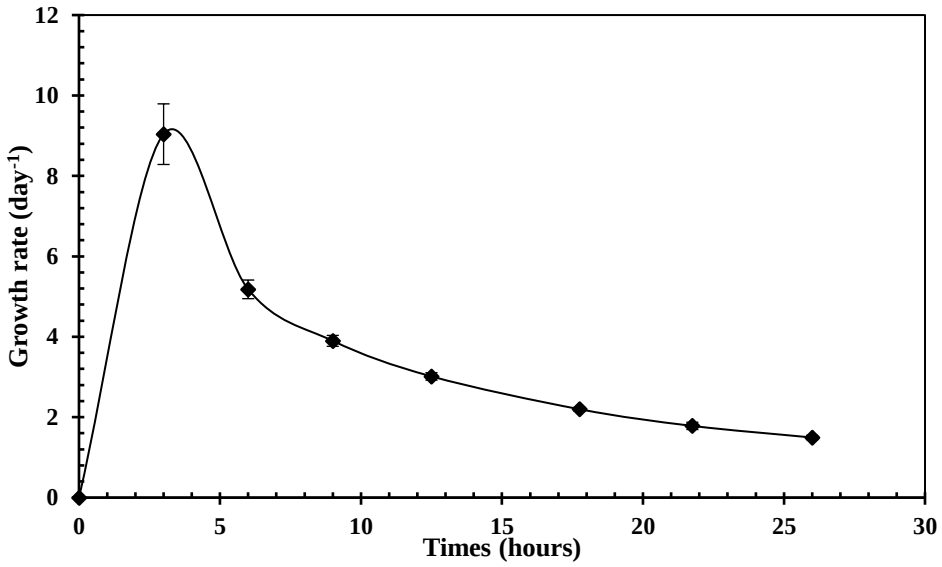

Figure (5-2): Type I Methanotrophs Growth Rate Over Time

The specific growth rates (μ) calculated at different substrate (methane) concentrations ($C_{aq., CH_4}$) were accurately fitting with Monod equation, expressed at **equation (5-11)**, resulting in coefficient of determination (R^2) of 0.9487. Hence, the maximum specific growth rate (μ_{max}) and

Monod half-saturation constant (K_s) were attained using lineweaver-Burk correlation, demonstrated at **equation (5-12)**. The attained maximum specific growth rate (μ_{max}) is equal to 0.358 hr^{-1} (8.592 day^{-1}). The Monod half-saturation constant (K_s) of 1.037 mg-$CH_{4,\ aq.}$/L.

Furthermore, the maximum specific substrate (methane) utilization rate (q_{max}) was calculated using **equation (5-13)**. It is equal to 0.605 g-$CH_{4,\ Total}$/g-DCW/hr^{-1}. The reported values in the literature ranging from 0.076 to 0.367 g-$CH_{4,\ Total}$/g-DCW/hr^{-1} (López et al., 2014; Ordaz et al., 2014; Rostkowski et al., 2013). It is noteworthy that the reported values were attained using type II pure cultures known for lower methane uptake (Kalyuzhnaya et al., 2015; Pfluger et al., 2011) which elucidate the obvious difference in the values. It should be highlighted that higher substrate utilization rates (q) are more desirable in the case of targeting substrate (methane) removal. Whereas, it is preferable to achieve lower methane utilization rates (q) while targeting the bacterial growth and biotechnological application. However, the low methane utilization rates should be associated with higher growth yields to maintain higher growth rates.

To best of our knowledge, type I methanotrophs mixed culture enriched from waste activated sludge attained in this study have the highest maximum specific growth rate (μ_{max}) in comparison with other mixed cultures. The maximum specific growth rates (μ_{max}) (hr^{-1}) reported previously using mixed cultures were equal to 0.08 – 0.22 (Heijnen and Roels, 1981), 0.054 (Boiesen et al., 1993), and 0.018 (Delhoménie et al., 2008). In addition, it is higher than the maximum growth rates obtained using type II pure cultures 0.09 – 0.299 hr^{-1} (Ordaz et al., 2014; Rostkowski et al., 2013). On the other hand, a very wide range of Monod half saturation constant (K_s) have been reported in the literature. It was reported to range from 0.05 to 6.13 mg$CH_{4,\ aq.}$/L for mixed cultures (Boiesen et al., 1993; Delhoménie et al., 2008; López et al., 2014; Ménard et al., 2014).

The K_s obtained in this study is comparable to the literature and lower than most of the reported values.

The endogenous decay rate (K_d) was re-calculated by running a substrate free experiment for twenty hours. No methane was detected both by the start and end of the incubation. The K_d was determined using **equation (5-9)**. The endogenous decay rate (K_d) is equal to 0.0108 hr^{-1} which is equal with the value throughout the previous experiment (K_d = 0.0109 hr^{-1}). The average K_d determined in this study is slightly higher but still comparable to the values obtained previously (0.002 - 0.008 hr^{-1}) (Boiesen et al., 1993).

The energy generation electrons fraction (f_e) and cell synthesis electrons fraction (f_s) were determined under methane sufficient environment. By the addition of 200 mL of methane to the headspace, the substrate (methane) concentration is higher than Monod half saturation constant and methane sufficient environment achieved. The molar ratio between the oxygen and methane consumed (O/M) was equal 1.302 ± 0.011. from methane Stoichiometry, f_e is equal the O/M molar ratio minus 1, while, f_s is equal 0.99 multiplied by the growth yield (Y_x). Thus, the f_e and f_s equal to 0.302 and 0.586, respectively. Those values result in stoichiometric closure error of 11.2% which is acceptable considering the effect of the associated microbial strains in the methanotrophic mixed culture.

The substrate (methane) concentration at which the microorganisms reached the stationary phase is the substrate threshold concentration (S_{min}). below this concentration, the endogenous decay rate (K_d) will be higher than the specific growth rate (μ) (Yu et al., 2017). the substrate threshold concentration (S_{min}) is computed according to **equation (5-14)**.

$$S_{min} = \frac{K_d \cdot K_s}{\mu_{max} - K_d} \qquad \text{(Eq. 5-14)}$$

Based on **equation (5-14)**, the substrate threshold concentration equals to 0.033 mg-$CH_{4,\ aq.}$/L. The S_{min} low concentration shows methanotrophs type I has a very high methane affinity as the net microbial growth can be maintained under very low methane aqueous concentration.

