# EVALUATION OF METHANOTROPHIC ACTIVITY AND GROWTH IN A METHANOTROPHIC-HETEROTROPHIC CO-CULTURE

by

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# ABSTRACT

Methane is a potent greenhouse gas (GHG) and accounts for 20-30% of the GHG emissions globally. In nature, methane is utilized as a sole carbon and energy source by a group of bacteria referred to as methanotrophs. Methanotrophs have been reported to have the ability to form close associations with other microorganisms such as heterotrophic bacteria in the environment. Therefore, understanding methanotrophic activity and growth in a microbial consortium with heterotrophic bacteria is of interest from an environmental and biotechnology perspective. In this study, a methanotroph; *Methylocystis sp.* NLS7 and a heterotrophic bacterium, *Pseudomonas chlororaphis,* were co-cultivated in a methane-fed bioreactor with a dialysis membrane device used to separate the species physically. It was hypothesized that the co-culture would exhibit enhanced methanotrophic activity and microbial growth of NLS7 in NLS7- *P. chlororaphis* co-culture. The methane-oxidation rate and microbial growth rate of NLS7 were evaluated as a functional response variable to the presence of *P. chlororaphis*. In addition, the effects of NLS7 growth were evaluated on the growth of *P. chlororaphis*. Our findings indicated that the presence of *P. chlororaphis* does not have any beneficial effects on *Methylocystis sp.* NLS7 activity and growth. However, the growth of *P. chlororaphis* in the co-culture with solely methane as a carbon source indicated that *P. chlororaphis* is likely gaining carbon and energy from by-products of methane oxidation by *Methylocystis sp.* NLS7 since *P. chlororaphis* could not utilize methane as a carbon and energy source. The results of this study give us an important insight into the activity and the growth of methanotrophic consortia in methane-driven ecosystem.

# CHAPTER ONE

# INTRODUCTION

## Project Rationale

Methane is one of the most important greenhouse gases and significantly contributes to global warming and climate change. The Intergovernmental Panel on Climate Change reports that methane emissions contribute 16% of GHGs emissions on a $CO_2$ equivalent basis (Hoffert 2002, Bernai 2013, Allen 2016). Methane is emitted from various sources, including coal mining, agriculture, landfills, and oil and gas operations (Semrau 2011, Haynes 2014). Methane has gained significant interest as a cheap and abundant feedstock in methane conversion technologies to value-added products (Taifan 2016). The current methane conversion technologies to value-added products such as methanol include processes with intensive energy requirements and large capital investments (Usachev 1993, Taifan 2016).

However, a group of bacteria in nature, referred to as methanotrophs, utilize methane as their sole carbon and energy source at ambient conditions (Semrau 2010, Semrau 2011). Methanotrophs play a key role in the global carbon cycle by transforming methane into methanol, formate, formaldehyde, and subsequently $CO_2$. Therefore, methanotrophs are considered key organisms due to their ability to degrade greenhouse gas methane and metabolize it into various organic compounds from an environmental and a biotechnological point of view (Singh 2019).

Methanotrophic activity and growth can be affected by various factors, including abiotic (e.g., temperature, oxygen, nutrients, and moisture) and biotic (e.g., animals, plants, protists, fungi, and bacteria) factors. Previous studies mainly focused on the effects of abiotic factors on

methanotrophic activity and growth (Ho 2013, Veraart 2015, Semrau 2018). Most microbial processes are not performed by single species but by a microbial consortium with diverse microorganisms (Wang 2019, Baas 2020). In methane-driven ecosystems, methanotrophs form the basis of the food web by oxidizing methane, and releasing by-products into the environment (Murase 2007). Heterotrophic bacteria in these environments utilize methane oxidation by-products for growth and metabolism (Murase 2007, Ho 2016). Stable isotope probing (SIP) studies revealed metabolic interactions among methanotrophic and non-methanotrophic bacteria (Hutchens 2004). The recent studies on methanotrophic-heterotrophic interactions have reported that microbial consortia, compare to their individual strains, is more efficient for biomass accumulation and catalytic functions of methane oxidation (Singh 2019) (Table 1.1). Table 1.1 summarizes the previous studies on methanotrophic activity and growth in methanotroph-heterotroph co-cultures. In a recent study, volatile-mediated interactions on methanotrophic activity and growth were evaluated in a methanotrophic-heterotrophic co-culture (Veraart 2018). In that study, Veraart *et al*. reported that the methanotrophic activity and growth of *Methylobacter luteus* were stimulated by the presence of specific heterotrophs such as *Pseudomonas mandelii.* The mixture of volatiles secreted by the heterotrophic bacterium (e.g., dimethylsulfide, dimethyldisulfide, and bicyclic sesquiterpenes) was reported as potential growth-promoting agents for the methanotroph in this consortium (Veraart 2018). In another study, the effects of density-dependent co-cultivation of *Methylocystis sp.* M6 and *Sphingopyxis sp.* NM1 was evaluated on the methanotrophic activity and growth of M6 (Jeong 2014). The presence of NM1 enhanced the methanotrophic activity and methanotrophic population density (Jeong 2014). Table 1.1

summarizes the previous studies on methanotrophic activity and growth in methanotroph-heterotroph co-cultures.

Table 1.1. Effects of heterotroph presence to methanotrophic activity and growth in methanotroph-heterotroph co-cultures. Table modified from a review paper on methanotrophic-heterotrophic interactions by Sing *et al*. (Singh 2019).

| **Methanotroph** | **Heterotroph** | **Impact of Interaction** | **Keynote** | **Reference** |
|---|---|---|---|---|
| *Methylovulum miyakonense* | *Rhizobium* sp. | Stimulation of growth. | Cobalamin (Vitamin B12) secreted by the heterotrophic bacterium was reported as a potential growth-promoting agent for the methanotroph. | (Iguchi 2011) |
| *Methylomonas sp.* | *Cupriavidus taiweninsis* | Stimulation of growth. | 3-fold increase in maximum density of the co-culture. | (Stock 2013) |
| *Methylomonas methanica* | *Rhizobium, Ochrobactrum, Pseudomonas, Escherichia coli* | Stimulation on growth. | | (Ho 2014) |
| *Methylocystis sp.* M6 | *Sphingopyxis sp.* NM1 | Stimulation on growth and methanotrophic activity. | Dialysis membrane used to inoculate the co-culture.1.34-fold increased methane oxidation and 2.4-fold increase in population density at 1:9 (M6: NM1 v/v) and in physical contact. | (Jeong 2014) |
| *Methylocystis parvus* | *Pseudomonas mandelii* | Stimulation on growth. | A complex blend of volatiles, including dimethylsulfide, dimethyldisulfide, and bicyclic sesquiterpenes secreted in the co-culture were reported as potential methanotroph growth promoting agent. | (Veraart 2018) |
| *Methylobacter luteus* | *P. mandelii* | Stimulation on growth and methanotrophic activity. | Two bicyclic sesquiterpenes production potentially by the methanotroph in this co-culture was reported. | (Veraart 2018) |

Though there is a great interest and progress in methanotrophic-heterotrophic interactions, our scientific knowledge is still limited. Therefore, this study aimed to establish a model co-culture system including a methanotrophic and a heterotrophic bacterium to evaluate the effects of heterotroph presence on methanotrophic growth and activity in a methane-based bioreactor. We hypothesized that the presence of heterotrophic bacteria would stimulate the methanotrophic activity and the microbial growth of both the methanotroph and the heterotrophic bacterium. Furthermore, the heterotrophic bacterium would utilize the by-products of methane oxidation to grow in this co-culture. A methanotroph isolated from a landfill, *Methylocystis sp.* NLS7, and a heterotrophic bacterium, *Pseudomonas chlororaphis,* were selected in this study to assemble this co-culture. The two organisms, NLS7 and *P. chlororaphis* were selected since they represented species isolated from the same environment. A fed-batch stirred tank reactor (STR) was designed for cultivation, and the gas mixture of methane and air (1:20 v/v) was supplied continuously. The methane-oxidation rate (MOR) and the specific growth rate of NLS7 were evaluated as a functional response variable to the presence of *P. chlororaphis*. In addition, the effects of NLS7 growth were evaluated on the growth of *P. chlororaphis*. This study focused on two main objectives:

(1) Evaluating methanotrophic activity and growth of only *Methylocystis sp.* NLS7 in a methane-based fed-batch STR. The methanotrophic activity and growth of only NLS7 culture were investigated based on the specific growth rate, methane-oxidation and $CO_2$-production rates (MOR and CPR), and cell-based MOR and CPR. In addition to that, the growth of *P. chlororaphis* in the filtered spent medium of NLS7 was investigated. The filtered spent medium was free from NLS7 cells and only contained the by-products of methane oxidation.

(2) Determining the co-cultivation effects of *Methylocystis sp.* NLS7 and *P. chlororaphis* on methanotrophic activity and microbial growth of both organisms. The NLS7 activity and growth were evaluated based on the specific growth rate, in addition to rates of methane oxidation and $CO_2$ production to understand the impact of *P. chlororaphis* presence on NLS7. In addition, the growth of *P. chlororaphis* on active NLS7 growth was also evaluated. The co-cultivation experiments were designed by utilizing a dialysis membrane. The dialysis membrane device was used to inoculate *P. chlororaphis* cells by physically separating the two organisms and promoting a high cell density of *P. chlororaphis*.

To test these hypotheses, *Methylocystis sp.* NLS7 was cultivated with and without the presence of *P. chlororaphis* in fed-batch STR by culturing the species in a physical and non-physical contact.

## Methane

Methane is the second-largest contributor as a greenhouse gas right after $CO_2$ and is 30 times more potent than $CO_2$ due to its higher radiative forcing (Bernai 2013, Allen 2016). Methane is emitted from both anthropogenic and natural sources. Methane emissions can be classified into three main categories: pyrogenic, thermogenic, and biogenic (Neef 2010, Monteil 2011). Pyrogenic methane is formed from the incomplete combustion of biomass, biofuels, fossil fuels, and soil carbon (13-25% of global emissions). Thermogenic methane is produced through various geological processes over millions of years. It is vented into the atmosphere from the subsurface of natural features such as terrestrial seeps, marine seeps, mud volcanoes (25-55 % of global

emission). Biogenic methane emission sources include anaerobic environments such as wetlands and rice paddies, organic waste deposits (manure, landfills, and sewage), the digestive system of ruminants and termites, and methane-generating microbes (methanogens) (55-70 % of global emission) (Neef 2010, Monteil 2011).

The primary methane sink is the oxidation of methane by hydroxyl radicals (OH) in the troposphere (90% of global sink). Additional methane oxidation sinks consist of aerobic methane-utilizing bacteria (methanotrophs) (4% of global sink), reactions of methane with chlorine and oxidation of methane with atomic oxygen in soils (3% of global sink), and reactions of chlorine radicals and sea salt in the marine boundary layer (3% of global sink). Since the industrial era, the difference between the sources and sinks is getting higher due to mainly human-related activities (Fung 1991, Zhuang 2004, Curry 2007).

## Current Strategies to Convert Methane to Value-added Products

Methane is an important energy source and is currently used as a fuel, for electricity generation, in transportation and industrial applications (Strong 2015, Gür 2016). The current technologies for the conversion of methane to higher value-added products such as methanol mainly depend on physicochemical processing. The state-of-the-art chemical conversion of methane into methanol can be classified into two main routes: direct and indirect (Horn 2015, Kondratenko 2017, Schwach 2017). The direct methane conversion route consists of reactions at 370-470 $^{o}$C and 8 MPa (de Klerk 2015) .The indirect conversion route includes the transformation of methane to syngas, a mixture of $CO_2$ and hydrogen ($H_2$), and various conversion processes of syngas to methanol at 700-1100 $^{o}$C and 1-25 bar (Hu 2021). Thus, the chemical methane conversion can be defined as a complex and energy-intensive process. Biological methods offer a

simpler and more environmentally friendly alternative to traditional physicochemical approaches and can be performed at ambient conditions (Dürre 2015, Strong 2016, Hwang 2018). Therefore, there has been a great interest in biological methane oxidation to produce value-added products and fuels (Kalyuzhnaya 2015, Jeon 2019). The research on high value-added products from methane includes various products such as methanol, biopolymers (e.g., polyhydroxyalkanoates), single-cell proteins, and ectoine (Fei 2018, Zhao 2020). The current industry on methane bioconversion technologies targets various products such as ethanol (e.g., LanzaTech, China), biopolymer (e.g., Mango Materials, USA), and single-cell protein (e.g., Calysta, USA and Unibio A/S, Denmark).

## Methanotrophs

Methanotrophs, a group of gram-negative proteobacteria, use methane as a sole carbon and energy source. The aerobic methanotrophs can be categorized into three major groups: Type I, Type II, and Type X based on formaldehyde assimilation pathways (Figure 1.1) (Hanson 1996). Type I methanotrophs utilize formaldehyde via ribulose monophosphate (RuMP) pathway, while Type II methanotrophs utilize serine cycle for formaldehyde assimilation (Bowman 1993). However, Type X methanotrophs are distinguished from other type methanotrophs by utilizing the RuMP pathway and ribulose-1,5-bisphosphate simultaneously and usually growing at high temperatures than other types (Bowman 2005). The knowledge of methanotrophic diversity has been expanded with the characterization of methanotrophs in the phylum of *Verrucomicrobia* (Dunfield 2007, Pol 2007). However, knowledge on *Verrucomicrobia*, in general, is relatively limited (Wagner 2006). Based on 16S rRNA gene sequence analyses, Type I methanotrophs grouped in gamma-proteobacteria include *Methylobacter, Methylococcus, Methylomicrobium,*

*Methylomonas, Methylocaldum, Methylohalobius, Methtyhlothermus, Methylosarcina, Methylosoma*, and *Methylosphaera.* Type II methanotrophs grouped in alpha-proteobacteria include *Methylocystis, Methylosinus Methylocella,* and *Methylocapsa* (Dedysh 2002, Dedysh 2004, Heyer 2005, Rahalkar 2007, Kalyuzhnaya 2015).

The initial step in aerobic methane metabolism is the oxidation of methane to methanol by an enzyme called methane monooxygenase (MMO) (Figure 1.1). MMO can be found in two different forms: soluble (sMMO) or particulate(pMMO) (Lee 2016). Methanol is then oxidized to formaldehyde by methanol dehydrogenase enzyme (MDH), which can be incorporated into cellular metabolites or further oxidized to formate and $CO_2$ by formaldehyde dehydrogenase (FADH) and formate dehydrogenase (FDH) enzymes respectively. The further oxidation of formaldehyde to formate and $CO_2$ is used to supply energy to the cells in the form of reducing agents (Hwang 2018).

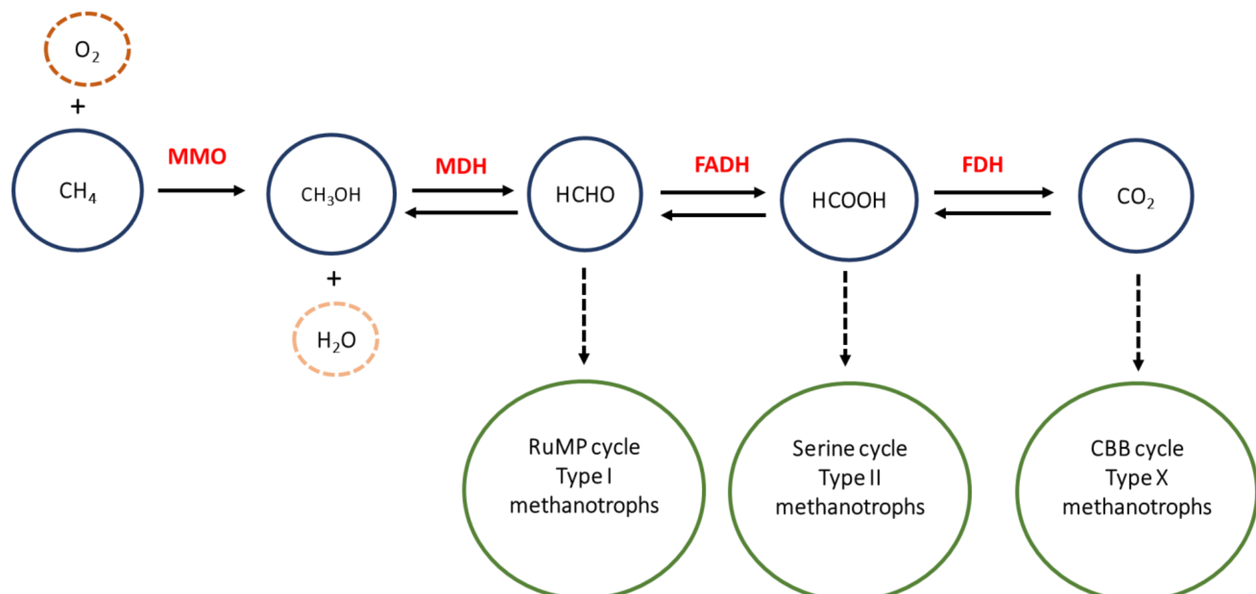


Figure 1.1. Pathway of methane oxidation and carbon assimilation in methanotrophs. MMO (methane monooxygenase), MDH (methanol dehydrogenase), FADH (formaldehyde dehydrogenase), and FDH (formate dehydrogenase).

Interactions of Methanotrophs with Other Bacteria

In methane-driven ecosystems, methanotrophs have been reported to have the ability to form close associations with other non-methanotrophic bacteria (e.g., heterotrophs) (Ho 2016). Previous studies utilizing SIP in methane-driven ecosystems reported that methane-derived carbon could be incorporated into non-methanotrophic bacteria when cultivated together (Hutchens 2004, Qiu 2008, Shrestha 2008). In such a context, methanotrophs can be considered as primary producers for non-methanotrophic bacteria, and methanotrophs may benefit from interactions with other bacteria in the form of essential nutrients (e.g., cobalamin) (Iguchi 2011, Stock 2013). Iguchi *et al.* (Iguchi 2011) reported that specific heterotrophs (e.g., *Rhizobium sp. Sinorhizobium sp., Mesorhizobium sp., Xanthobacter sp.,* and *Flavobacterium sp.*) supply essential nutrients such as cobalamin to methanotrophs when they are co-cultivated. In that study, they inoculated *Methylovulum miyakonense* HT12 with nine heterotrophic bacteria. Cobalamin (Vitamin $B_{12}$) was identified as a growth-promoting factor for the growth of an alpha-proteobacteria, *Methylovulum miyakonense*, when co-cultivated with *Rhizobium sp.* (Iguchi 2011). To best our knowledge, NLS7 and *P. chlororaphis* have not reported with their ability to produce cobalamin yet. In addition, in our study with NLS7 and *P. chlororaphis,* the liquid media supplemented with Vitamin $B_{12}$ prior to inoculation therefore the potential exchange of Vitamin $B_{12}$ during co-culturing was eliminated. A previous study with co-culture of NLS7 and *P. chlororaphis* investigated methanotrophic activity and growth in the lack of Vitamin $B_{12}$ and any exchange potential of Vitamin $B_{12}$ was not reported (Moon 2020). In a broader study, Stock *et al*. (Stock 2013) incubated nine methanotrophic strains with 25 different heterotrophs, total of 225 pairwise co-culture. Four-fold increase in maximum density of *Methylomonas sp.* M5 with the presence of *Cupriavidus taiwanensis* was

reported. In the same study, the cultivation of *Pseudomonas aeruginosa* with *Methylobacterium radiotolerans* inhibited methanotrophic growth. That indicated a strain-specific interaction between methanotrophs and heterotrophs (Stock 2013).

In contrast to these findings, Ho *et al.* (Ho 2014) reported that heterotrophic richness increases the methanotrophic growth and activity rather than the single species present in the culture medium. In that study, they assembled artificial microbial communities using *Methylomonas methanica* as a model methanotroph and ten different heterotrophs (Ho 2014) and reported that the heterotrophic richness stimulates the methanotrophic activity and growth rather than the strain-specific interactions.

Several studies reported the use of methanotrophic consortium and mixed cultures of methanotrophs in value-added product development. For example, recent studies focused on biopolymer accumulation utilizing methanotrophic microbial consortia or mixed cultures in methane-based bioprocesses (Helm 2006, Helm 2008, Zúñiga 2011, Rahnama 2012, Chidambarampadmavathy 2015, Fergala 2018, Zhang 2018). Therefore, the current knowledge of methanotroph-heterotroph interactions is still limited and needs further research to develop effective and sustainable strategies targeting climate change and global warming potential as well as the use of methanotrophs and methane in industrial applications.

In this study, methanotrophic activity and growth in a methanotrophic (*Methylocystis sp.*)-heterotrophic (*Pseudomonas chlororaphis*) co-culture were evaluated in a methane-based bioreactor. Moreover, the possible impact of NLS7 active growth on *P. chlororaphis* was investigated. The two organisms, NLS7 and *P. chlororaphis* were selected since they represented species isolated from the same environment. A gas-phase fed-batch stirred tank reactor (STR) was

designed to cultivate this co-culture; a dialysis membrane device was further used to assess whether the interaction depends on the direct contact. The methanotrophic activity and growth were evaluated based on the specific growth rate, methane-oxidation, and $CO_2$-production rates. The heterotrophic growth was also evaluated based on the cell count results of *P. chlororaphis*.

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# CHAPTER TWO

## METHANE-BASED BIOREACTOR CONFIGURATIONS IN VALUE-ADDED PRODUCT DEVELOPMENT: A REVIEW

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## Abstract

Methane is the second most significant greenhouse gas after $CO_2$, with a global warming potential 25 times higher than $CO_2$ due to its higher radiative forcing. Methane is released from various human-made and natural sources, mainly wetlands, sediments, fossil fuel burning, and livestock farming. Methane is also considered an alternative raw material for producing diverse higher value-added products due to its thermodynamic properties, high abundance in nature, and low commodity price. From a chemical engineering perspective, methane plays a significant role in conversion technologies. The chemical methane conversion technologies require intensive energy input and complex physicochemical processing due to high temperature and pressure requirements. However, in nature, a group of bacteria called methanotrophs can utilize methane as a sole carbon and energy source at ambient conditions. Due to their potential utility in methane conversion, methanotrophs have gained significant interest in biotechnological research and development. Previous studies have reported producing various value-added products by methanotrophs such as methanol, biopolymers, single-cell protein, and ectoine. In those studies, various bioreactor configurations and operational parameters have been reported with different yield and methane conversion efficiencies. Here, we review the existing literature on methanotrophic production of the aforementioned value-added products. The various bioreactor configurations and operational parameters were evaluated on reported values for yield and methane conversion efficiency. Studies reported higher yield of ectoine and biopolymer in a continuous stirred tank reactor at high $CH_4$ concentrations. At the low $CH_4$ concentrations, pneumatically agitated bioreactors such as bubble-column reactor and airlift reactor are the choices for higher yields of single-cell protein and methanol production. This review will give an important insight into methane-based bioreactor configuration to produce value-added products by utilizing methane as a feedstock.

# Introduction

## Methane and Current Technologies to Convert Methane

Methane, a potent greenhouse gas (GHG) (Strong 2015, Cantera 2018), is emitted from both natural and anthropogenic sources (Bousquet 2006, Strong 2015) and is responsible for about 25% of global warming (Cantera 2018). Natural sources include wetlands, rivers, lakes, permafrost, terrestrial and marine geological sources, oceans, estuaries, gas hydrates, wildfires, vegetation, terrestrial arthropods, and wild animals (40 % of total global emission, 208 Tg CH4/year) (Bousquet 2006, Kinnon 2018). Primary anthropogenic sources account for most global methane emissions (60 % of total global emission, 566 Tg CH4/year), including burning fossil fuels, livestock farming, landfilling, and biomass burning (Bousquet 2006). Since the industrial revolution, methane emissions from those activities have increased significantly (Strong 2015).

The combustion of methane is highly exothermic, energy and heat are released (Eq.1). This favorable thermodynamic propriety makes methane a good candidate as a fuel alternative. Methane is currently utilized to generate electricity in gas turbines and steam boilers in addition to domestic heating and cooking as a main component of natural gas (Mac Kinnon 2018). Although methane could be considered a potential transportation fuel due to its favorable thermodynamic properties (e.g., high calorific value) (Eq. 1), its high volatility does not allow it to be utilized within the current infrastructure of liquid transportation fuels. However, there is a significant interest in converting methane into liquid transportation fuels that benefit from current transportation infrastructure and existing engine design (Conrado 2014).

$$CH_{4(g)} + 2O_{2(g)} \rightarrow CO_{2(g)} + 2H_2O_{(l)} \quad \text{(55.7 kJ per g of } CH_4 \text{ at 1 atm and } 15^{o}C) \quad \text{(Eq.1)}$$

The rapid increase of global methane production and capture from both engineered processes and natural environments has enhanced its accessibility on the global market and reduced the commodity price of natural gas and methane-rich biogas (Fei 2014). This has triggered a significant interest in utilizing methane as a carbon source to produce value-added products. However, the current processes for thermochemical conversion of methane to value-added products (e.g., methanol and syngas) perform at high temperatures and pressures (370-450 $^{o}$C and 8 MPa) in addition to large capital investments exceeding one billion USD (Vosloo 2001, Wood 2012).

This review will explore a biological alternative to convert methane to value-added products utilizing methane-oxidizing bacteria. Evaluation of the literature will focus on bacterial strain selection, bioreactor setup, and configuration in addition to operational conditions. Previous reviews on biotechnological applications of methanotrophs have covered the various aspects of methane conversion, such as the biological conversion of methane to potential products (Strong 2015, Lee 2016), mostly on methanol and biodiesel (Park 2013, Fei 2014, Ge 2014), and a few of them focused on process development. For example, Fei *et al.*(Fei 2014) reviewed the existing literature on available bioprocess technologies using methane as a potential carbon source, including cultivation conditions, fermentation modes, bioreactor design, and microbial lipid extraction and upgrading (Fei 2014). Strong *et al.* (Strong 2015) also reviewed a scenario for a methanotroph-based biorefinery to generate multiple value-added products from a single fermentation process. This review will focus on two major topic in methane bioconversion technologies (1) available high-value added pordcuts from methane by methanotrophs and (2) common bioreactor configurations. The high value added products including methanol,

biopolymers (polyhydroxyalkanotes), ectoine, and single-cell protein will be introduced.The various types of bioreactors such as stirred-tank reactor, biotrickling filter, bubble column reactor, airlift bioreactor with internal loop, airlift bioreactor with external loop, membrane aerated biofilm bioreactor will be explained in detail based on their use in methane conversion technologies for the production of these aforementioned bioproducts. The production of value-added products from methane will be discussed critically based on methanotropic strain selection, operatinal parameters, methane concentration and yield.

Methane-utilizing Bacteria: Methanotrophs

In nature, a group of bacteria called methanotrophs utilize methane as a sole carbon source and electron donor while utilizing oxygen as an electron acceptor. Methanotrophs were first discovered by Söhngen *et al*. (Söhngen 1906) in 1906. In 1970, Whittenbury *et al.*(Whittenbury 1970) isolated and characterized over 100 novel methane-oxidizing bacterial strains (Whittenbury 1970). Since then, methanotrophic bacteria have been isolated mostly from sewage, wetlands, lake basins, bogs, and ruminants (Hanson1996).

Methanotrophs are classified into two major phylogenetic groups, gamma-proteobacteria (Type I and Type X) and alpha-proteobacteria (Type II), based on their formaldehyde assimilation pathways (Whittenbury 1970, Bowman 1993, Gilbert 2000). Gamma-proteobacteria (e.g., *Methylococcus, Methylomonas, Methylosphaera, Methylosoma, Methylomicrobium, Methylothermus, Methylohalobius, Methylosarcina,* and *Methylobacter*) utilize the ribulose monophosphate (RuMP) cycle, whereas alpha-proteobacteria (e.g., *Methylosinus, Methylocapsa, Methylocella,* and *Methylocystis*) utilize the serine cycle to assimilate formaldehyde (Whittenbury 1981, Bowman 1993). Recently, a novel group of thermo-acidophilic, aerobic methanotrophs

belongs to *Verrucomicrobia* phylum instead of *Proteobacteria* (can grow at temperatures 55-60 $^{o}$C and pH 1- 4), capable of assimilating formaldehyde with the Calvin Benson Bassham (CBB) cycle have been characterized (Dunfield 2007, Pol 2007, Khadem 2012). Methanotrophic bacteria convert methane to methanol with methane monooxygenase (MMO), then to formaldehyde with methanol dehydrogenase (MDH), and further to formate and carbon-dioxide by formaldehyde and formate dehydrogenase enzymes, respectively (Buswell 1952, Hamer 1967, Anthony 1982, Anthony 1986, Hanson 1990, Dijkhuizen 1992, Jiang 2010, Fei 2014). MMO can be present within a cell as soluble (sMMO, cytoplasmic) and particulate (pMMO, membrane-bound) forms. Most methanotrophs can express pMMO in the presence of copper, while the expression of sMMO is improved in copper-limited environments (Anthony 1986, Dijkhuizen 1992, Hanson 1996, Park 2013). Soluble MMO synthesis is inhibited by higher $Cu^{+2}$ concentrations ( >4 µM) (Han, Su et al. 2008), while pMMO is efficiently expressed at 5 µM of $Cu^{+2}$ (Stanley 1983, Choi 2003). The sMMO expressed by various alpha- and gamma-proteobacteria in nature has a broader substrate range than pMMO, including ethane, propane, ethene, propene, cyclohexane, benzene, toluene, naphthalene, diethyl ether, and CO (Colby 1977, Burrows 1984, Brusseau 1990). The pMMO can oxidize terminal alkanes to 2-alcohols,1,2- epoxides and C1-C5 n-alkanes other than methane (Burrows 1984, Smith 1989, Jiang 2010, Miyaji 2011). Type I methanotrophs expressing pMMO and using the RuMP pathway exhibit higher growth rates than the Type II and X, methane-oxidation rates (Pieja 2017), and carbon capture efficiency (Bowman 2006, Kalyuzhnaya 2015, Karthikeyan 2015).

For aerobic methanotrophs, a wide range of specific growth rates ($\mu_{max}$) (0.018-0.34 $h^{-1}$) have been reported (Boiesen 1993, Rostkowski 2013, Menard 2014, Ordaz 2014, AlSayed 2018).

Most of the methanotrophs reported optimally grow at 10-35 $^{o}C$ and pH 5-8 (Eshinimaev 2004, Bowman 2006, Houghton 2019). A nitrate mineral salt (NMS) medium is preferred to cultivate methanotrophs, utilizing either nitrate or ammonium as a nitrogen source. However, ammonia and its intermediates (e.g., hydroxylamine and nitrite) can inhibit the activity of the MMO enzymes for some methanotrophs (Auman 2001). Since MMO can not only oxidize methane but also ammonia to nitrite, ammonia presence will reduce the methane oxidation rate of the methanotrophs in a concentration-dependent way (Schnell 1994). In addition, hydroxylamine and nitrite can be toxic to methanotrophic bacteria, inhibiting methane oxidation (Schnell 1994). Another important thing to note, the addition of high amounts of ammonium salts can cause an inhibitory effect on methanotrophs due to osmotic stress (Whalen 2000).

Type II methanotrophs can fix molecular nitrogen and survive under dry conditions better than Type I methanotrophs. Therefore, Type II methanotrophs do not depend on dissolved inorganic nitrogen in the environment; thus, they can utilize lower oxygen and higher methane concentrations than Type I methanotrophs (Graham 1993, Amaral 1995). Methanotrophs are also affected by $CO_2$ concentrations in the environment as they are capable of fixing $CO_2$. Type I methanotrophs can produce up to 15% of their biomass from $CO_2$, while Type II methanotrophs can integrate up to 55 % of their biomass from $CO_2$ (Auman 2001).

## Potential Value-added Bioproducts from Methane by Methanotrophs

### Methanol

Methanol ($CH_3OH$) has become a widely used feedstock in producing many value-added products such as olefins, propylene, formaldehyde, and other potential organic acids and alcohols (Zhen 2015, Strong 2016). Methanol's chemical and physical simplicity allows it to be produced

with a wide range of chemical processes (Cantera 2019). The current commercially available process to produce methanol from methane is via synthesis gas. The synthesis gas also referred as syngas is a mixture of $H_2$ and CO. The conversion of methane to methanol via syngas is a two-step process perform at high temperatures (~900 °C) and pressures (~3 MPa) (Wood 2012). This commercial production is not favorable due to intensive energy requirements, need for expensive equipment and low conversion and selectivity (Cantera 2019).

As aforementioned, methanotrophic bacteria can convert methane to methanol at ambient conditions. The initial oxidation of methane to methanol is a single-step process catalyzed by the MMO enzyme (Sheets 2016, Bjorck 2018). Methanotrophs will then further oxidize methanol to formaldehyde by the MDH enzyme (Bjorck 2018), which needs to be inhibited if methanol is the final product. Inhibitors of MDH include (EDTA>0.05 mM), cyclopropanol (>67 nM), sodium chloride (>100 mM), magnesium chloride (>5 mM), ammonium chloride (>40 mM) and high concentrations of phosphate (>40 mM) (Sheets 2016, Bjorck 2018). Carbon dioxide ($CO_2$) or sodium bicarbonate addition to the culture medium has been reported to prevent complete oxidation of methane to methanol and only allowing partial oxidation by inhibiting MDH enzyme (Bjorck 2018). The MDH enzyme needs to be inhibited in the presence of an additional carbon source(s) such as formate (15-40 mM) and formaldehyde to retain the methanotrophic activity (Pieja 2017). Xin *et al.* (Xin 2007) reported methanol concentration up to 0.004 µmol methanol/mg dry cell weight (DCW) by *Methylosinus trichosporium* IMV 3011 with $CO_2$ addition in batch flasks at 30 °C (Xin, Zhang et al. 2007). Some studies have reported increased methanol concentration by encapsulating the cells into alginate and silica-gel (Patel 2018) as well as immobilizing them in polyvinyl alcohol (Patel 2020) and chemically modified chitosan (Patel

2020). The pure culture of *Methylosinus trichosporium* OB3b is typically used to convert methane to methanol using phosphate, magnesium chloride, cyclopropanol, or a mixture of phosphate and EDTA as successful MDH inhibitors (Sugimori 1995, Duan 2011).Methanotrophs use in methane to methanol conversion can be an efficient and cost-effective strategy to reduce the need for the chemical processing which is performed at high temperatures and pressures.