Accordingly, **Table (5-1)** summarizes type I methanotrophs attained growth kinetics.

Table (5-1): Type I Methanotrophs Growth Kinetics

Kinetic Parameter	Unit	Value
Maximum Specific Growth rate (μ_{max})	day^{-1}	8.59
Maximum Specific Methane Utilization rate (q_{max})	g-$CH_{4,\ Total}$/g-DCW/day^{-1}	14.52
Monod half saturation constant (K_s)	mg-$CH_{4,\ aq.}$/L	1.04
Endogenous Decay rate (K_d)	day^{-1}	0.262
Cellular Growth Yield (Y_x)	g-DCW/g-CH_4	0.592
Methane Threshold concentration (S_{min})	mg-$CH_{4,\ aq.}$/L	0.033

5.4.2 Design Parameters of Type I Methanotrophic Bioreactor

The growth kinetics identified in this study confirm the great potential for type I methanotrophs to be employed in multiple resources recovery processes from waste streams. Various bioreactor design parameters can also be deduced on the attained kinetics. The SRT is estimated using **equation (5-15)** (Tchobanoglous et al., 2003). Hence, knowing the substrate (methane) concentration, the SRT can be calculated using the growth kinetics constants obtained throughout this study.

$$\frac{1}{SRT} = \mu - K_d = \mu_{max}\frac{S}{K_s + S} - K_d \qquad \text{(Eq. 5-15)}$$

Where S is the methane concentration ($C_{T,\ CH_4}$) in the reactor and the effluent (mg-CH_4/L). Relying on gaseous electron donor and acceptor, the hydraulic retention time (HRT) is determined based on the nitrogen uptake. Throughout chapter 3, It was determined that the

maximum HRT is 128 hours. After 128 hours, the growth rate declines due to the nitrogen, in the form of sodium nitrate, deterioration. On the other hand, the HRT can be minimized for feasible bioreactor design. The minimum HRT would be estimated based on the growth rate to eliminate biomass washout. Hence, the minimum HRT will be equal to the SRT estimated in **equation (5-15)**. Furthermore, the methane loading rate (MLR) (mg-CH_4/hr) can be determined based on the maximum specific substrate utilization rate (q_{max}). building the mass balance for continuous methane utilization, the methane loading rate can be estimated using **equation (5-16)**.

$$MLR = \frac{S_o}{t} = \frac{S}{t} + X\, q_{max} \frac{S}{K_s + S} \qquad \text{(Eq. 5-16)}$$

Where t is the gas residence time, X is the biomass concentration in the reactor (mg-cells/L), S_o methane concentration in the influent gas (mg-CH_4/L).

5.5 Conclusion

Type I methanotrophs are of interest in the resources recovery of waste streams due to their multiple biotechnological applications potential integrated with their methane mitigation capacity. The understanding of type I methanotrophs growth kinetics is pivotal for better development of methanotrophic bioreactor. Throughout this study, all Monod kinetic parameters have been reported, for the first time, for Type I methanotrophs mixed culture enriched from waste activated sludge: μ_{max} 8.59 day-1, q_{max} 14.52 g-$CH_{4,\ Total}$/g-DCW/day^{-1}, Ks 1.04 mg-$CH_{4,\ aq.}$/L, K_d 0.262 day-1, Y_x 0.592 g-DCW/g-$CH_{4,}$ and S_{min} 0.033 mg-$CH_{4,\ aq.}$/L. Furthermore, different methanotrophic design parameters such as SRT and MLR can be obtained based on the attained growth kinetics, **equations (5-14, 5-15, and 5-16)**. The high maximum specific growth rate (μ_{max}) and low methane affinity (S_{min}) confirm the prominence and feasibility of type I methanotrophs to be employed in various resources recovery processes from waste streams.

Chapter 6

Enhancement of Methane Bio-Hydroxylation Process using Mixed Culture of Type I Methanotrophs as Biocatalyst Enriched from Waste activated sludge [5]

6.1 Introduction

Recently, resources recovery from the wastewater streams is of industrial interest. It was reported in a study performed on WWTP located in Toronto, Canada, that the wastewater has energy content (in the form of organics) up to 9 times higher than energy consumed for its treatment (Logan, 2008). As demonstrated within chapter 2, methanol is as a multiple use commodity with a prominent role as an efficient and sustainable substitute for biomethane produced within WWTPs what makes the process of methane bio-hydroxylation is more feasible.

Methanotrophs can be employed as a biological catalyst for methane hydroxylation. Owing to the possession of MMO enzyme, methanotrophs catalyze methane oxidation into methanol. One oxygen molecule and two electrons should be incorporated in methane hydroxylation. Under normal conditions, the produced methanol is not accumulated and instantly oxidized into formaldehyde in a reaction catalyzed by MDH enzyme. Therefore, MDH activity should be inhibited for extracellularly methanol accumulation.

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As elucidated previously, type I methanotrophs is more advantageous to be manipulated in methanol production. Supportively, a recent study obtained the highest reported methanol concentration by using novel type I methylomonas isolated from brewery waste sludge (Hur et al., 2016). To best of our knowledge, only one study has used mixed cultures for the conversion of methane into methanol which was enriched from landfill cover soil samples (Han et al., 2013). Even though, pure cultures are not imitating the real conditions in methane producing facilities which are pollutant control facilities such as WWTPs.

Various factors were reported to affect methanol production such as -but not limited to- methanol MDH inhibitors, headspace gaseous composition, and biomass density. However, the results reported in the literature are contradicted mainly due to the use of different strains at each study (Ge et al., 2014). Therefore, the optimization of such factors using mixed culture from waste activated sludge is a major breakthrough towards the scaling up of methane bio-hydroxylation process in WWTPs. In this study, the influence of different MDH inhibitors, formate concentration, headspace gaseous composition, copper concentration, and biomass density on methanol accumulation using proteobacteria phylum methanotrophs (type I methanotrophs) enriched from waste activated sludge as biocatalyst were investigated and optimized.

6.2 Materials and Methods

6.2.1 Type I Methanotrophs Mixed Culture Enrichment

Type I methanotrophs was enriched by the suspension of filtered waste activated sludge in modified nitrate mineral salt (NMS) solution. The used sludge was obtained from Humber wastewater treatment plant (Toronto, Canada). The modified NMS composition was as following (mg/L): $NaNO_3$, 3400; $MgSO_4.7H_2O$, 1000; $CaCl_2.6H_2O$, 200; KH_2PO_4, 272; K_2HPO_4, 610;

Ferric EDTA, 4. 1 mL/L; and 1 mL of trace elements solution (Disodium EDTA, 500; $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; H_3BO_3, 30; $Na_2MoO_4.2H_2O$, 3; $FeSO_4.7H_2O$, 200; $NiCl_2.6H_2O$, 2; $CoCl_2.6H_2O$, 20); and 20 µM of copper sulfate. The enrichments were performed in 250-mL serum bottles with 50 mL as liquid volume. Initial biomass density of 0.7 ± 0.1 measured as optical density (OD_{600}) was used for 3 bottles. Methane and oxygen were added on daily bases, while, cultures were transferred every two to three days into fresh media. After the third transfer, Cultures started to shift into the pinkish color recognized for type I methanotrophs. After two weeks, cultures showed stable growth and methane consumption confirming methanotrophs existence. Thereafter, the cells were harvested and re-suspended in 800 mL of modified NMS solution running in 2L Bottle at 165 rpm and room temperature. On daily basis, methane and oxygen were added to the headspace, after being evacuated, with volumetric ratio of 1:1. cells were transferred to fresh media every three days with initial OD_{600} of 0.818 ± 0.107 to ensure that any used biomass for methanol production are in the exponential phase.

6.2.2 Methanol Accumulation

All the batch optimization experiments were carried out in duplicate using 250 mL sealed serum bottles capped with rubber stoppers. Unless otherwise mentioned, the reaction media volume was equal to 50 mL and consists of the modified NMS, MDH inhibitors, and Sodium formate with varied concentrations. After being evacuated for 5 mins, methane (50%) and oxygen (50%) were added to the headspace. The bottles were incubated at room temperature with shaking speed of 165 rpm. At each experiment, all bottles have similar initial cell density to eliminate its effect. Methane, oxygen and biomass density were measured both at the end and start of any experiment. Moreover, by the end of each experiment, cultures were centrifuged at 4200 rpm for 20 mins at 4°C to ensure reaction immediate stoppage and the supernatant was collected to

measure methanol concentrations. All methanol accumulation experiments were 6 to 8 hours long.

6.2.3 Reaction Media Optimization

The influence of various concentrations of MDH were evaluated while using 100 mM of formate. Phosphate buffer (as NaH_2PO_4/ Na_2HPO_4 with pH of 7) concentration was optimized by evaluating different concentrations (50, 100, 200, and 500 mM). Thereafter, EDTA (0.1, 0.5, 1, and 5 mM), NaCl (100, 200, and 300 mM), and $MgCl_2$ (10, 25, 40, and 50 mM) were added to the phosphate buffer optimized concentration to promote MDH disruption efficiency. After the optimization of MDH inhibitors, different formate concentration (0, 20, 40, 80, 100, 120, 160, and 200 mM) were assessed as alternative electron donor. Furthermore, the effect of modified NMS addition on methanol accumulation was investigated. Finally, the copper effect was evaluated by applying five different copper concentrations of 0, 5, 10, 20, 40 mM added as copper sulfate. By the end of this phase, methanol accumulation was observed over time using three different formate concentrations (80, 120, and 200 mM) with the optimized concentrations obtained throughout this phase. Methane, oxygen, methanol, and biomass density were periodically measured. In addition, the effect of inhibitors and formate addition after reaching the maximum methanol concentration in order to promote methanol productivity was also assessed.

6.2.4 Biomass Density Optimization

Various initial biomass densities with the same methane, oxygen, reaction medium concentrations were used in this phase. Cell densities ranged from ≈0.25 to 3 measured as OD_{600}. The optimized values obtained previously for the reaction medium were employed. The change in biomass density while maintaining the same reaction medium concentration resulted in different formate to microorganisms (FO/M) and Inhibitors to microorganisms (I/M) ratios.

Therefore, the effect of increasing both the formate as well as the inhibitors concentrations to maintain the same FO/M and I/M ratio on higher biomass density was investigated.

6.2.5 Gaseous Headspace Optimization

Different methane to oxygen ratios (v/v) were added to the bottles headspace. to evaluate the effect of increasing oxygen concentration, the ratios applied were 1:1, 1:2, 1:3, and 1:4. The experiments were performed while maintaining the same methane concentration to eliminate its effect. Thereafter, the ratio was increased to 2:1 and 4:1 by increasing methane concentration while oxygen concentration was maintained constant. Furthermore, the influence of hydrogen addition to the headspace was also investigated. Hydrogen concentrations of 10% and 20% were added to the headspace. All the experiments were running using the optimized concentrations obtained throughout this study for the reaction medium and biomass density, whereas, methane and oxygen were added with 1:1 volumetric ratio.

6.2.6 Analytical Methods

Gaseous samples were withdrawn from the serum bottles headspace using gas tight syringe. subsequently, it was analyzed using SRI 8610C gas chromatography (SRI instrumentation, Torrance, USA) equipped with thermal conductivity detector (TCD), methanizer and 6' molecular sieve column (Restek, Bellefonte, PA.) to obtain methane and oxygen peaks. The peaks were compared to previously calibrated values using pure gases to be converted into concentrations. The temperature program was as following: injector, 80°C; Oven, 80°C; FID, 300°C; TCD, 155°C and helium gas was used as carrier gas with flowrate of 20 mL/min. For biomass density measurements, optical density at 600 nm were measured using a DR 3900 Benchtop Spectrophotometer (HACH Company, Loveland, Colorado, USA). To obtain the dry cell weight (DCW), a correlation equation between OD_{600} and DCW was developed. DCW was

measured after the filtered samples (TSS Glass Fiber Filter, Pore Size 1.5 µm, Diameter 47 mm, 100/pk) dried overnight in the oven at 105˚C. for methanol measurement, liquid samples were harvested from the supernatant after cultures centrifuge. Thereafter, it was injected to the GC equipped with MXT-WAX column (Restek, Bellefonte, PA.), whereas, the flame ionization detector (FID) was used. Injector and oven temperature were set constantly at 60˚C. the temperature of the FID was set to 300˚C and Helium was used as the carrier gas. Methanol concentrations were correlated with externally calibrated curve obtained using pure methanol. To calculate methanol productivity (mg/L/day), methanol concentration (mg/L) was divided by experiment duration in days. number of methane moles consumed was divided by number of methanol moles produced to determine the methane to methanol conversion efficiency.