Biopolymers: Polyhydroxyalkanoates (PHAs)

PHAs such as poly(3-hydroxybutyrate) (PHB) and copolymer PHB (PHBV) are biopolymers produced intracellularly by various microorganisms. They serve as a source of carbon and reducing agent under nutrient limiting conditions such as low O, N, P, S, Mg, Ca, and Fe and in the presence of excess carbon (Pieja 2017). PHAs are biodegradable polyesters which make them a potential alternative to petrochemical plastics (Myung 2017). Several companies in Europe and the US manufacture PHAs up to 10 kt to 300 kt annually (Chen 2015, Cantera 2018). The PHA-producing heterotrophic microorganisms in the biopolymer industry mainly include *Ralstonia eutropha*, *Bacillus megaterium,* and *Alcaligenes latus.* The processes utilize common expensive feedstocks such as glucose and fructose, comprising 30-40 % of the total production cost (Cantera 2018).

Methane is emerging as a cheap and abundant feedstock to reduce the cost of PHA production (Pieja 2017, Cantera 2018). Mango Materials and Newlight Technologies are pioneering companies utilizing methanotrophs in methane based PHB production technologies. *Methylocystis, Methylosinus,* and *Methylocella* have been considered the main methanotrophic PHA producing genera under nutrient limiting conditions (N, P, or Mg) (Cantera 2018). Sundstorm *et al*. (Sundstrom 2015) reported increased PHB concentration from 0.69 g $L^{-1}$ up to 3.43 g $L^{-1}$ by

*Methylocystis parvus* OBBP under optimized copper (5 μM) and calcium (7.2 μM) concentrations. Optimized copper and calcium conditions also decreased the doubling time from 10.6 to 8.6 h.

Osmoprotectants: Ectoine

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid), a cyclic amino acid, and its hydroxy- derivatives (e.g., hydroxyectoine) are produced by salt-tolerant, or halotolerant, bacteria to maintain the osmotic integrity of cells at high salt concentrations (Pastor 2010). Ectoines are widely used in pharmaceutical and cosmetic industries due to their unique characteristics as stabilizers and heat protectors for enzyme(s), DNA, protein, and nucleic acids. The current retail cost of ectoine in the pharmaceutical industry is around US$1000 $kg^{-1}$ with an annual demand of 15,000 tons (Strong, Kalyuzhnaya et al. 2016). Ectoine is currently produced on a large scale by a heterotrophic halotolerant bacteria, *Halomonas elongate* (Sauer and Galinski 1998, Kunte, Lentzen et al. 2014). The large-scale production of ectoine from the halotolerant bacteria is called bacterial milking or bio-milking, which includes a long fed-batch fermentation at varying concentrations of NaCl (15-20 % (w/v)) at 25-40 $^{o}$C (depends on the desired product, e.g., ectoine, hydroxyectoine or coproduction of ectoine and hydroxyectoine) in 1,000-3,500 L fermenters. Once the maximum cell growth is achieved, bacteria are exposed to a hypo-osmotic shock (e.g., a sudden decrease from 15 % to 3 % (w/v) NaCl) to release ectoine into the culture medium. The product solution, including ectoine, is then further purified by electrodialysis, chromatography, filtration, and evaporation while the cells of *H. elongate* are recycled back to the process (Sauer 1998, Melmer 2009). The current commercial upstream process requires further improvements such as a decrease in the demand for common expensive feedstocks such as glucose, sucrose, sodium glutamate which account for 30% of the total production cost of ectoine.

Recent advances in the isolation of methane-utilizing halotolerant bacteria from high-salinity environments provide an alternative to *H. elongate* in commercial ectoine production. As an abundant and inexpensive carbon source, methane promises to replace common expensive feedstocks used in the traditional bioproduction of ectoine (e.g., glucose). Methanotrophs belonging to the *Methylomicrobium* genera have been reported as having the ability to produce ectoine intracellularly by utilizing methane as the only carbon and energy source. Khmelenina *et al.* (Khmelenina 2000) illustrated that *Methylomicrobium alcaliphilium* 20Z can accumulate intracellular ectoine up to 230 ± 20 mg ectoine g biomass $^{-1}$ in batch cultures with 9 % (w/v) NaCl and high methane concentrations in the headspaces (50 % (v/v)) (Khmelenina 2000). Further studies investigated optimized growth conditions for higher ectoine concentrations by *M. alcaliphilium* 20Z under high methane concentrations. Cantera *et al.* (Cantera 2016) reported that environmental conditions; $CH_4$ (2–20 % (v/v)), $Cu^{2+}$ (0.05–50 μM) and NaCl (0–9 % (w/v)) concentrations at 25–35 °C play a key role to increase methane-oxidation rate and ectoine synthesis (Cantera 2016).

Single-cell proteins (SCPs)

SCPs are referred to as crude or refined proteins derived from the cells of various microorganisms such as yeast, fungi, algae, and bacteria and used as animal feeds and human food supplements (Ritala 2017). The production of SCPs has gained significant interest in research and industry due to the ever-increasing human population and decreasing cultivable land because of global warming. SCPs derived from bacteria generally account for 50-80% protein on a DCW basis (Anupama 2000). The SCP production in biotechnology is dominated by algae-based SCP (Anupama 2000). However, the growth of algae is highly dependent on the landscape and the

climatic conditions. Therefore, the use of bacteria in the production of SCPs is highly preferable since the bacteria can easily grow in bioreactors and utilize a wide selection of feedstocks. Methanotrophs can be a highly advantageous alternative for SCP production since they can degrade GHG methane, which is cheap and highly abundant in nature (Anupama 2000). Methane or natural gas is currently serving as feedstock for the production of SCPs. Calysta Inc. (Menlo Park, CA, US) and UniBio A/S (Denmark) use methane or natural gas as a carbon source in their fermenters to produce animal feed. Calysta Inc. is commercially producing and distributing their methane-based protein, FeedKind ®, for various applications in the aqua-mariculture and animal feed industries. A mixed culture dominated by *Methylococcus capsulatus* Bath is used to derive the protein in the production of their patented SCP (Silverman 2015). Although the commercial applications are still limited due to the slow enzyme kinetics of methanotrophs, synthetic biology technologies (e.g., genetically engineered methanotrophs) are a step towards utilizing methane as a cheap stock for SCP production.

## Bioreactor Configurations for Bioconversion of Methane to Value-added Bioproducts

Due to the recent increased demand for the removal of GHGs from the environment, biological methane conversion has gained significant interest in various fields of biotechnology such as bioenergy, pharmaceuticals, cosmetics, bioplastics, and aquaculture (Conrado 2014). However, three main technical issues limit the commercialization of biological methane conversion and need to be considered to design an effective and efficient bioreactor and bioprocess:

1.) The slow enzyme kinetics of methanotrophs cause low volumetric productivity. Slow enzyme kinetics of methane oxidation leads to slow growth because it is the primary

metabolic and energy-generating process for these organisms. (Lee 1996, Shah 1996, Arcangeli 1999, Xing 2006, Han 2009).

2.) The gas flammability of methane limits the operational conditions of methane-based bioreactors. The lower explosive limit for $CH_4$ in the air is 4.4%, and the upper explosive limit is 16.4%; therefore, mixtures of $CH_4$ and $O_2$ can be explosive (Hoffert 2002). Due to the potential risk of any gas leakage from the system, operation above the UEL is usually avoided (Zlochower 2009, Su 2020). Interestingly, this creates a feedback to issue #1, since the lower methane concentrations limit the rate of oxidation and growth.

3.) In the bioconversion of methane by methanotrophs, $CH_4$ and $O_2$ act as electron donor and acceptor, respectively. However, the gaseous nature of $CH_4$ and $O_2$ makes the solubility of those gases extremely limited in the liquid culture medium. Therefore, the growth of methanotrophs is limited by the low gas solubility and rate of $CH_4$ and $O_2$ mass transfer into the liquid medium (Vega 1990).

Bioreactors are designed to deliver optimum conditions for microbial growth and biochemical processes to occur. The size of the reactor, configuration, and operation mode are critical components to design an efficient bioprocess. In addition, the operational parameters such as pH, temperature, the concentration of the carbon source(s), availability of electron donor(s), macro- and micro- nutrients to gain high yields, and efficiency are critical components that need to be taken into account (Zhong 2011). The choice of the bioreactor, bioprocess, and operating strategy in methane bioconversion applications needs to be justified with respect to the aforementioned technical issues. Bioreactor selection directly impacts methanotrophs' cultivation and growth,

which determines the final product yield, carbon conversion efficiency, and biomass productivity (Zhong 2011).

Operational Modes

Batch bioprocessing consists of microbial incubation in a closed system for a certain amount of time. The end product is harvested at the end of the batch time. Batch operation is usually preferred when small production (2-10 $m^3$) quantities are needed (Hessel 2012, Noorman 2018). In methane bioconversion, the headspace is filled with a methane-based gas mixture with either $O_2$ or air when aerobic methane oxidation is desired. However, in such an operational mode, the surface area between the gas and liquid phase is relatively small, limiting the effective mass transfer rate of the gas substrates.

In fed-batch processing, substrates are added either continuously or periodically to a closed-system bioreactor inoculated with microorganisms (Noorman 2018). In methane bioconversion, fed-batch processing is usually defined as the continuous feeding of methane or methane gas mixture into a batch vessel. In this operational mode, the gaseous substrate(s) are usually supplied by sparging into liquid suspension culture to prevent substrate depletion. The main drawback of the fed-batch mode is the potential loss of methane. That may present the need to have a system to capture and recycle the gas stream.

In continuous processing, the fresh culture medium is added continuously as it is removed. In gas-based systems both liquid and gas phases are continuously replenished. This operational mode is generally suitable when maintaining constant operational conditions (e.g., substrate and nutrient concentrations) is important. A significant requirement for continuous processes is that both the

inlet and outlet flow rates need to be less than the doubling time of microorganisms during the operation (Zhong 2011).

Bioreactor Configurations

In this chapter, different bioreactor configurations (Figure 2.1) including stirred-tank reactor (STR), biotrickling filter (BTF), bubble column reactor (BCR), airlift bioreactor with internal loop (internal loop position different), airlift bioreactor with external loop, membrane aerated biofilm bioreactor (MABR) and their utilization in methane bioconversion technologies were discussed.

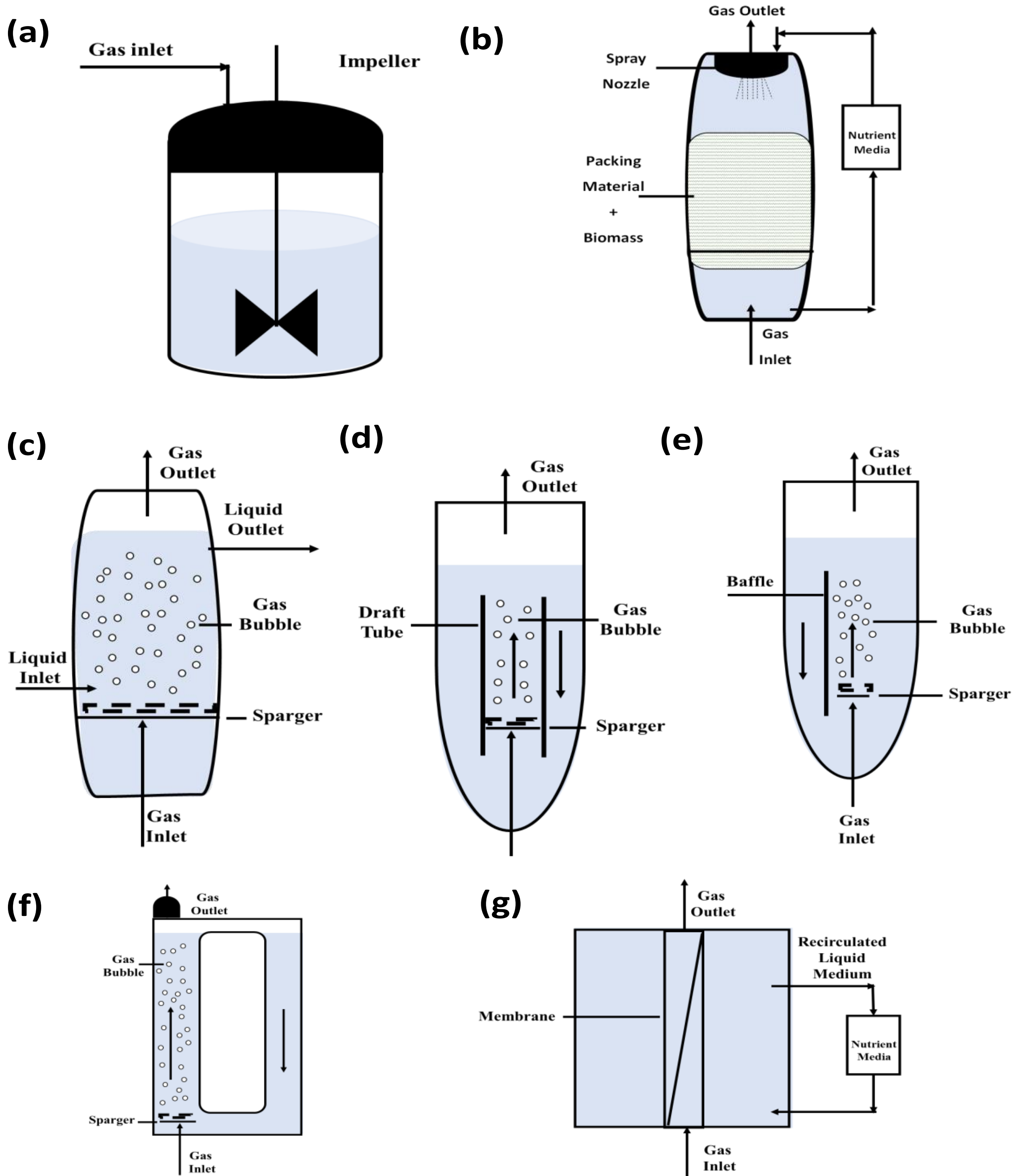


Figure 2.1. Schematic diagrams of (a) stirred-tank reactor (STR), (b) biotrickling filter (BTF), (c) bubble column reactor (BCR), (d)-(e) airlift bioreactor with internal loop (internal loop position different), (f) airlift bioreactor with external loop, (g) membrane aerated biofilm bioreactor (MABR).

<u>Stirred Tank Reactors (STRs)</u>

STRs (Figure 2.1 **a**) usually include a tank fed with culture medium, microbial inoculum, and a gas feed to supply oxygen or air for aerobic processes. STRs can be operated in batch, fed batch, and continuous modes. They are used to obtain a well-mixed environment by internal mechanical agitation among all phases. Internal mechanical agitation is usually provided by an agitator, impeller or stir blade and baffle to achieve adequate mixing in the reactor (Garcia-Ochoa 2009). It is the most conventional reactor type in bench and industrial-scale applications.

The convenience for scale-up, good fluid mixing properties, gas transfer ability, and easy compliance with good manufacturing practices (GMP) make STRs a beneficial configuration for bioprocessing applications (Burcham 2018). The main advantages of STRs consist of the efficient and uniform gas transfer due to constant mechanical mixing. However, the main limitation of STRs is that the high amount of mechanical agitation can cause shear stress on microbial cells and high energy input for large-scale operations (Zhong 2011).

STRs have been used extensively in methane bioconversion studies (Cantera 2016, Cantera 2017, Tikhomirova 2021). The batch operational mode in a STR is usually preferred due to easier operational handling. That knowledge is usually further applied for scaling up production of value-added bioproducts (Cantera 2016, Cantera 2017, Tikhomirova 2021).

In an STR, increasing the agitation rate is one of the strategies to overcome the potential inefficient gas transfer issue due to the low solubility of methane and oxygen. The stirring in an STR allows breaking down the bubbles into smaller sizes and increases the gas-liquid interfacial area which results in a more efficient gas transfer to the liquid medium. Fei *et al.* (Fei 2018) reported that the increased agitation rate (500 to 1000 rpm) resulted in a higher cell density of *M.*

*buryatense* 5GB1. In that study, thirty percent enhancement in cell density was reported compared to the values reported in the literature for 5GB1. However, the lipid concentrations were higher at the lowest agitation rate (500 rpm) (Fei 2018). This indicates that increasing the agitation rate might adversely affect cell productivity and morphology. Therefore, before changing the operational parameters the downstream effects need to be understood on a single cell basis.

The use of continuous STRs (CSTRs) can be limited due to the slow growth rates of methanotrophs. The dilution rates in CSTRs need to be less than the specific growth rates of the methanotrophic bacteria (Han 2009). The fed-batch operation in STRs allows higher cell densities and carbon conversion efficiencies by the methanotrophic bacteria (Kraakman 2011).

Mehta *et al.*(Mehta 1987) reported methanol accumulation up to 2.7 μmol $mg^{-1}$ $h^{-1}$ by *Methylosinus trichosporium* using STRs ranging from 500 to 2,000 mL with batch feeding of 1:1 (v/v) methane: air mixture. Increased cell density (above 3 mg $mL^{-1}$) did not favor methanol accumulation possibly due to the low solubility of methane and oxygen in the liquid medium (Mehta 1987). Cantera *et al*. (Cantera 2017) reported ectoine production up to 82.5 mg ectoine g $biomass^{-1}$ in a CSTR with *M. alcaliphilium* (6% NaCl and 4% $CH_4$) (Cantera 2017).