6.3 Results and Discussion

6.3.1 MDH Inhibitors Screening

Unless the PQQ linked MDH enzyme is inhibited, almost no methanol would be accumulated (Han et al., 2013). Various inhibitors have been reported in the literature. Even though, the addition of 0.67 µM cyclopropanol had reported to efficiently inhibit MDH activity and accumulate methanol. Cyclopropanol was not evaluated in our study because of its irreversible inhibitory effect on the cells and unsteadiness aerobically (Furuto et al., 1999; Kim et al., 2010). Furthermore, NH_4Cl has negative on methane uptake caused by the co-substrate rivalry between ammonium and methane on the MMO (López et al., 2013; Sundstrom and Criddle, 2015). Thus, only phosphate buffer, $MgCl_2$, EDTA, and NaCl was evaluated in this study.

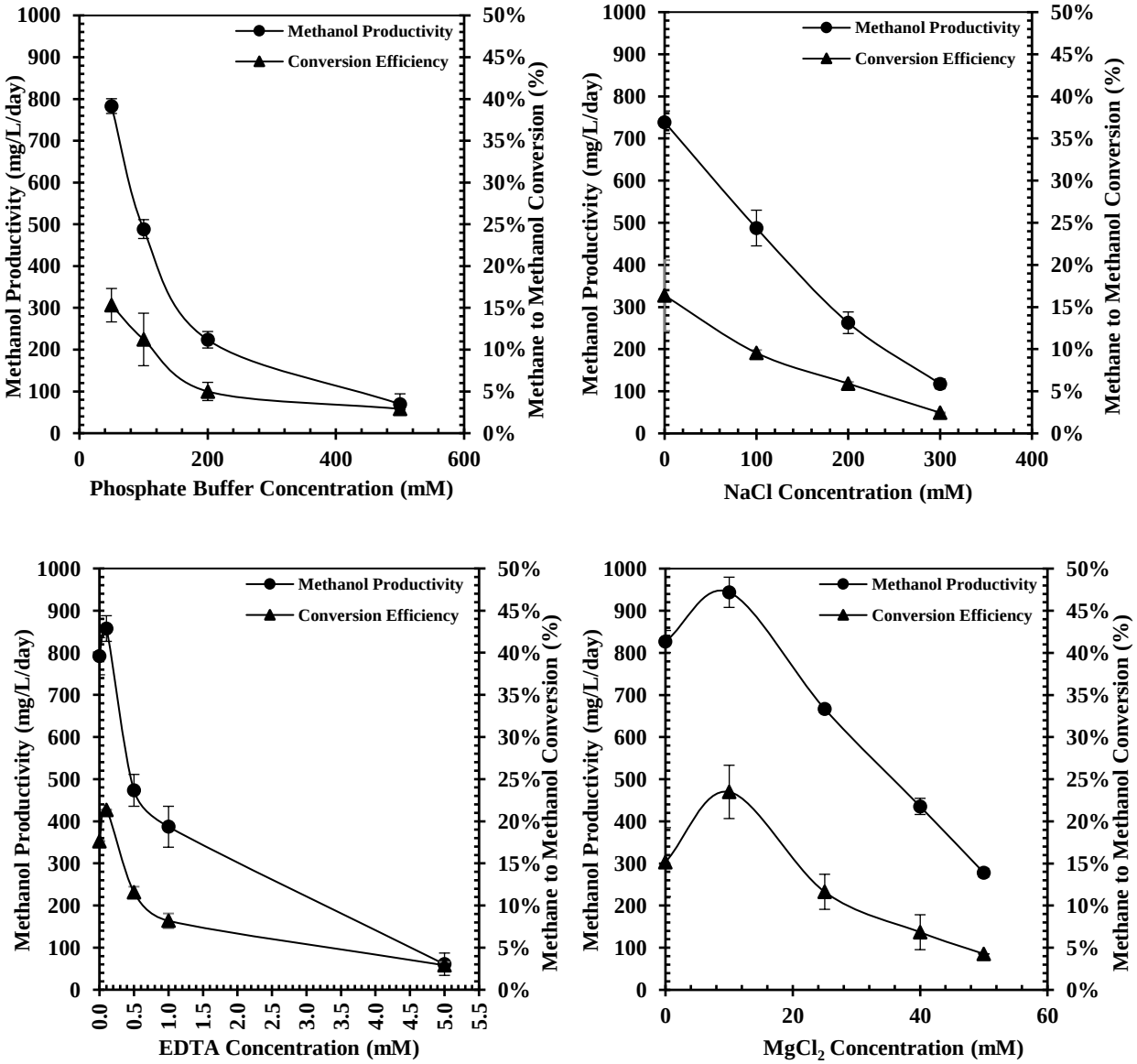

Figure (6-1): The Effect of Different MDH Inhibitors on Methanol Productivity (mg/L/day) and Methane to Methanol Conversion Ratio (%)

(a) Phosphate Buffer, (b) Sodium Chloride, (c) EDTA, (b) Magnesium Chloride.

As shown in **Figure (6-1a)**, a methanol productivity of 783 ± 17 mg/L/day was achieved at 50 mM of phosphate buffer. Increasing the phosphate buffer concentration above 50 mM results in 40% decrease in methanol productivity. Whereas, trace concentration of methanol (≈ 20 mg/L)

was observed by reaching the concentration of 500 mM. In agreement with our results, the optimum phosphate buffer concentration reported ranges from 40 to 100 mM (Ge et al., 2014). It was found that starting from 60 mM of phosphate buffer a decline in MMO activity was observed (Han et al., 2013). Agreeably, methane uptake declined by more than 20% by increasing phosphate buffer above 50 mM. Due to the aforementioned inhibitory effect at high phosphate buffer concentrations, other inhibitors are usually added to the reaction medium in association with the phosphate buffer.

NaCl as inhibitor is more profitable due to its lower cost and abundance in comparison with other inhibitors. Regrettably, addition of concentrations from 100 to 300 mM of sodium chloride did not result in any increase in methanol concentration or methane to methanol conversion efficiency, **Figure (6-1b)**. On the other hand, the addition of 0.1 mM of EDTA to the 50-mM phosphate buffer promoted methanol productivity from 792 ± 44 to 857 ± 30 mg/L/day, **Figure (6-1c)**. Moreover, the methane to methanol conversion efficiency has increased by 22%. Further increase in EDTA concentration resulted in a decline in methanol productivity even lower than the productivity obtained with no EDTA addition. As illustrated in **Figure (6-1d)**, the highest methanol productivity was obtained by using 10 mM $MgCl_2$ in which cultures yielded methanol concentration of 236 ± 13 mg/L after 6 hours. Also, methane to methanol conversion efficiency was elevated from 15 ± 4% to 23 ± 3%. As shown in **Figure (6-1)**, the addition of 10 mM $MgCl_2$ results in methanol productivity of 943 ± 55 mg/L/day which is higher than 0.1 mM of EDTA and 100 mM of NaCl by 11% and 93%, respectively. $MgCl_2$ effect on MDH activity have not been elucidated yet, while, it was reported that Mg^+ ions support methanotrophs cellular growth (Duan et al., 2011). In contrast, EDTA was reported to negatively affect MMO activity (Ge et al., 2014). Supportively, Mardina et al. stated that EDTA have higher inhibitory effect on MDH

activity but higher methanol can be accumulated using $MgCl_2$ because it has lower inhibitory effect on the MMO (Mardina et al., 2016). Collectively, the combination of 50 mM phosphate buffer and 10 mM $MgCl_2$ is the optimum MDH inhibitor for methanol accumulation.

6.3.2 Formate Addition Effect on Methane Bio-Hydroxylation

As illustrated previously, continuous supply of reducing power is crucial for methanotrophs to simultaneous methane uptake and methanol accumulation. Either formaldehyde or formate can be externally added to be oxidized and provide the cells with the needed electrons. Owing to formaldehyde toxicity, formate is commonly used as alternative electron source. Ideally, the oxidation of one mole of formate into carbon dioxide produce one mole of reducing equivalent (i.e., NAD(P)H). Whereas, one mole of reducing power is needed for the uptake of one mole methane and to produce one mole of methanol. Hence, the sufficient amount of formate in the reaction medium needs to be optimized. As shown in **Figure (6-2)**, only trace amount of methanol yielded in the presence of 20 and 40 mM of formate and were equal to 50 ± 12 and 75 ± 5 mg/L after 6 hours. This concentration has increased by three folds by adding 80 mM of formate. The maximum methanol productivity was obtained at 120 mM of formate and was equal to 1109 ± 30 mg/L/day. Increasing formate concentration above 120 mM resulted in a decline in methanol concentration. At 200 mM, methanol concentration was 27% less than the concentration obtained at 120 mM. This can be elucidated that formate increase resulted in the production of more carbon dioxide. The increase of any reaction products (carbon dioxide in methanotrophs) makes the reaction not thermodynamically in favour for the microorganisms which can affect methane uptake. Supportively, methane to methanol conversion ratio increased even above 120 mM, whereas, methanol productivity declined. This observation was in association with a decline in methane uptake which confirm our hypothesis.

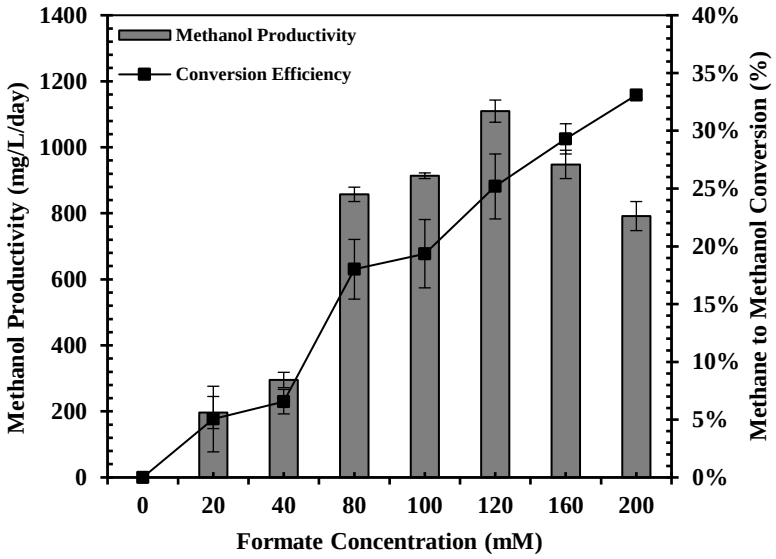

Figure (6-2): The Effect of Different Formate Concentrations on Methanol Productivity (mg/L/day) and Methane to Methanol Conversion Ratio (%).

Formate addition may not only affect methanol concentration but also the production duration. Hence, methanol concentration was monitored over 50 hours as shown from **Figure (6-3)**. After 8 hours, methane to methanol conversion efficiency was equal to 33 ± 0% and 40 ± 4% in the presence of 120 and 200 mM formate, respectively. However, methanol concentration was almost the same at both concentrations of 120 mM. those findings confirm our hypothesis about the decline of methane uptake at higher formate concentration. On the other hand, increasing formate concentration from 80 to 200 mM had a limited effect on methanol accumulation after 5 hours. Methanol concentration was equal to 277 ± 14, 281 ± 16, and 264 ± 33 mg/L at 80, 120, and 200 mM of sodium formate, respectively. At 8 hours, methanol accumulation and methane uptake have notably declined at 80 mM formate cultures.