Biotrickling Filter (BTF) Reactors

BTF (Figure 2.1 **b**) reactors include a column packed with high specific surface area materials (e.g., glass rings, porous ceramics, perlite, open-pore polyurethane foam, etc.), on which biofilm formation is promoted. Biofilm is defined as aggregates of microbial cells attached to solid surfaces. The substrates in the gas phase are forced through the packed bed while the liquid media is trickled or recycled through the packing material to supply nutrients and moisture for the biofilm formation on the solid surface. One of the advantages of BTFs when the electron donors and

acceptors are in the gas phase is the large gas-liquid surface area allows high mass transfer rates and higher cell productivity (Porté 2019).

BTFs are one of the most common reactor types used in the bioremediation applications of methane, not currently preferred in bioconversion technologies. BTFs are mostly used to simulate the conditions of anthropogenic methane sources such as landfills, livestock farming, and animal husbandry (contain less than 3% methane in their gas emissions) (Estrada 2014, Stone 2017). Methanotrophs are usually embedded within biofilms on a carrier material. The liquid culture medium consists of essential nutrients that are fed to the filter usually on a per-day basis while substrate gases (usually waste gas containing methane) is continuously fed to the filter (Estrada 2014). The gas-phase substrates in a BTF are recirculated in the pore space surrounding the whole packing material as well as the biofilm. That recirculation creates an independent superficial gas velocity. The superficial gas velocity (cm $s^{-1}$) is calculated by dividing the volumetric flow rate of the gas ($cm^3$ $s^{-1}$) to the cross-sectional area of the vessel ($cm^2$) (Strübing 2017, Ullrich 2019). However, for hydrophobic gases such as methane, hydrogen, carbon dioxide, and noble gases, the aqueous medium surrounding the biofilm may prevent gas transfer and result in inefficient gas-liquid mass transport. Based on Henry`s law, the dissolved gas concentration in the aqueous phase is proportional to its partial pressure in the gas phase, thus increasing the gas pressure can overcome that issue (Muñoz 2007, Ullrich 2019).

Membrane Aerated Biofilm Reactors (MABRs)

MABRs (Figure 2.1 **g**) mainly include a gas-permeable membrane that supplies a gaseous electron donor or acceptor substrate (e.g., methane, oxygen, and hydrogen). This delivery of the substrate promotes biofilm formation on the membrane (Nerenberg 2016). The MABR technology

had gained interest mostly in wastewater treatment applications where air or oxygen is supplied through the membrane (Martin 2012). The significant interest in methane bioconversion to value-added products have promoted the use of methane based MABRs recently. The MABRs offer the advantage of supplying gas substrates across the membrane without forming any gas bubbles in the reactor, which reduces the loss of gaseous substrate in the system. The methane based MABRs have primarily been reported for the conversion of methane to methanol (Xin 2004, Duan 2011, Pen 2016). The methanol production in MABR applications reported with yields ranging from 0.1-6.8 % wt. by *Methylosinus sp.* strain on methane (20-50 % v/v) (Xin 2004, Duan 2011, Pen 2016). However, a significant disadvantage of MBRs has been fouling of the membrane caused by biofilm growth, which can decrease the mass transfer rate of the substrate over time and increase the cost of substrate supply, membrane cleaning, and replacement (Meng 2009).

Pneumatically Agitated Bioreactors

Reactors agitated by gas pressure are referred to as pneumatically agitated reactors. The pneumatically agitated bioreactors mainly include two different types (1) bubble column reactors (BCRs) (Figure 2.1 **c**) and (2) airlift bioreactors (Figure 2.1 **d**, **e** and **f**). In this type of reactor, gas is sparged from the bottom; thus, the buoyancy of the rising bubbles causes mixing. During the operation of the reactor, the flow pattern of bubbles depends on the superficial gas velocity. The bubbles rise uniformly at the gas velocities below 1-4 cm $s^{-1}$ and create a homogenous flow. At higher velocities, bubbles distribute heterogeneously at the very base of the vessel and coalesce. That back mixing and coalescence of the gas bubbles cause local differences in fluid density and circulatory (heterogenous) flow (Merchuk 1994, Stanbury 2017). The gas velocities need to be adjusted in these types of reactors for successful biological methane conversion.

BCRs consist of a cylindrical vessel (usually tower-shaped) in addition to the gas sparging system at the bottom (Kantarci 2005). However, one limitation in BCR applications is that they are prone to limitations of gas-liquid mass transfer and poor mixing of the liquid phase, primarily at high cell densities (Chisti 1989).

Kim *et al.* (Kim 2010) reported optimized conditions in a lab-scale bubble column reactor for methanol production. They reported methanol accumulation up to 0.85 mmol $h^{-1}$ (1.41 µmol $mg^{-1}$ $h^{-1}$) by *M. trichosporium* OB3b (0.6 mg dry cells $mL^{-1}$) in 3,000 mL fed-batch BCR with NMS and 1:1 (v/v) methane: air mixture. On a large scale, Wendlandt *et al*. (Wendlandt 2001) reported PHB production of 4.2 g /L in pressurized BCRs by *Methylocystis* sp. GB 25 under P-deficient non-sterile conditions (Wendlandt 2001). Rahnama *et al.* (Rahnama 2012) evaluated the performance of BCRs for PHB production by *Methylocystis hirsuta* and achieved 51.6 % g PHB/ g DCW from natural gas (Rahnama 2012). Ectoine production together with hydroxyectoine about 13 mg g $biomass^{-1}$ was reported with up to 79.4 - 94.2 mg ectoine g $biomass^{-1}$ with *M. alcaliphilum* 20Z and an enriched haloalkaliphilic consortium in bubble-column reactor under 6% NaCl and 4% $CH_4$ (Cantera 2018).

Airlift bioreactors (Figure 2.1**c**) also include the gas sparging system at the bottom but with an internal loop (draft tube) (Martínez 2013).In an airlift bioreactor, gaseous substrates are passed through a sparger ring into the bottom of a central draft tube or outside of the internal loop. Gas bubbles flow up (riser), some of the bubbles coalesce and leave from the top of the column and others circulate down (downcomer). Turbulence is usually greater in the riser section instead of the downcomer. Airlift bioreactors are advantageous in contrast to STRs in terms of gentle shear stress on the cell growth, and easy to construct since they do not have any removable parts

(Martínez 2013). However, the poor liquid circulation of airlift bioreactors made them a less attractive option for methane fermentation (Yazdian 2005).

Unibio A/S, a Danish biotechnology company, produces animal protein from natural gas with their patented U-loop technology. U-loop bioreactor is a unique design as a pneumatically agitated bioreactor which includes a low-flow(vertical-down) and up-flow(vertical-up) part in addition to a horizontal connection part from the ends of the low-flow and up-flow portions. The horizontal connection has a larger diameter than the other parts. That creates a liquid circulation in the U- part of the fermenter as well as multiple points for the gas injection that allows higher gas to the liquid contact area.

Table 2.1. Methane-based operational systems in different value-added bioproduct production by methanotrophs (wt; weight; T, temperature; D, dilution rate; V, volume; P, pressure).

| **Operational System** | **Operational Mode** | **Product** | **Organism** | **$CH_4$ % (v/v)** | **Yield (wt %)** | **Operational Conditions** | **Reference** |
|---|---|---|---|---|---|---|---|
| STR | Continuous | Ectoine | *Methylomicrobium alcaliphilum* 20Z | 4 | 7 | T=25 ºC pH=9.0<br>300-600 rpm<br>3-6 % NaCl | (Cantera, 2017) |
| | Two stage (Continuous & Batch) | PHB | *Methylocystis sp.* GB 25 | 20 | 4-51 | T=38 ºC pH=5.7<br>$D=0.17\ h^{-1}$<br>N limitation | (Wendlandt, 2001) |
| | Two stage (Continuous & Batch) | PHB | *Methylocystis sp.* GB25 (mixed culture) | 25 | 10-33 | V= 7-70 L<br>$P \leq 0{\cdot}6$ MPa<br>$D=0.17\ h^{-1}$<br>Potassium limitation | (Helm, 2008) |
| | Batch | PHB | *Methylosinus trichosporium* OB3b | 50 | 50 | T=30 ºC<br>pH=6.8-7.2<br>10 µM $Cu^{+2}$ | (Shah, 1996) |
| BCR | Continuous | Ectoine | *Methylomicrobium alcaliphilum* | 4 | 10 | T=25ºC<br>pH=9.0<br>V= 2 L<br>$Mg^{2+}$ limitation | (Cantera, 2018) |
| | Continuous (Recycled) | PHB | *Methylocystis hirsuta* | 4 | 34 | T=25ºC<br>pH=7.3± 0.2<br>V= 2.5 L<br>250 rpm<br>Time = 25 days<br>$D=0.1\ h^{-1}$<br>N limitation | (García-Pérez, 2018) |

Table 2.1 (continued). Methane-based operational systems in different value-added bioproduct production by methanotrophs (wt; weight; T, temperature; D, dilution rate; V, volume; P, pressure).

| **Operational System** | **Operational Mode** | **Product** | **Organism** | **$CH_4$ % (v/v)** | **Yield (wt %)** | **Operational Conditions** | **Reference** |
|---|---|---|---|---|---|---|---|
| BCR | Continuous | PHB | *Methylomicrobium alcaliphilum* 20ZR | 50 | - | T=25°C<br>pH=9<br>V= 2 L<br>$Mg^{2+}$ limitation | (Cantera, 2018) |
| | Continuous | SCP | *Methylomonas* (mixed culture) | *60 | 69.3 | T=32 °C<br>pH=6.8-7.2<br>V=750 mL | (Yazdian, 2005) |
| | Continuous | PHB | *Methylocystis hirsuta* | 30-33 | 14.5 | T=25°C<br>pH= 6.8<br>V= 2.5 L<br>500 rpm<br>Time = 5-8 days | (Rodríguez, 2020) |
| MBR | Fed-batch | Methanol | *Methylosinus trichosporium* OB3b | 50 | **0.092 | T= 30 °C<br>pH= 6.3<br>V= 500 mL<br>Time = 40 h | (Duan, 2011) |
| | Continuous | Methanol | *Methylosinus trichosporium* OB3b | 50 | - | T=25 ± 5 °C<br>P= 20 ± 1 kPa<br>pH= 7.0 ± 0.1<br>V=3 L<br>Time = 22-48 h | (Pen, 2016) |
| | Continuous | Methanol | *Methylosinus trichosporium* IMV 301 | 20 | **6.864 | T= 32 °C<br>P= 12 ± 1 kPa<br>V=100 mL<br>150 rpm<br>D=0.167 $h^{-1}$<br>Time = 198 h | (Xin, 2004) |

Table 2.1 (continued). Methane-based operational systems in different value-added bioproduct production by methanotrophs (wt; weight; T, temperature; D, dilution rate; V, volume; P, pressure).

| **Operational System** | **Operational Mode** | **Product** | **Organism** | **$CH_4$ % (v/v)** | **Yield (wt %)** | **Operational Conditions** | **Reference** |
|---|---|---|---|---|---|---|---|
| Airlift with external loop | Continuous | PHB | *Methylocystis hirsuta* | 45 | 51.6 | T= 30 ºC<br>pH=7.0<br>V=1 L | (Rahnama, 2012) |
| | Continuous | Methanol | Two methanotrophic strains | 50-70 | 94 | T= 28-32 ºC<br>1500 rpm<br>V=1 L<br>Time= 3:30 h | (Ghaz-Jahanian, 2018) |

*ng., natural gas; **cd., calculated

## Potential Advancements

In the design and development of methane-based bioreactors, there are two main challenges, (1) slow enzyme and growth kinetics of methanotrophs and (2) low mass transfer rate of methane that needs to be addressed to have more efficient processes and higher yields. The most popular approach to solve these aforementioned problems in industrial applications is the use of STRs with fed-batch operation mode under controlled operating conditions such as nutrient feeding, pH, temperature and dissolved oxygen concentration (Harding 2007). However, this approach is limited due to the high energy and cost requirements of intensive mechanical agitation.

Another approach is the addition of oxygen delivery vectors into the culture medium such as oil (Han 2009), hydrocarbons (Lai 2002, Galaction 2004, Galaction 2005), and perfluorocarbons (Menge 2001). Those non-toxic antifoam agents enhance oxygen transfer into the cells. For example, Han *et al*. (Han 2009) reported that 5% paraffin oil addition to *Methylosinus trichosporium* OB3b culture in 5-L methane-based fermenter significantly increased the cell density and the growth rate. In this proof of concept study, seven times higher cell densities than the control group were reported (Han 2009). Even though these aforementioned delivery vectors promote higher mass transfer rate of methane in liquid medium, the removal of these compounds are likely to increase the cost of downstream processes and the use of potent GHGs (e.g., perfluorocarbons) might not be favorable in the removal of GHG methane.

In pollutant conversion technologies, the use of biofilm reactors is a common approach to increase microbial cell density. Biofilms are defined as attached cells onto solid surfaces and can be cultivated in different types of reactors such as STRs, BTFs, packed-bed reactors (PBRs), and airlift bioreactors (Tyagi 1982, Qureshi 1988, Demirci 1997). In methane bioconversion

applications, usually, MBRs were preferred to support the growth of methanotrophic biofilms (Rishell 2004, Chen 2016, Xu 2020). However, even though MBRs offer several advantages in the mass transfer of methane due to higher surface area and increased cell densities of methanotrophs, the use in the industry is still limited due to membrane fouling (Meng 2009).

The potential use of microbial consortia has gained significant interest in the biotechnology industry. Recent studies on microbial consortia reported enhanced biomass accumulation and biocatalytic functions (Kouzuma 2015, Jiang 2017, Bhatia 2018). Recently, Iguchi *et al*. reported increased methanotrophic growth in a co-culture system with 9 different heterotrophic bacteria and only one methanotrophic strain (Iguchi 2011). In another study, Ho *et al.* previously reported an increased methane oxidation rate as a function of heterotrophic richness (Ho 2014). The use of microbial consortia is an alternative approach for methane bioconversion applications to overcome the low microbial cell density limitations.

## Conclusion

In methane bioconversion applications, the right selection of operating system and mode play a key role in the final product yield. Continuous STR is one of the most common reactor types to give a higher yield of ectoine and PHB at the high $CH_4$ concentrations. At the low $CH_4$ concentrations, pneumatically agitated bioreactors such as BCR and airlift are the choice for higher yields of SCP and methanol. The common methanotrophic reactor designs presented attempt to optimize both methane and oxygen mass transfer as well as cell growth rates. It is important to consider the requisite rates of substrate delivery and growth for a given product or application, in order to effectively design a bioreactor for methanotrophic cultivation. One other important thing to note that the abiotic factors (e.g., nutrients, oxygen, temperature, medium composition) can

greatly impact the growth rate of methanotrophs and final product yield. The configurations of the bioreactors need to be justified with the desired abiotic component selection.

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# CHAPTER THREE

# EVALUATION OF METHANOTROPHIC ACTIVITY AND GROWTH IN A METHANOTROPHIC-HETEROTROPHIC CO-CULTURE

## Contribution of Authors and Co-Authors

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## Abstract

In nature, a group of bacteria referred to as methanotrophs utilize greenhouse gas methane as a sole carbon and energy source. In methane-driven ecosystems, methanotrophs degrade methane into various organic compounds and form the basis of the food web. Previous studies revealed that methanotrophs form close associations with other bacteria, such as heterotrophic bacteria. Recent studies on microbial consortia use in bioprocessing have been reported with enhanced catalytic functions of microorganisms and increased biomass accumulations. Therefore, understanding methanotrophic interactions are crucial from an environmental and a biotechnological point of view. In this study, we hypothesized that methanotrophic cultivation with heterotrophic bacterium enhances methanotrophic activity and growth. Therefore, we established a co-culture system with a methanotroph, *Methylocystis sp*. NLS7 and a heterotrophic bacterium, *Pseudomonas chlororaphis,* in a methane-fed stirred tank reactor (STR). Methane-oxidation rate (MOR) and microbial growth rate of NLS7 were evaluated as a functional response variable to the presence of *P. chlororaphis.* In addition, the impact of NLS7 growth on *P. chlororaphis* was investigated. A methane fed STR with a dialysis membrane was designed to cultivate this microbial consortium. The dialysis membrane device was used to inoculate *P. chlororaphis* and discern the effects of physical contact on microbial growth and methanotrophic activity of NLS7. In this study, the presence of *P. chlororaphis* had any beneficial or harmful effects on methanotrophic growth and activity of NLS7 in methane fed STR. The consistent increase of *P. chlororaphis* cell concentration in the co-culture with NLS7 whether in physical or non-physical contact, indicated that *P. chlororaphis* could grow in methane fed co-culture with NLS7. These findings revealed that the relationship between NLS7 and *P. chlororaphis* is likely commensal rather than a symbiotic one. The results of this study give us an important insight into the activity and the growth of methanotrophic consortia in methane-driven ecosystems. The knowledge can be expanded into methane bioconversion technologies where high value-added product development is desired.