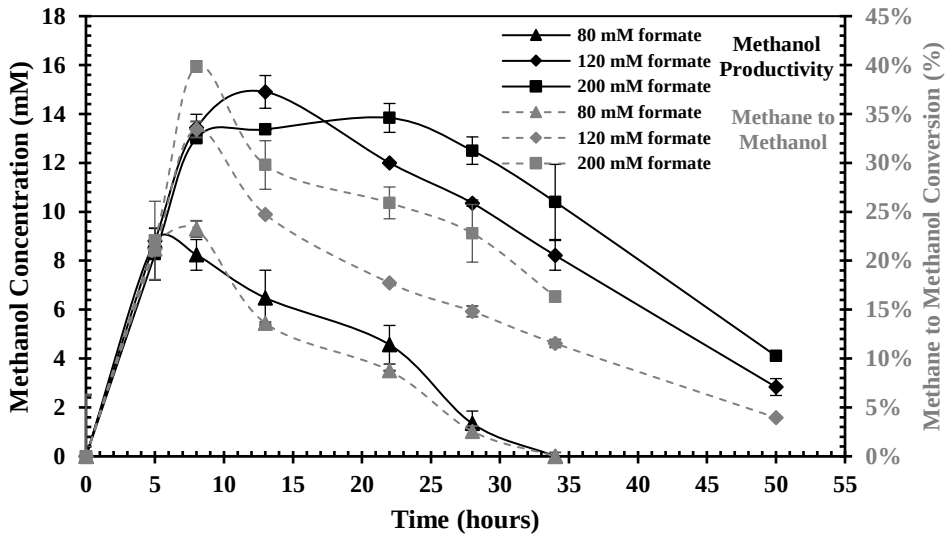

Figure (6-3): Time Course Methanol Accumulation (mM) and Methane to Methanol Conversion Ratio (%) Under Three Different Formate Concentrations.

At 120 mM of formate, the maximum methanol concentration was equal to 477 ± 21 mg/L observed after 13 hours. Whereas, the highest methanol concentration of 442 ± 18 mg/L was obtained after 22 hours in the case of addition of 200 mM formate. Thus, the optimum formate concentration to be added is 120 mM of formate but a decline is expected after 6 to 12 hours. Therefore, the addition of higher formate concentrations (or any other electron source) result in lower methanol production and methane uptake rate but would maintain higher methanol concentration for longer duration. The decline observed was expected to be due to formate depletion. The experiment was repeated while formate was re-added to the cultures after 12 hours in order to eliminate its negative effect on the production rate. However, no enhancement in methanol accumulation was observed. Therefore, the decline can be referred to the biomass inactivity rather than formate depletion. The methanol concentration observed within this experiment is 2 times higher than the maximum reported methanol concentration using mixed culture (Han et al., 2013). Moreover, it is the fourth highest methanol concentration in comparison with results obtained using pure cultures (Ge et al., 2014; Hur et al., 2016).

6.3.3 Nutrients Influence on Methane Bio-Hydroxylation

Copper presence has a regulatory effect on MMO expression. At copper concentrations above 1µM, methanotrophs express the particulate MMO known for its higher methane affinity. On the other hand, soluble MMO is expressed at copper concentrations below 1 µM (Hanson and Hanson, 1996). Copper removal from the reaction medium resulted in the lowest methanol productivity which was equal to 424 ± 25 mg/L/day, **Table (6-1)**. The addition of 5 µM to the culture resulted in 35% increase in methanol accumulation. Further increase in copper concentration resulted in slight decrease in methanol concentration. It is noteworthy that the low methanol concentrations observed throughout this experiment is due to the low biomass density employed which was equal to 0.574 ± 0.078 OD_{600}.

Table (6-1): The Influence of Different Copper Concentrations

Copper Concentration (µM)	Initial TSS (mg/L)	Methanol Concentration (mg/L)	Methanol Productivity (mg/L/day)	Conversion ratio (%)
0.00	268.10±31.90	106.03±26.43	424.14±25.82	20±2
5.00	271.43±20.00	146.29±9.18	585.15±36.72	21±3
10.00	267.86±9.29	137.11±0.00	548.43±0.00	25±1
20.00	265.48±7.86	139.42±2.31	557.67±9.24	26±6
40.00	307.14±2.86	134.79±2.32	539.14±9.29	21±1

On the other hand, nutrients are crucial for microbial growth and activity (Madigan et al., 2015). Hence, its effect on methanol accumulation was investigated. The results showed that nutrients addition have significant on methanol production. Cultures with modified NMS resulted in 60% higher methanol concentration in comparison with nutrients free cultures. This resulted can be referred to their effect on the cellular activity.

6.3.4 Biomass Density Influence on Methane Bio-Hydroxylation

Biomass density significantly affects the performance of methanotrophs due to its direct effect on the gaseous substrates diffusion throughout the liquid culture. The increase in biomass density from 0.5 to 3 measured as OD_{600nm} resulted in 2.26 times higher methanol accumulation, **Figure (6-4)**. After 6 hrs, cultures with optical density of 3 accumulated methanol with concentration of 365 ± 2 mg/L. Furthermore, methane to methanol conversion efficiency of 35 ± 4% was obtained at optical density of 3. However, the methanol specific productivity was declining by increasing the biomass density. Methanol specific productivity declined from 2.90 ± 0.10 to 1.07 ± 0.11 $g_{methanol}/g_{DCW}$/day in association with the biomass density increase from 0.5 to 3 OD_{600}.