## Introduction

Methane ($CH_4$) is the second most significant greenhouse gas (GHG) right after $CO_2$ and is accountable for 20-30% of global warming potential (Solomon 2007). However, due to the higher ability of methane to absorb radiation, it is considered a more potent GHG than $CO_2$ (~25 times) over a 100-year horizon (Shindell 2009). Methane is emitted anaerobically from the degradation of organic matter. It is released from both anthropogenic (~60% of global methane emission) and natural sources (~40% of global methane emission) (Karakurt 2012). Anthropogenic sources mainly include burning fossil fuels and biomass, livestock farming, and landfills (EPA 2012, Karakurt 2012). Natural sources include wetlands, rivers, lakes, permafrost, terrestrial and marine geological sources (Bousquet 2006). The global methane sources and sinks are in balance at a similar magnitude; however, the strength of the total methane sources is slightly bigger than total methane sinks, which has caused steady increase in atmospheric methane concentration since the industrial era (Lelieveld 1998, Wang 2004). The major methane sinks include photochemical oxidation of methane, diffusion into the stratosphere, and microbial methane oxidation (Conrad 2009). Therefore, understanding of microbial methane oxidation is relevant to predicting the balance of the greenhouse gas methane in the environment.

In nature, methane is consumed by a group of *Proteobacteria* called methanotrophs as an only carbon and energy source. Most of the methanotroph strains isolated to this day are aerobic and play a key role in atmospheric methane mitigation (Whittenbury 1970, Hanson 1996). Methanotrophs have been reported to form close associations with other bacteria in methane-driven ecosystems, such as heterotrophs (Hršak 1998, Hršak 2000, Van Der Ha 2013). In such environments, methanotrophs can be considered primary producers. They degrade methane and

release methane-derived organic compounds into the environment and form the basis of the methane-driven food web. Previous studies reported that heterotrophs in methane-driven ecosystems can consume by-products of methanotrophs. They can utilize the methane-derived organic compounds as carbon source(s) (Hutchens 2004, Murase 2007, Qiu 2009, Dumont 2011), such as exopolymers (Hou 1979, Wilshusen 2004) and formaldehyde (Bussmann 2006). The interaction between methanotrophs and heterotrophs can be mutualistic, symbiotic or commensal, providing a stable and optimal environment for all species (Raghoebarsing 2005, Petersen 2009). The previous studies showed that methanotrophic and heterotrophic bacteria co-cultivation can benefit methanotrophic activity and growth in various ways (Iguchi 2011, Stock 2013). For example, Iguchi *et al.* (Iguchi 2011) reported that selective heterotrophs (e.g., *Rhizobium sp.*, *Mesorhizobium sp.* and *Sinorhizobium sp*.) were found to deliver essential nutrients to methanotrophs (e.g., *Methylovulum, Methyloparacoccus, Methylomonas*) when co-cultivated. In that study, the strong stimulation of methanotrophic activity and growth was reported in a co-culture with heterotrophic bacteria. The organic compound delivered by the heterotrophic bacterium was identified as cobalamin (Vitamin $B_{12}$). These methanotroph strains showed no growth or very weak growth when cobalamin was not introduced to the culture medium. In addition these methanotrophs did not produce cobalamin by themselves (Iguchi 2011). In another study by Stock *et al*. (Stock 2013), nine different methanotrophic bacteria co-cultivated with 25 heterotrophic strains as two-species consortia in total of 225 methanotrophic-heterotrophic co-cultures. A three-fold increase in maximum cell density was reported in the co-cultivation of *Methylomonas sp.* M5 with *Cupriavidus taiwanensis* LMG 19424 (Stock 2013). Ho *et al*. (Ho 2014) reported the heterotrophic richness rather than single heterotroph addition as a strong

stimulation factor for methanotrophic activity and growth (Ho 2014). Moreover, Jeong *et al.*(So-Yeon 2018) recently reported the benefits of heterotroph presence to methanotrophic activity and growth in a methanotrophic-heterotrophic co-culture assembled by utilizing a dialysis membrane tube. The dialysis membrane experiment revealed that the physical proximity between the methanotroph and heterotrophic bacterium is required for methane removal enhancement (So-Yeon 2018). Although the previous studies give us important insight into methanotrophic activity and growth in a methanotrophic consortium, more research is still needed to expand the knowledge of methanotrophic activity and growth in methane-driven ecosystems.

In this study, our main goal was to investigate the interaction between a methanotroph and a heterotrophic bacterium in a methane-based system. We hypothesized that methanotrophic growth and activity are enhanced by the presence of a heterotrophic bacterium. Therefore, a model co-culture system in a methane-based bioreactor was established to achieve this goal and to expand the current knowledge of methanotrophic activity and growth in methanotrophic-heterotrophic interactions. A methanotrophic strain, *Methylocystis sp*. NLS7 and a heterotrophic bacterium, *Pseudomonas chlororaphis,* were selected and used to assemble the co-culture. A methane-fed stirred tank reactor (STR) with a dialysis membrane was designed to cultivate this microbial consortium. The dialysis membrane device was used to inoculate *P. chlororaphis* and discern the effects of physical contact on microbial growth and methanotrophic activity of NLS7. The methane-oxidation rate (MOR), the microbial growth rate of NLS7, and MOR on a per cell basis were evaluated as a functional response variable to the presence of *P. chlororaphis.* In addition, the impact of NLS7 growth on *P. chlororaphis* was investigated.

# Materials and Methods

## Methanotroph Strain and Medium Composition

A live culture of *Methylocystis sp.* NLS7 strain was obtained from Dr. Lee Krumholz's Lab at University of Oklahoma, Norman, OK, USA. *Methylocystis sp.* NLS7 was isolated from a sediment sample from USGS (United States Geological Survey) landfill in Norman, OK, USA. Cultures were grown in sealed 160 mL serum bottles and modified nitrate mineral salts (NMS) medium, first described by Whittenbury *et al.* (Whittenbury, Phillips et al. 1970) with 2- 2.5 % (v/v) methane and ambient oxygen in the headspace. The modified NMS medium contains (per liter of distilled water): 1 g $KNO_3$, 0.27 g $KH_2PO_4$, 1 g $MgSO_4 \bullet 7H_2O$, 0.14 g $CaCl_2 \bullet 2H_2O$, and 0.23 g $Na_2HPO_4$, 0.05% (v/v) acidic trace element solution (AcTES), 0.02% (v/v) alkaline trace element solution (AlTES), and 1% vitamin solution (final pH 7) and 2 mM $NaHCO_3$. AcTES contains (per liter), 2 g $FeSO_4 \bullet 7H_2O$, 0.07 g $ZnSO_4 \bullet 7H_2O$, 0.5 g $MnCl_2 \bullet 4H_2O$, 0.12 g $CoCl_2 \bullet 6H_2O$, 0.01 g $NiCl_2 \bullet 6H_2O$, 0.5 g $CuSO_4$, 0.01 g $H_3BO_3$, 0.06 g $LaNO_3$, 0.06 g $CeNO_3$, and 100 mM HCl. AlTES contains (per liter) 0.07 g $SeO_2$, 0.05 g $Na_2NO_4$, 0.2 g $WO_4$, and 0.04 g NaOH. The vitamin solution contains (per liter) 5 mg 4-Aminobenzoic Acid, 2 mg D-Biotin, 2 mg Folic Acid, 10 mg Pyridoxine-HCl, 5 mg Riboflavin, 5 mg Thiamine-HCl x $2H_2O$, 5 mg Nicotinic Acid, 5 mg Calcium D-Pantothenate, 5 mg Thioctic Acid (α-Lipoic Acid), and 0.10 mg Vitamin $B_{12}$. Serum bottles with 50 mL liquid working volume were incubated in the dark at 150 rpm and 30 $^{o}$ C for 4-5 weeks. The methane concentration in the headspace of the bottles was measured every other day using a gas chromatograph equipped with a thermal conductivity detector (TCD) and a flame ionization detector (FID). The GC-FID was equipped with a 6-foot Haysep-D precolumn (SRI Multi-Gas 5 Chromatograph). Hydrogen gas at 20 psi, 300 $^{o}$ C, and nitrogen gas at 13 psi,

100 °C were used as external carrier gases and $O_2$ via an internal compressor. The oven of GC-FID operated at 50 °C. Headspace methane percentage was compared to the abiotic control. Bottles with no detectable methane were re-amended with 2-2.5% methane.

Heterotrophic Strain and Medium Composition

The heterotrophic bacterium strain *Pseudomonas chlororaphis* was obtained from Dr. Lee Krumholz's Lab at OU. *P. chlororaphis* was isolated from stream sediments at Honey Creek, Davis, OK, USA. Cryopreserved cells of *P. chlororaphis* were inoculated into 250-mL flasks containing 100 mL Luria-Bertani medium (Miller, Sigma-Aldrich, USA) at 30 °C and 150 rpm for 24 hours. 25 g LB broth dissolved in 1 L distilled (DI) water and autoclaved at 121 °C for 20 minutes prior to inoculation.

Fed-batch Stirred-tank Reactor (STR)

A fed-batch stirred-tank reactor (STR) was designed to evaluate the effects of co-culturing *Methylocystis sp.* NLS7 and *P. chlororaphis* on methanotrophic growth and activity. Cells were cultured in 1-L media bottles containing 750 mL NMS medium. All bottles were purged with (1:1 v/v) 5% $CH_4$ balanced with argon (95%) (American Welding & Gas, Billings, MT, USA) and air. The reactors continuously stirred at 150 rpm at room temperature (approximately 22 °C) for six days.

Experimental Design

*Methylocystis sp.* NLS7 cells were inoculated into the bulk liquid medium in all experimental groups. Methane (5% v/v) was constantly supplied to the liquid medium by bubbling. In NLS7-only culture (n=4), there was no *P. chlororaphis* addition. In NLS7-*P. chlororaphis* (in)

(n=5), *P. chlororaphis* was inoculated inside of a dialysis membrane device (Repligen, USA, Spectra/por, Ready-to-use Float-A-Lyzer G2 Dialysis Device, MWCO=8-10 kDa, 5 mL), and duplicate dialysis membrane were added to the bulk culture medium (Fig. 3.1).

In NLS7-*P. chlororaphis* (out) culture (n=5), the cells of NLS7 and *P. chlororaphis* were inoculated together into the bulk liquid medium.In addition, a control group of *P. chlororaphis* only and an abiotic control group were used to illustrate that methane and $CO_2$ changes were only due to the methanotrophic activity. In the *P. chlororaphis* only group, pure *P. chlororaphis* culture was inoculated by itself in NMS with only methane as a carbon source. The abiotic control group had no bacterial inoculation and demonstrated that the system was free from contamination.

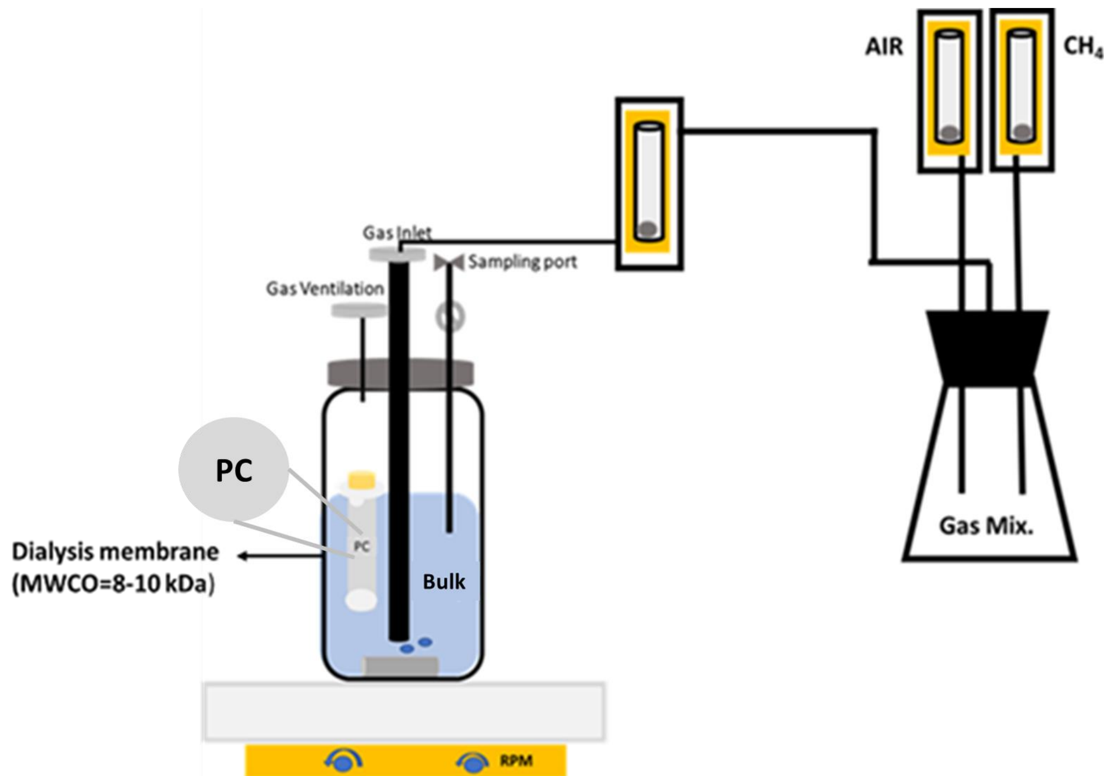


Figure 3.1. Schematic of fed-batch STR. The yellow boxes with cylinders represent air flow meters. Separate air flow meters were used to adjust the flow rates of 5% $CH_4$ balanced with argon and air. The equal volume of methane and air was mixed in an Erlenmeyer-flask. A separate airflow- meter was used to adjust the final mixed gas volumetric flow rate. The gas mixture was continuously sparged into liquid NMS medium from the gas inlet. The gas ventilation port was

used to continuously replenish the gas phase and sample the gas headspace during the experimental period. The sampling port was used to sample the bulk medium. *P. chlororaphis* **(PC)** culture was capsulated with a dialysis membrane device and inoculated as duplicates per bottle. Magnetic stirrer was used to provide continuous mixing.

Dialysis Membrane Device Preparation

To achieve maximum membrane permeability and to remove glycerin, the dialysis membrane device was pre-wetted and disinfected before the experiment. First, the membranes were soaked into 10% ethanol for 10-15 min and followed by thoroughly flushing and soaking in DI water. Afterward, the membranes were disinfected by soaking into 70% ethanol solution for 30 min and thoroughly flushing and soaking in filtered and autoclaved DI water, membranes were kept in autoclaved DI water until microbial inoculation to prevent any changes in the membrane structure.

The dialysis membrane device was inoculated with 6.5 mL of *P. chlororaphis.* Before the inoculation, 10 mL of *P. chlororaphis* grown in LB medium were centrifuged at 6000 rpm, 4 $^{o}$C for 5 min, and the supernatant was discarded. The cells were washed out with 10 mL autoclaved NMS media, the centrifugation and washing steps were repeated twice. The optical density (OD) of the culture was measured in a microplate reader at 600 nm ($OD_{600nm}$) (Synergy HT, BioTek). Two hundred-µL of the sample were placed into a well of 96-well plate. $OD_{600nm}$ of *P. chlororaphis* culture was adjusted to 0.045-0.05. The microbial suspension was loaded as 6.5 mL in a dialysis membrane device and inoculated as duplicates per fed-batch STR.

*Methylocystis sp.* NLS7 cells without cell washing step were inoculated in 750 mL bulk medium as 50 mL before the inoculation of *Methylocystis sp.* NLS7, $OD_{600nm}$ of the culture was measured with the microplate reader as described above and noted 0.1-0.12.

Cell Concentrations of *Methylocystis sp.* NLS7 and *P. chlororaphis*

Three mL of samples were taken daily from the liquid bulk medium to measure $OD_{600nm}$ over six days. A standard curve of $OD_{600nm}$ over NLS7 cell concentration (cells/mL) in addition to $OD_{600nm}$ over *P. chlororaphis* (CFU/mL) were made, and the equations from the linear fits were used to calculate cell concentrations at respective $OD_{600nm}$ measurements.

The direct cell count assay was performed to measure NLS7 cell concentration (cells/mL). The sample was serially diluted with a filter-sterilized NMS medium. One mL of serially diluted samples was filtered through a 0.22 µm black polycarbonate filter, 25 mm (Cytiva Whatman Nucleopore, USA) and stained with SYBR- gold nucleic acid gel stain (Invitrogen, ThermoFisher Scientific, USA). The filtered cells were preserved with 4 % v/v paraformaldehyde in Phosphate Buffer solution on the filters and stored at 4 $^{o}$C. The stained cells on the filter paper were counted under an epifluorescence microscope (Nikon, Eclipse E800). For statistical reliability, ten different fields of view (FOV) were examined with a maximum of 40 cells per FOV. An average number of the cell counts in the 10 different FOVs were taken and multiplied by the conversion factor to calculate the cell concentration. The conversion factor was calculated by considering the inner circle area (wetted area) on the filter paper (Area of inner circle =706.5 $mm^2$), the area of one FOV, and the dilution factor. The dimensions of the FOV were measured with a stage micrometer (Area of a FOV = 0.01 $mm^2$).

The cell concentrations of NLS7 in NLS7-only and NLS7- *P. chlororaphis* (in) cultures were calculated from $OD_{600nm}$ measurements. For NLS7 – *P. chlororaphis* (out) co-culture, NLS7, and *P. chlororaphis* were inoculated into the bulk NMS medium. Therefore, $OD_{600nm}$ readings represented the growth of NLS7 and *P. chlororaphis* together in this co-culture. Since

methanotrophic growth in LB medium is inhibited, the mixed samples were plated on LB medium, and the *P. chlororaphis* cell concentration was determined by plate counting on LB agar (e.g., viable cell counting assay). Therefore, *Methylocystis sp.* NLS7 cell concentration in this group of experiments was estimated by subtracting the viable *P. chlororaphis* cell count (CFU/mL) from the calculated total cell count (cells/mL).

The direct cell count assay results for NLS7 cell concentration were referred to as actual NLS7 cell concentration in NLS7-only, NLS7-*P. chlororaphis* (in) and NLS7- *P. chlororaphis* (out) groups (Appendix A). In this study, the actual cell counts of NLS7 were not presented due to unexplained errors in the NLS7 cell concentration in NLS7-*P. chlororaphis* (out) co-culture.

The concentration of *P. chlororaphis* in NLS7-*P. chlororaphis* co-culture was assessed via viable cell counting assays. First, the serially diluted samples were plated as 20 µL drops on LB agar (five replicates for each dilution group). Then, after 24 hours, viable colonies were counted and noted by multiplying the dilution factor to colony-forming units (CFU/ 20 µL). Finally, the CFU/20 µL results were converted to CFU /mL, multiplying them by 50. In NLS7-*P. chlororaphis* (in) co-culture, the cells of *P. chlororaphis* were inoculated inside a dialysis membrane device; the $OD_{600nm}$ readings and cell counting assays were only performed at the beginning and the end of the experiment due to the limited volume of the dialysis membrane device and to prevent contamination.

Batch Test

Batch tests were designed and performed to calculate each experimental group's methane-oxidation and carbon-dioxide production rates (MOR and CPR, respectively). After six days of continuous growth, the STRs were sealed and capped with rubber stoppers (DWK Life Sciences,

DURAN, GL 45). The gas headspaces were purged for 20 minutes with the same gas mixture used during the experimental run (1:1 (v/v) 5% $CH_4$ balanced with argon and air). The bottles were kept at the same operational conditions (room temperature and stirred at 150 rpm). The gas samples from the gas phase of the STRs were collected (1- mL) with a gas-tight syringe at 3 hours intervals for a total of 12 hours and analyzed in the GC-FID to determine MOR and CPR. The MOR (mmol $CH_4$ $h^{-1}$) and CPR (mmol $CO_2$ $h^{-1}$) were calculated from the slope of linear fit for methane and $CO_2$ mole concentrations over time. The rates were then normalized to the working volume of STRs (800 mL) (Eq. 8 and 9).

$$MOR\left(\frac{\mu mol}{L\,h}\right) = MOR\left(\frac{mmol}{h}\right) x\ \frac{1000\ \mu mol}{1\ mmol} x \frac{1}{Vliq.(L)} \quad \text{(Eq.8)}$$

$$CPR\left(\frac{\mu mol}{L\,h}\right) = CPR\left(\frac{mmol}{h}\right) x\ \frac{1000\ \mu mol}{1\ mmol} x \frac{1}{Vliq.(\,L)} \quad \text{(Eq.9)}$$

The MOR and CPR (mmol $h^{-1}$) were then normalized to the final calculated cell concentration of NLS7 (cells $mL^{-1}$) to calculate MOR and CPR on a per cell basis ($10^{-12}$ µmol cell $^{-1}$ h $^{-1}$).

Analytical Measurements

$CH_4$ and $CO_2$ concentrations were measured using GC-FID. The instrument was calibrated with standard calibrated gas tanks (Scott, Mini-Mix Portables, USA) by injecting 0.5- and 1-mL gas samples. The first standard gas tank contains 1% $CH_4$, 1% $CO_2$, 1% $O_2$, and 1% CO balanced with $N_2$. The second calibration gas tank contains 15% $CO_2$, 7% CO, 4.5% $CH_4$, and 4% $O_2$ balanced with $N_2$. Five replicates of the gas samples were measured in GC-FID. The peak area readings for each gas were recorded, and standard curves for both $CH_4$ and $CO_2$ were made.

Gas samples (1 mL) were collected from the headspace of each STR daily, the peak areas for $CH_4$ and $CO_2$ were measured and recorded. The mole percentages of $CH_4$ and $CO_2$ in the headspace were calculated from the linear fit of the standard curves. The moles of $CH_4$ and $CO_2$ in the headspaces were calculated by taking into account the ideal gas law (1 mole =22.4 L), working gas volume of the media bottles ($V_{gas}$ =350 mL), and the mole % of both gases in the headspace (Eq.3 and Eq.4).

$$CH_4\ (mmol) = \frac{\text{mmole}\ \%\ CH_4}{\text{mmole}\ \%\ total}\ x \frac{1\ mmole}{22.4\ mL}\ x\ Vgas(mL) \qquad \text{(Eq.3)}$$

$$CO_2\ (mmol) = \frac{\text{mmole}\ \%\ CO_2}{\text{mmole}\ \%\ total}\ x \frac{1\ mmole}{22.4\ mL}\ x\ Vgas(mL) \qquad \text{(Eq.4)}$$

The methane amount in the STRs was calculated by considering methane diffusion from gas to the liquid phase ($V_{liq.}$ = 800 mL) (Eq. 5 and 6). The diffusion calculations were made using Henry's Law constant (29.4 mmol $mL^{-1}$ at 25°C and 1 atm).

$$CH_4tot.(mmol) = Vgas(mL)\ x\ CH_4gas\ \left(\frac{mmol}{mL}\right) + Vliq.(mL)\ x\ CH_4liq.\left(\frac{mmol}{mL}\right) \qquad \text{(Eq. 5)}$$

$$CH_4liq.\ \left(\frac{mmol}{mL}\right) = \frac{CH_4\ gas\left(\frac{mmol}{mL}\right)}{29.4\ \frac{mmol}{mL}} \qquad \text{(Eq. 6)}$$

*P. chlororaphis* Inoculation in Filtered Spent Medium

In NLS7-only culture, *Methylocystis sp.* NLS7 was inoculated in fed-batch STR. After six days, the spent medium, including NLS7 cells, was filtered out aseptically using a 0.2 µm filter. Before the inoculation, 5 mL of *P. chlororaphis* grown in LB medium were centrifuged at 6000 rpm, 4 °C for 5 min, and the supernatant was discarded. The cells were washed out with 10 mL autoclaved NMS media, the centrifugation and washing steps were repeated twice. The *P.*

*chlororaphis* cells (0.5 mL) were inoculated in 250-mL Erlenmeyer flasks filled with 50 mL filtered spent medium.

Data Analysis for Specific Growth Rate Calculation

The specific growth rates of NLS7 in all three different experimental groups were determined from the calculated NLS7 cell counts over six days. The lag and stationary periods were removed from the data sets, and only the exponentially increasing region was observed and noted. The linear regression analysis with 95% confidence level was further applied to the natural log of the data set (exponentially growing region) to calculate the specific growth rate of NLS7 in each experimental group (Eq. 7). In Eq. 7, X, t and μ represents cell concentration, time and specific growth rate respectively.

$$\frac{dX}{dt} = \mu X \qquad \text{(Eq.7)}$$

Statistical Analysis

Mixed-effects model and Tukey pairwise comparison tests (Milton 1990) were performed to determine whether MOR, CPR, specific growth rate of NLS7 and MOR, and CPR on a per-cell basis differed among only-NLS7 culture, NLS7-*P. chlororaphis* (in) and NLS7- *P. chlororaphis* (out). Analysis of variance (ANOVA) was conducted to determine the effect of *P. chlororaphis* addition to the MOR, CPR, and growth rate of NLS7 in co-culture experiments. Tukey pairwise test was performed using Minitab Software.

## Results

### *Methylocystis sp.* NLS7 Growth

*Methylocystis sp*. NLS7 activity and growth were initially evaluated by cultivating NLS7 pure culture in the fed-batch STR for six days while continuously supplying methane (1-1.5 % v/v). $OD_{600nm}$ measurements of NLS7 from the reactor indicated consistent growth over the duration (Figure 3.2 **A**). The $OD_{600nm}$ readings were converted into calculated NLS7 cell concentration (cells/mL) from the standard curve of NLS7 cell concentration over $OD_{600nm}$ readings (Figure 3.2 **B**). The specific growth rate of NLS7 was calculated from the calculated cell counts, lag (0-24 h), and stationary (120-144 h) periods were excluded from the data set, and only the slope of the exponentially growing region (48-120 h) was used to calculate the growth rate (Figure 3.2 **B**). The specific growth rate of NLS7 in NLS7-only culture was $0.03 \pm 0.007\ h^{-1}$. Previous studies reported specific growth rates of various *Methylocystis sp.* strains ranging from 0.03 to 0.07 $h^{-1}$ on methane (5- 15% v/v) in serum bottles in which methane was fed only at the beginning of the experiment (Baani 2008, Belova 2013, Bordel 2019).

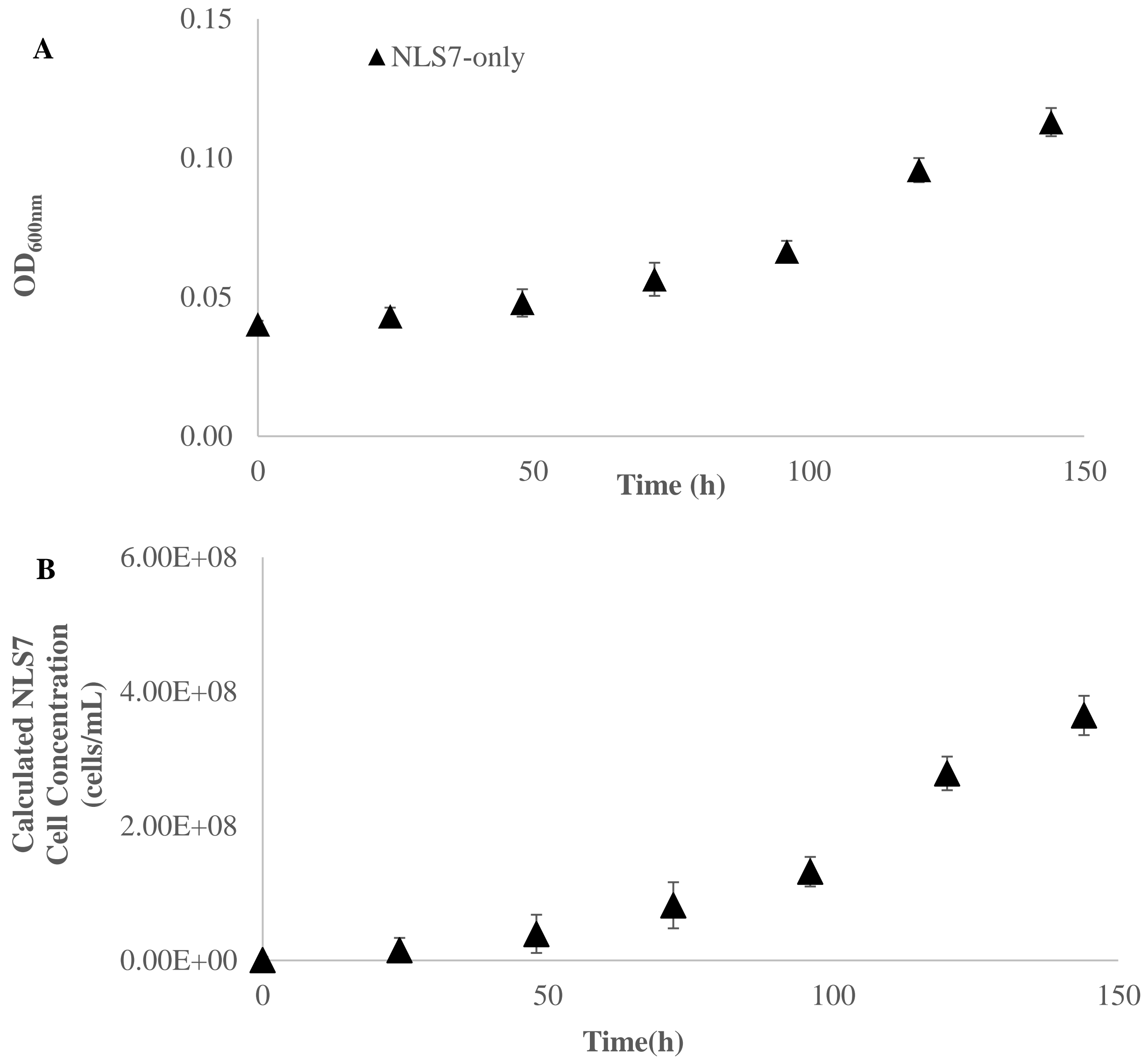


Figure 3.2. (**A**) Average $OD_{60nm}$ changes of NLS7 with standard deviations in NLS7-only culture over time. (**B**) Average calculated direct cell counts of NLS7 with standard deviations in NLS7-only culture (n=4).

Figure 3.3 **A** and **B** illustrate the $CH_4$ and $CO_2$ changes respectively in the reactors during the batch test. The changes were consistent over time and showed a linear decrease for $CH_4$ and an increase for $CO_2$. (Figure 3.3 **A** and **B**). The batch test was performed after six days of NLS7 growth on methane at 1-1.5% v/v. The *P. chlororaphis* only control group was used to show that

*P. chlororaphis* cannot oxidize methane and produce $CO_2$. The abiotic control group showed that the system was free from contamination and the changes in methane and $CO_2$ are only due to the methanotrophic activity of NLS7.

The MOR was calculated from changes in aqueous methane concentration over time, calculated from the gas phase measurements. The MOR was 3.5 ± 0.8 µmol $L^{-1}$ $h^{-1}$ in NLS7-only culture with $10^8$ per mL cell concentration. The previous studies reported methane oxidation rate of a *Methylocystis sp*. strain ranging from 0.4 to 0.8 µmol $L^{-1}$ $h^{-1}$ on methane (5-10% v/v) with up to $10^8$ cells per mL as initial cell concentrations in batch serum bottles with one initial injection (Jeong 2014, So-Yeon 2018).

The CPR was calculated from the $CO_2$ changes in the headspaces of the reactors and was 0.6 ± 0.2 µmol $L^{-1}$ $h^{-1}$. The ratio of CPR to MOR was 0.17 µmol $CO_2$/ µmol $CH_4$. That indicates methane was not only oxidized to $CO_2$ but also it incorporated into the biomass of NLS7.

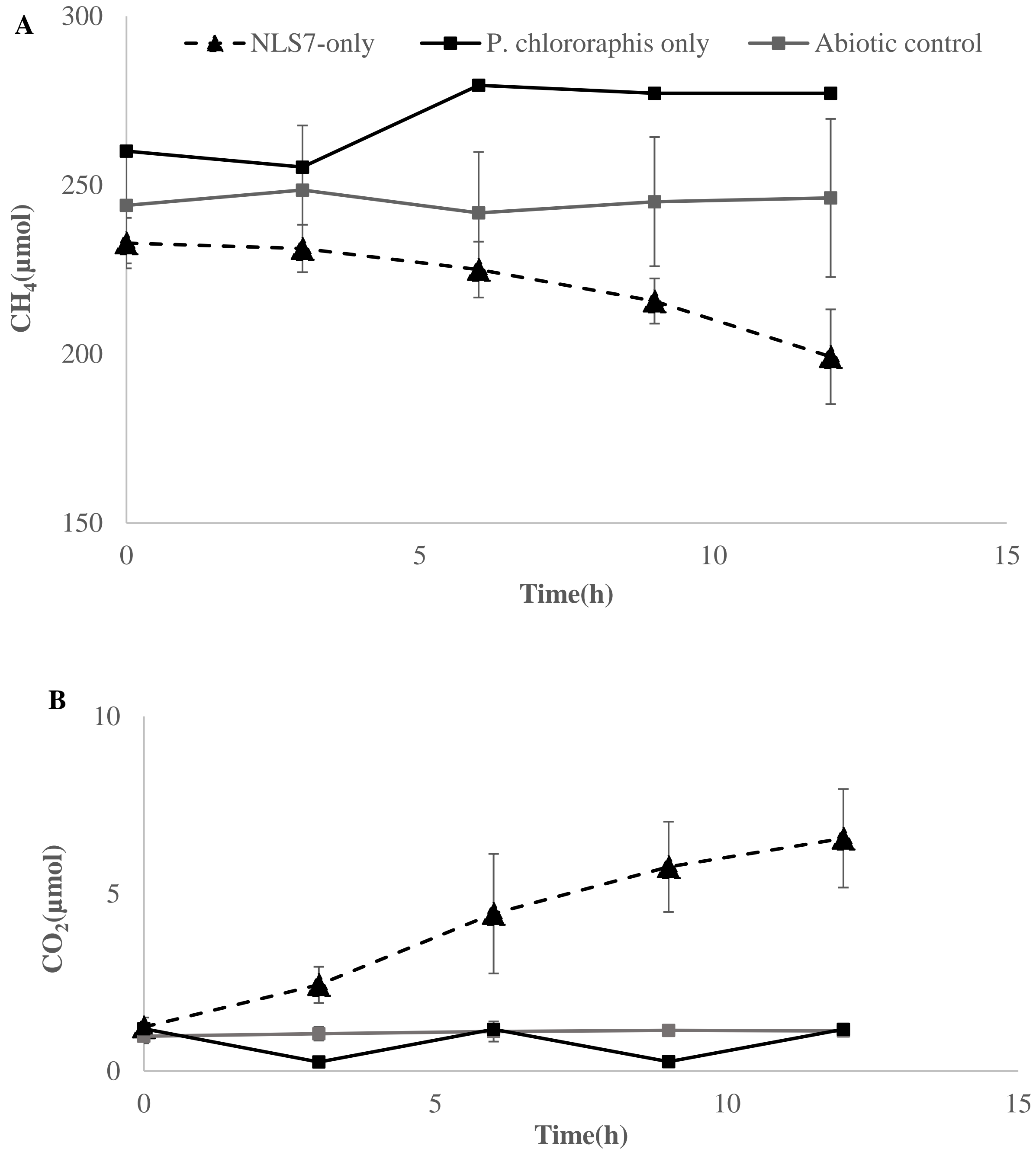


Figure 3.3. Average (**A**) $CH_4$ (μmol) and (**B**) $CO_2$ (μmol) changes with standard deviations in NLS7-only cultures in fed-batch STR (n=4 and time=12 h). The black triangles with a dashed line represent NLS7-only culture. The dark gray squares with a straight line represent abiotic control. The black squares with a straight line represent *P. chlororaphis*-only inoculation. Cultures were purged with methane gas mixture, sealed, capped, and the gas-phase changes were recorded over 12 hours with 3 hours intervals.