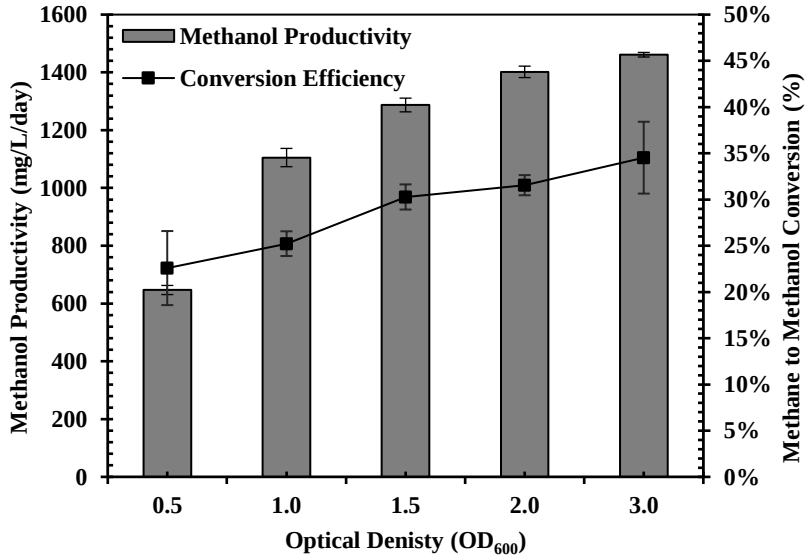

Figure (6-4): The Effect of Biomass Density on Methanol Productivity (mg/L/day) and Methane to Methanol Conversion Ratio (%)

The I/M and FO/M ratios declined by increasing the biomass density. Hence, cultures with optical density of 3 were employed while adding higher formate concentration (180 mM) in one culture to increase the FO/M ratio and another culture with 75 mM of phosphate buffer and 15 mM $MgCl_2$ to increase the I/M ratio. the results showed that increasing inhibitors concentration

does not enhance methanol accumulation at higher biomass densities. Whereas, increasing the FO/M resulted in 15% higher methanol accumulation. The methanol productivity obtained was equal to 1520 ± 32 mg/L/day which is 8% higher than productivity achieved at optical density of 2.

6.3.5 Gaseous Headspace Optimization

Oxygen is not only utilized as electron acceptor, but also, in methane hydroxylation step. Therefore, the optimization of methane to oxygen ratio is needed for methanol accumulation enhancement. As shown in **Table (6-2)**, increasing oxygen concentration does not result in enhancement in methanol accumulation or methane uptake. On the other hand, higher methanol concentrations are associated with the increase in methane to oxygen ratio. The optimum methane to oxygen was 4:1 in which 331 ± 4 mg/L of methanol was accumulated after 6 hours. However, lower methane to methanol conversion efficiency was observed at methane to oxygen of 4:1.

On the other hand, it was recently reported that addition of hydrogen to the headspace by 10 to 20% may enhance methanol production (Patel et al., 2017, 2016b). Even though, the enhancement mechanism has not been reported yet, it can be hypothesized that to the microorganisms have utilized the hydrogen as reducing power for NAD(P)H generation. However, the addition of hydrogen gas by 10 and 20% resulted in a decline in methanol accumulation by 13 and 25%. Whereas, the methane to methanol conversion ratio was slightly increased to 45 ± 4% by adding 20% of hydrogen to headspace.

Table (6-2): Optimization of Gaseous Headspace Composition

	Initial TSS (mg/L)	Methane Consumed (mg)	Methanol Conc. (mg/L)	Methanol Productivity (mg/L/day)	Conv. ratio (%)
1:1	611.90±21.90	18.96±0.12	307.93±9.89	1231.73±39.54	41±0%
1:2	586.19±6.19	15.36±0.88	205.94±2.37	823.78±9.48	34±2%
1:3	575.71±0.00	10.86±0.85	139.58±7.11	558.31±28.43	32±4%
1:4	564.76±26.19	10.82±2.02	137.21±4.74	548.83±18.96	33±5%
1:1	564.29±0.00	18.83±0.19	308.66±6.21	1234.64±24.83	41±0%
2:1	624.29±0.00	22.23±0.55	316.91±10.31	1267.63±41.24	36±0%
4:1	601.43±0.00	29.35±5.06	331.32±4.10	1325.27±16.39	29±5%
H_2-10%	670.24±5.48	14.50±0.29	230.59±8.62	922.38±34.48	40±1%
H_2-20%	635.95±52.62	13.89±0.42	247.72±12.80	990.89±51.21	45±4%

6.3.6 Time Course Methanol Production under the Optimized Condition

By the end of the study, the optimized conditions were applied to one culture. The initial optical density was equal to 3.066 ± 0.050 OD_{600}. Methane and oxygen were added to the headspace with the ratio of 4:1. Whereas, formate was added with the concentration of 180 mM and 50 mM of phosphate buffer and 10 mM of $MgCl_2$ were added as MDH inhibitors. Moreover, NMS with 5 µM of copper were added to the reaction medium. As shown in **Figure (6-5)**, a methanol productivity of 2.11 ± 0.81 g/L/day were obtained after 3 hours' operation. Furthermore, methanol concentration of 485.08 ± 21.43 mg/L was achieved after 8 hours which the highest methanol concentration observed throughout this study. Whereas, methane to methanol conversion ratio of 38 ± 1% was achieved. Thereafter, a drop in methanol concentration was observed due to the reducing power depletion.

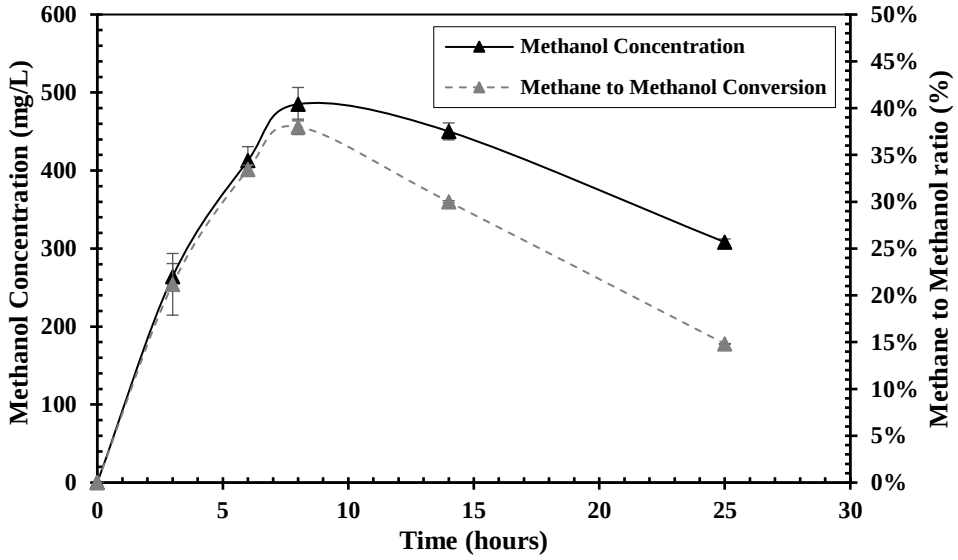

Figure (6-5): Time Course Methanol Accumulation (mM) and Methane to Methanol Conversion Ratio (%) under the Optimized Conditions.