The methanotrophic activity on a per cell basis was calculated from the MOR (Table 3.1), and the final calculated cell concentration of NLS7 at 144 h (Figure 3.2 **B**). The MOR on a per cell basis (Table 3.2) in NLS7-only culture was $7.5 \pm 1.3 \times 10^{-12}$ µmol cell $^{-1}$ h $^{-1}$ with the final cell concentration $3.6 \pm 0.3 \times 10^{8}$ cells per mL. A previous study with *Methylocystis sp.* SC2 strain reported a MOR on a per cell basis of $0.4 \times 10^{-12}$ µmol cell $^{-1}$ h $^{-1}$ with 10-15 % v/v methane in a serum bottle with up to $10^{8}$ cells per mL (Baani 2008).

The CPR on a per cell basis (Table 3.2) was calculated from the CPR (Table 3.1) and the final calculated cell concentration of NLS7 at 144 h (Figure 3.2). The CPR on a per cell basis in NLS7-only culture was $1.6 \pm 0.5$ $10^{-12}$ µmol cell $^{-1}$ h $^{-1}$ (Table 3.2).

In summary, the specific growth rate and the MOR on a per cell basis in NLS7-only culture indicated that the methane fed STR provided suitable environment for NLS7 cells to grow.

Effect of *Methylocystis sp.* NLS7 on *P. chlororaphis* Growth

The filtered spent medium of NLS7 was used to cultivate *P. chlororaphis* to evaluate whether *P. chlororaphis* can utilize the organic compounds secreted by NLS7 and accumulated during its growth in the methane-fed STR. The filtered spent medium only consisted of the by-products of methane oxidation in NMS medium and did not contain NLS7 cells or any additional carbon source(s). Figure 3.4 shows the average $OD_{600nm}$ data of *P. chlororaphis* growth in the filtered spent medium of NLS7 for four days. The consistent increase of $OD_{600nm}$ indicated that *P. chlororaphis* could grow in the filtered spent medium of NLS7 and likely utilized the soluble metabolites formed, excreted, and accumulated by NLS7 during its growth in the fed-batch STR. The abiotic control group was used to show that there was no additional microbial growth that could affect the *P. chlororaphis* growth in the filtered spent medium. In *P. chlororaphis* in fresh

medium with methane control group, *P. chlororaphis* was cultivated in the fresh sterile NMS medium with methane in the headspace. The straight line for the $OD_{600nm}$ of *P. chlororaphis* indicated the lack of *P. chlororaphis* growth in fresh medium containing only methane as a carbon source which illustrated that *P. chlororaphis* growth was only due to the by-products of NLS7.

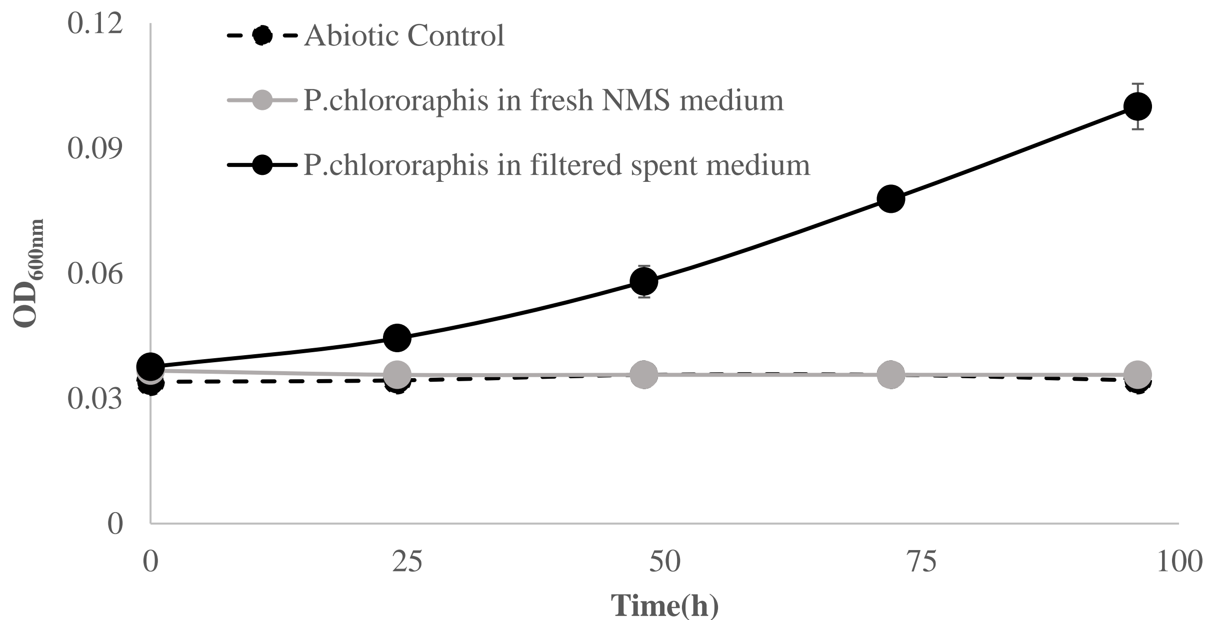


Figure 3.4. Average $OD_{600nm}$ readings of *P. chlororaphis* with standard deviations in the filtered spent medium of NLS7-only (n=3 and time=96 h). The black circles represent $OD_{600nm}$ readings of *P. chlororaphis* in the filtered spent medium. The gray circles represent *P. chlororaphis* inoculation in the abiotic filtered spent medium and the data with dotted line represent the abiotic control.

In NLS7- *P. chlororaphis* co-culture experiments, the impact of simultaneous co-cultivation of *P. chlororaphis* with NLS7 was evaluated. In NLS7- *P. chlororaphis* (in) co-culture, *P. chlororaphis* was inoculated into a dialysis membrane device. The use of dialysis membrane prevented the dilution of *P. chlororaphis* cell concentration within the bulk medium since the capacity of the dialysis membrane device was smaller by 123 times than the total bulk liquid volume (1:123 mL device/ mL bulk medium). However, the capacity of the dialysis membrane

also limited the amount of sampling. Therefore, the $OD_{600nm}$ measurement for *P. chlororaphis* was only performed at the beginning and end of the experiment (t=0 and 144 h). The cell concentration of *P. chlororaphis* was calculated from $OD_{600nm}$ measurements and the standard curve of *P. chlororaphis* cell concentration over $OD_{600nm}$ (Appendix A). In the co-culture of NLS7-*P. chlororaphis* (in), the cell concentration of *P. chlororaphis* started at $5.3 \pm 0.8 \times 10^7$ calculated CFU per mL and reached $4.1 \pm 1.2 \times 10^8$ calculated CFU per mL (n=10), a 7-fold increase in the cell density of *P. chlororaphis* indicated that *P. chlororaphis* was able to grow with NLS7 simultaneously without any direct contact. In *P. chlororaphis*-only control group, the final cell concentration of *P. chlororaphis* was similar to the initial inoculum concentration, indicating that *P. chlororaphis* does not grow on methane or in the absence of NLS7. These findings indicated that the only source for *P. chlororaphis* to grow in this co-culture was the organic compound(s) secreted by NLS7 since *P. chlororaphis* could not utilize methane as a carbon and energy source.

The cell concentration changes of *P. chlororaphis* in NLS7- *P. chlororaphis* (out) culture were shown in Figure 3.5. In the co-culture of NLS7- *P. chlororaphis* (out), *P. chlororaphis* grew when both species were inoculated into bulk NMS medium together. Since both species were inoculated and grown together in bulk NMS medium, viable cell count assay was used to quantitively separate the two species. The methanotrophic growth on LB agar is inhibited therefore the viable cell count assay only revealed the cell concentration of *P. chlororaphis* over time. Therefore, the viable cell count assay also allowed us to measure *P. chlororaphis* cell concentration in the co-culture of NLS7- *P. chlororaphis* at lower cell concentrations. The cell density of *P. chlororaphis in* this set of experiments increased up to 160-fold over the six days of cultivation. The increase in the cell concentration was consistent over the duration.

In summary, the cell concentration of *P. chlororaphis* increased whether it interacted with NLS7 through direct contact or not. That indicated co-culturing of *P. chlororaphis* with NLS7 allowed *P. chlororaphis* growth in a solely methane fed system. In the co-culture experiments, the initial cell concentration of *P. chlororaphis* to NLS7 was 1:100 calculated CFU/ calculated cell. Although total initial *P. chlororaphis* cell amount was similar in these two different experimental groups, the initial *P. chlororaphis* cell concentration in dialysis membrane experiment was 100-fold higher than the NLS7-*P. chlororaphis*(out) group due to the limited volume of the dialysis membrane. Higher cell concentration of

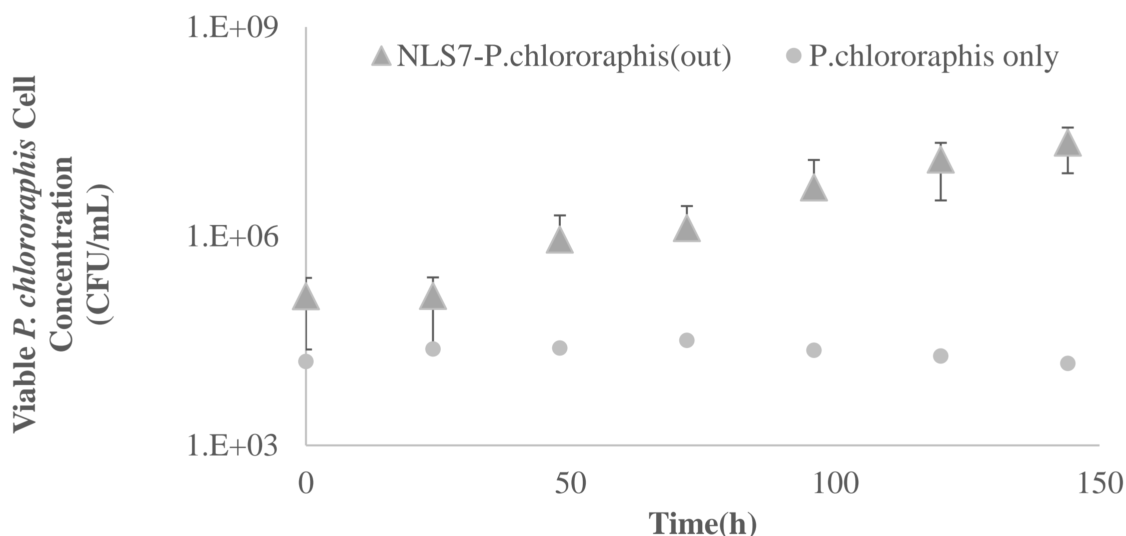


Figure 3.5. Average viable cell count data of *P. chlororaphis* with standard deviations in NLS7-*P. chlororaphis* (out) culture (n=5 and time=144h). Cultures were fed with 5% $CH_4$ balanced with argon and air as equal volume mixtures. The gray triangles represent viable *P. chlororaphis* cell count in the co-culture of NLS7 and *P. chlororaphis.* The gray circles represent only *P. chlororaphis* control group.

Co-culture of NLS7 and *P. chlororaphis*

The co-culture experiments were designed to understand the effects of *P. chlororaphis* presence on the growth and activity of NLS7. The specific growth rate, MOR, CPR, along with MOR and CPR on a per cell basis, were compared among all groups (Table 3.1 and 3.2).

The consistent increase in $OD_{600nm}$ readings indicated the active growth of NLS7 in all experimental groups (Figure 3.6 **A**). The cell concentrations of NLS7 were calculated from the $OD_{600nm}$ changes over time and the standard curve of $OD_{600nm}$ over NLS7 cell concentration Therefore, the specific growth rate of NLS7 in co-culture experiments was calculated from the exponentially growing region of calculated NLS7 cell concentration (Figure 3.6 **B**), the lag and stationary periods were excluded from the calculated cell counts. The specific growth rates of NLS7 in co-culture experiments were similar to NLS7 growth rate in NLS7-only culture (Table 3.1). Tukey pairwise comparison test also supported that finding and revealed that the specific growth rate among all groups did not differ significantly. That indicated that the presence of *P. chlororaphis* did not increase the specific growth rate of NLS7 with six days of continuous methane feeding.

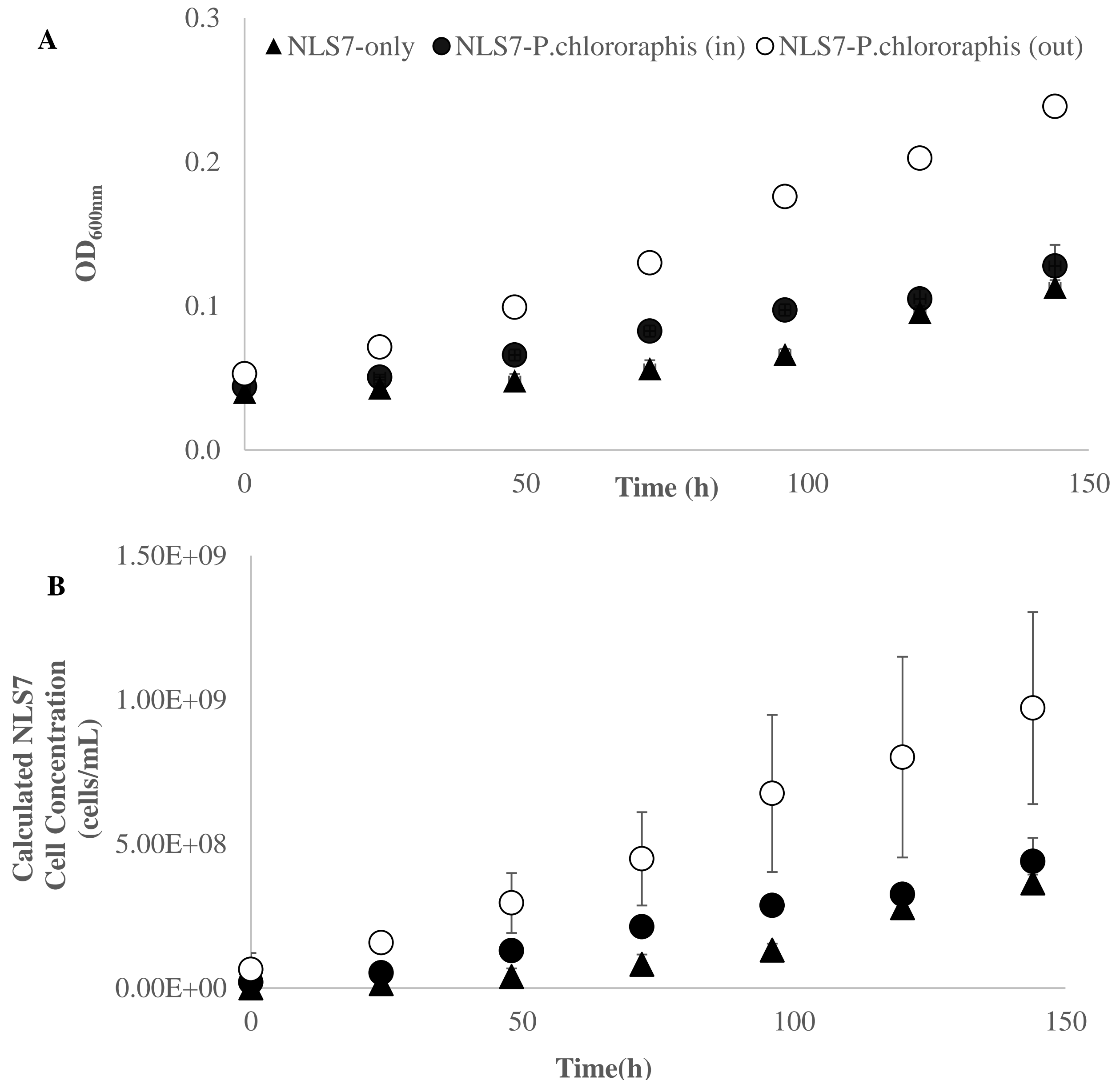


Figure 3.6. (**A**) Average $OD_{600nm}$ readings of NLS7 with standard deviations in NLS7-only (n=4) and NLS7- *P. chlororaphis* (in) (n=5) cultures for 144 hours. In NLS7- *P. chlororaphis* (out) (n=5) culture, $OD_{600nm}$ changes were due to the growth of NLS7 and *P. chlororaphis*. (**B**) Average calculated cell count results (cells/mL) of NLS7 with standard deviations in NLS7-only and the co-culture experiments. The black triangles represent NLS7-only culture, while the filled black circles represent NLS7- *P. chlororaphis* (in) culture. The black circles represent NLS7- *P. chlororaphis* (out) culture. In NLS7- *P. chlororaphis* (out) co-culture, the total cells count was calculated from $OD_{600nm}$ readings and the standard curve for $OD_{600nm}$ over NLS7 cell concentration. In NLS7- *P. chlororaphis* (out) co-culture, *P. chlororaphis* cells were counted via

viable cell count assay. Therefore, the NLS7 cells were calculated by subtracting the total cell count from the viable cell count of *P. chlororaphis.*

Figure 3.7 **A** shows the mole changes of methane over time in the headspaces of the STRs in all three groups. A linear decrease in methane was observed in only NLS7 experimental replicates, implying that only NLS7 performed the methane-oxidation. The abiotic and only *P. chlororaphis* control group resulted no changes in OD readings, methane and $CO_2$ in STRs. Therefore, the changes in methane were only due to the microbial activity of NLS7, not *P. chlororaphis*. The MOR was higher in the co-culture of NLS7-*P. chlororaphis* (in) than in NLS7-only and NLS7-*P. chlororaphis* (out) (Table 3.1). However, the statistical analysis in ANOVA revealed that the differences in MOR between experimental groups were not statistically significant.