This methanol productivity is the third highest methanol productivity in comparison with cultures obtained using pure cultures (Hur et al., 2016; Mehta et al., 1991) and higher than the previously obtained in mixed cultures by 2.2 times (Han et al., 2013). The resulted methane to methanol conversion efficiency was equal to 35% which needs to be furtherly enhanced. however, it is comparable to the values reported in the literature which range from 25% to 80% (Ge et al., 2014; Sheets et al., 2016).

6.4 Conclusion

A mixed culture dominated with type I methanotrophic bacterial was successfully enriched from waste activated sludge. Furthermore, the enriched cultures have efficiently converted methane into methanol. To the best of our knowledge, this study is the first investigate and report biological methanol production using mixed culture enriched from waste streams. Throughout this study, the reaction medium composition as well as the gaseous headspace were successfully optimized. After the optimization of multiple factors, a methanol productivity of 2115 ± 81

mg/L/day was obtained which is the third highest reported methanol productivity in comparison with pure culture results. Furthermore, methanol concentration of 485 ± 21 mg/L was achieved throughout this study. This concentration is higher than the reported methanol concentration obtained using mixed culture by two folds. These achieved results are a pivotal milestone on the way of developing an efficient bioprocess that not only mitigate greenhouse gases such as methane but also recover resources from WWTPs like methanol.

Chapter 7
Conclusion and Future Work

7.1 Conclusion

Methanotrophs have recently attracted the attention not only to mitigate the methane as GHG, but also, to be employed in recovering value added products from methane. Furthermore, it was demonstrated that methanol is an efficient and sustainable substitute for biomethane produced within WWTPs. The methane bio-hydroxylation system would consist of two main bioreactors; biocatalysts (type I methanotrophs) cultivation reactor and methanol production reactor. Throughout the thesis, multiple operational parameters that are affecting methanotrophs cultivation have been optimized.

Two different perspectives can be adopted to assess methanotrophs behavior according to the targeted bio-application. The first perspective is based on methane mitigation as the target in which methane uptake and removal ratio is the decisive parameter. The other one is targeting methanotrophs cultivation as a first step for further biotechnological application. From the latter prospective, the growth rate and growth yield are more critical because the target is to have higher growth with the minimum methane uptake and the lowest possible retention time to achieve less pumping requirement while maintaining the targeted SRT.

In **chapter 3**, it was concluded that the optimum nitrogen and copper concentrations for type I methanotrophs is 40 mM in the form of sodium nitrate and 20 µM of copper sulfate. Moreover, the addition molar ratio of methane to oxygen was found to be more feasible at 1:1 ratio due to the higher methane loading and lower aeration requirements. Additionally, the maximum overall nitrate uptake rate was obtained at cell density of 0.91 ± 0.01 OD_{600} and was equal to 3.51 ± 0.07 mgN-NO_3/L/hr. Therefore, it was determined that HRT up to 128 hours can be applied to Type I methanotrophs with limited effect on the bacterial growth.

In **chapter 4**, the decline in microbial activity at higher cell densities was investigated. In contrast to the literature, it was concluded that the microbial is affected mainly by the decline in the F/M ratio associated with the biomass density increase. Whereas, the biomass density has lower effect on the microbial growth. Furthermore, it was confirmed that both N/M and C/N ratios have no effect on the microbial activity of type I methanotrophs. Therefore, better microbial activity is achievable at high growth rate by increasing the methane loading rate. Those findings promote the potential of methanotrophic-based biotechnological applications.

In **chapter 5**, the kinetic parameters for type I methanotrophs mixed culture show a notable enhancement in microbial activity in comparison with the values reported in the literature. The attained maximum specific growth rate (μ_{max}) (0.358 hr^{-1}) and maximum specific methane biodegradation rate (q_{max}) (0.605 g-$CH_{4,\ Total}$/g-DCW/hr^{-1}) were the highest reported in mixed cultures. Whereas, the low substrate threshold concentration (S_{min}) (0.033 mg-$CH_{4,\ aq.}$/hr) attained shows that the microbial activity can be maintained at a very low methane aqueous concentrations. Furthermore, different methanotrophic design parameters such as SRT and MLR can be obtained based on the attained growth kinetics

In **chapter 6**, for the first time, methanol production using mixed culture from wastewater sludge was demonstrated. Optimization of methanol dehydrogenase inhibitors, sodium formate, and copper concentrations, as well as, the gaseous headspace composition and biomass density resulted in a significant enhancement in methanol production. The maximum methanol concentration achieved in this study was 2115 ± 81 mg/L/day. This is the third highest reported methanol productivity in comparison with pure culture results. Whereas, methanol concentration of.0.485 ± 0.021 g/L was attained which the highest reported methanol concentration from mixed culture.

7.2 Direction of Future work

The optimization of methanotrophs cultivation and methanol production processes, as well as, the determination of methanotrophs growth kinetics were an important milestone towards the development of sustainable and feasible methane bio-hydroxylation system. Multiple obstacles are still unsolved to make the system feasible. Most importantly, the external addition of expensive electron donor such as sodium formate is still problematic. Hence, the next step should be the exploration of alternative and more feasible electron donors. Thereafter, the bench-scale methane bio-hydroxylation system based on the data attained from this thesis as well as the alternative electron donors research. In this phase, multiple systems to enhance methane delivery should be evaluated. Furthermore, the suitable hydraulic regime to maximize methanol production needs to be explored.

On the other hand, it was observed in several experiments the high denitrification potential of methanotrophs type I mixed culture. This observed denitrification activity needs to be furtherly investigated. There is no confirmation about the microorganism responsible of such activity. it

might be carried out by methanotrophs or associated denitrifies. Moreover, the effect of oxygen elimination on the anoxic process needs to be studied as well. Lastly, nitrogen removal while producing methanol should be investigated. Such potential can be explored in batch mode. Thereafter, a bench scale system can be developed based on the obtained results.

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