Figure 3.7 **B** illustrates $CO_2$ changes over time during the batch test. The linear increase in $CO_2$ (μmol) over time indicated active methane oxidation by NLS7 and indicated microbial activity of NLS7 and *P. chlororaphis*. The CPR in the co-culture of NLS7-*P. chlororaphis* (in) was greater than in NLS7-only and NLS7-*P. chlororaphis* (out). (Table 3.1). However, the Tukey pairwise comparison test illustrated no significant difference in CPR among all experimental groups. The MOR to CPR ratio in NLS7 -*P. chlororaphis* (in) and NLS7 -*P. chlororaphis* (out) was 0.15 µmol $CH_4$/ µmol $CO_2$ while the ratio was 0.17 µmol $CH_4$/ µmol $CO_2$ in NLS7-only.

In summary, the findings on MOR, CPR, and MOR to CPR ratio indicated that the addition of *P. chlororaphis* did not significantly impact the methanotrophic activity or the growth of NLS7 when co-cultivated with *P. chlororaphis*.

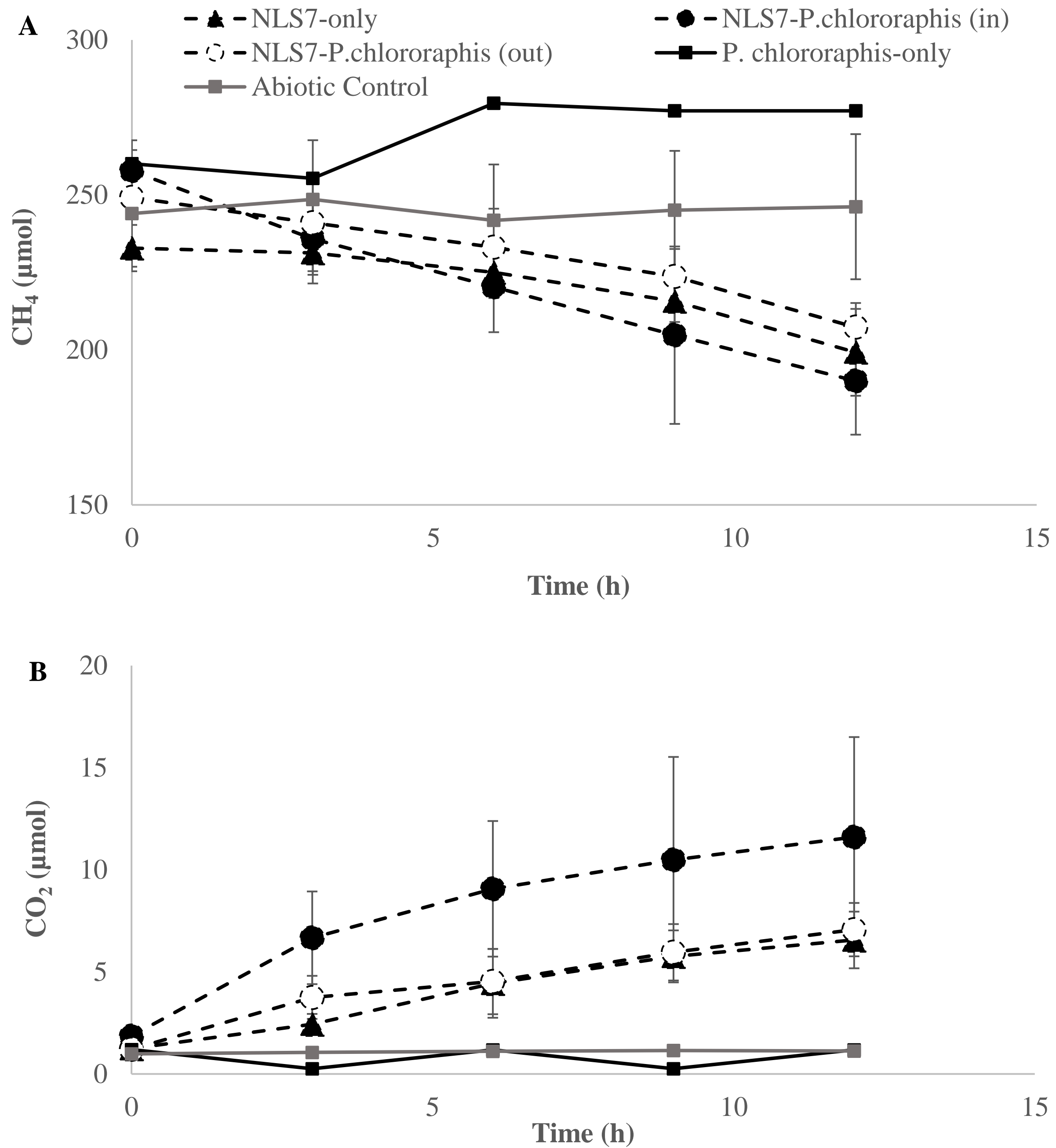


Figure 3.7. Average (**A**) $CH_4$ (µmol) and (**B**) $CO_2$ (µmol) over time with standard deviations in NLS7-only (n=4), NLS7- *P. chlororaphis* (in) (n=5), and NLS7- *P. chlororaphis* (out) (n=5) cultures (Time=12 h). The black triangles with a dashed line represent NLS7-only culture. The filled black circles represent NLS7- *P. chlororaphis* (in) culture, while the open circles represent NLS7- *P. chlororaphis* (out). The dark gray squares with a solid line represent abiotic control. The black squares with a solid line represent *P. chlororaphis* only group. Cultures were purged with methane gas mixture, sealed, capped, and the concentrations in gas phase were recorded over 12 hours with 3-hour intervals.

In summary, NLS7`s specific growth rate, MOR, and CPR were evaluated as functional response variables to the presence of *P. chlororaphis* in the co-culture experiments (Table 3.1 and 3.2). The statistical analysis in ANOVA and the pairwise comparison test with Tukey`s method revealed that the specific growth rate of NLS7, MOR, and CPR in the co-cultures did not significantly differ among all groups ($p>0.05$).

Table 3.1. Table summarizes the average specific growth rate of NLS7, MOR, and CPR with standard deviations in all three experimental groups.

| **Group Name** | **Specific Growth Rate ($h^{-1}$)** | **MOR (μmol $L^{-1}h^{-1}$)** | **CPR (μmol $L^{-1}h^{-1}$)** |
|---|---|---|---|
| **NLS7** | 0.03 ± 0.007 | 3.5 ± 0.8 | 0.6 ± 0.2 |
| **NLS7- *P. chlororaphis* (in)** | 0.03 ± 0.007 | 6.9 ± 2.7 | 1.0 ± 0.5 |
| **NLS7- *P. chlororaphis* (out)** | 0.02 ± 0.004 | 4.2 ± 2.2 | 0.6 ± 0.1 |

The MOR and CPR were normalized to the final cell concentration of NLS7 to investigate the methane-oxidation and $CO_2$-production activity on a per cell basis. Table 3.2 summarizes the MOR and CPR on a per cell basis, and the average calculated NLS7 cell concentrations in the liquid medium at the end of the six days of continuous methane feeding. The Tukey pairwise comparison test for MOR and CPR on a per cell basis among all groups also indicated that *P. chlororaphis* addition did not impact the methanotrophic activity of NLS7 compared to the pure NLS7 culture.

Table 3.2. Table summarizes the average cell concentration of NLS7 (cells/ mL) at six days, MOR on a per cell basis, and CPR on a per cell basis in all three groups with standard deviations.

| **Group Name** | **NLS7 cell concentration at 6 days (cells/mL)** | **$10^{-12}$ µmol $CH_4$ cell $^{-1}$ h $^{-1}$** | **$10^{-12}$ µmol $CO_2$ cell $^{-1}$ h $^{-1}$** |
|---|---|---|---|
| **NLS7-only** | $3.6 \pm 0.3 \times 10^8$ | $7.5 \pm 1.3$ | $1.6 \pm 0.5$ |
| **NLS7- *P. chlororaphis* (in)** | $4.4 \pm 0.8 \times 10^8$ | $12.9 \pm 5.4$ | $2.5 \pm 1.9$ |
| **NLS7- *P. chlororaphis* (out)** | $9.7 \pm 3.3 \times 10^8$ | $4.4 \pm 3.7$ | $0.7 \pm 0.3$ |

## Discussion

In natural environments, methanotrophs interact with various other organisms in different ways. Methanotrophs have been reported to have predator-prey relationships with zooplankton and protozoa as bacterivores (Gonzalez 1990), mutualistic relationships with marine invertebrates as endosymbionts (Petersen 2009), synergistic relationships with algae and mosses by exchanging $CO_2$ and $O_2$ (Gonzalez 1990, Petersen 2009, van der Ha 2011), and commensal and endosymbiotic relationships with non-methanotrophic bacteria (Wilkinson 1974, Hrsak 1998, Stock 2013, Jeong 2014).

In this study, we hypothesized that methanotrophic activity and growth are stimulated by the presence of heterotrophic bacteria in a methanotrophic-heterotrophic co-culture. To test this hypothesis NLS7 and *P. chlororaphis* were selected since they represent the species isolated from same environment, potentially methane driven ecosystem. In this study, the specific growth rate of NLS7 was $0.03 \pm 0.07$ $h^{-1}$ and similar to values reported earlier for various *Methylocystis sp.*

strains methane (Jeong 2014, So-Yeon 2018). However, the MOR of NLS7 (3.5 ± 0.8 µmol $L^{-1}h^{-1}$) in NLS7-only culture on 1-1.5% v/v methane was reported higher than the previously reported values (0.4-0.8 µmol $L^{-1}h^{-1}$) on 5-15% v/v methane (Jeong 2014, So-Yeon 2018). That indicated the fed-batch STR was an efficient system to cultivate NLS7 cells on methane and promote methanotrophic activity.

The statistical analysis illustrated that the specific growth rate, MOR, CPR, in addition to MOR and CPR on a per cell basis did not differ significantly among groups ($p>0.05$). Therefore, this study did not reveal any beneficial or harmful effects for NLS7 when co-cultivated with *P. chlororaphis*.

The increase in *P. chlororaphis* cell concentration in the co-culture experiments revealed that *P. chlororaphis* could grow in methane fed co-culture with NLS7. In NLS7-*P. chlororaphis* (out) co-culture, 160-fold increase in the cell concentration of *P. chlororaphis* was observed while 7-fold increase in the dialysis membrane experiment was noted. However, it should be noted that the initial cell concentration of *P. chlororaphis* in NLS7- *P. chlororaphis*(in) group was higher than in NLS7- *P. chlororaphis*(out). However, the total cell amount with respect to the volume of inoculation location was similar. In addition, initial NLS7 to *P. chlororaphis* cell ratio was 100:1 in the two different co-culture experiments. Thus, the growth of *P. chlororaphis* was likely limited due to initial high cell concentration and that potentially triggered stationary phase. Furthermore, the consistent growth of *P. chlororaphis* over time in the filtered spent medium of NLS7 also indicated that *P. chlororaphis* can utilize the accumulated carbon sources by the methane oxidation and that its growth in this co-culture does not solely depend on the active growth of NLS7.

The findings of this study imply that the co-cultivation of NLS7 and *P. chlororaphis* benefits only *P. chlororaphis* growth, indicating a likely commensal relationship rather than a symbiotic one since the methane oxidation and growth of NLS7 were not impacted when co-cultured with *P. chlororaphis* but benefited the growth of *P. chlororaphis*. A similar study with NLS7 and *P. chlororaphis* reported similar methane-fed co-culture findings (2 % v/v). They reported a 100-fold increase in *P. chlororaphis* cell concentration when in physical contact with NLS7 cells while no beneficial effects for NLS7 in the co-culture with *P. chlororaphis* in 160 mL serum bottles with 50 mL liquid volume (Moon-Escamilla 2020). Even though this study does not reveal any enhanced growth and activity of NLS7, the results of this study give us an important insight into the activity and the growth of methanotrophs in methane-driven ecosystems. However, further investigation is still needed to better understand the relationship between NLS7 and *P. chlororaphis* from the perspective of heterotrophic growth in methane-driven ecosystems. Future research in this interaction can target to identify the exchange of resources for *P. chlororaphis* when cultivated with NLS7 in methane fed STR using high-resolution liquid chromatography and stable isotope labeling. *P. chlororaphis* previously tested with methanol (100 mM) as a by-product of methane oxidation (data not shown) however the growth on methanol at 100 mM concentration did not observe, indicating that *P. chlororaphis* cannot utilize methanol at that concentration. Previous studies reported that the organic compound exchange between methanotrophs and non-methanotrophs is a critical interaction mechanism for microbial growth and methanotrophic activity (Iguchi 2011, Jeong 2014, So-Yeon 2018). It has been hypothesized and studied that non-methanotrophic bacteria can enhance the methanotrophic activity and growth by secreting growth/activity promoting agents (Iguchi 2011). In that study, cobalamin (vitamin $B_{12}$) was

reported as a growth/activity-promoting agent for methanotrophs when cultivated with several non-methanotrophic bacteria. The cobalamin stimulated methanotrophic activity has been reported in the co-culture of *Rhizobium* with methanotrophs belonging to *Methylococcaceae*, *Methylomonas*, and *Methylobacter* strains (Iguchi 2011). In this study, the bulk liquid medium was supplemented with cobalamin (Vitamin $B_{12}$) prior to inoculation of the co-cultures indicating there was potentially less likely cobalamin-driven interaction between these two organisms. Moon et al. also tested this interaction in the lack of cobalamin and did not report any enhanced methanotrophic activity and growth in NLS7-*P. chlororaphis* co-cultures. In another study, volatile chemical compound exchange was reported as a stimulating factor for methanotrophic growth and activity in methanotrophic-heterotrophic co-cultures. (Veraart 2018). These findings suggested that methanotrophic growth and activity can be impacted in methanotrophic-heterotrophic interactions through different mechanisms such as direct chemical compound exchange, volatile and/or soluble organic compound exchange.

In methane bioconversion applications, methanotrophs remove GHG methane from the environment and degrade it into value-added products such as methanol (Bjorck 2018), biopolymers (e.g., PHBs) (Rahnama 2012), ectoine (Cantera 2017), and single-cell proteins (Pieja 2017). However, slow enzyme and growth kinetics of methanotrophs restrict their use in industrial applications (Lawton 2016). In bioprocessing, the utilization of microbial consortia has been reported more advantageous over pure culture usage due to higher biomass accumulation and enhanced catalytic functions of a consortium (Ghosh 2016). Moreover, *Pseudomonas* strains have been vastly investigated and studied in the field of genetic engineering (Arias-Barrau 2005, Weimer 2020). The genetically engineered strains of *Pseudomonas* with different functions such

as methylotrophic fluorescent strain can be used to assemble efficient model methanotrophic co-cultures where methane is desired to be used as a feedstock. The utilization of genetically engineered *Pseudomonas* strains can make the methanotrophic-heterotrophic co-culture a novel step towards methane as a feedstock in biotechnology.

Future research can focus on evaluating NLS7 growth with other heterotrophic bacteria to identify if these findings are unique to *P. chlororaphis*. For example, a recent study focused on the interaction of *Methylocystis sp*. M6 and *Microbacterium sp.* NM2 in a methane fed co-culture (Jeong 2018). In this study, Jeong *et al*. (Jeong 2018) reported stimulated methanotrophic activity and growth in *Methylocystis sp.* M6 and *Microbacterium sp.* NM2 co-culture (Jeong 2018). In addition, Schroeckh *et al.* (Schroeckh 2009) reported a stimulated *Methylocystis sp.* M6 growth and activity in the presence of *Sphingopyxis sp.* NM1 (Jeong 2014). Future work can test the effects of common heterotrophic bacteria in methane-driven ecosystems on the growth and activity of NLS7.

## Conclusion

In this study, we investigated the effects of *P. chlororaphis* presence on *Methylocystis sp.* NLS7 growth and methanotrophic activity when they were co-cultivated in methane fed STR. We hypothesized that the addition of a heterotrophic bacterium would stimulate the growth and/or activity of methanotrophic species. However, our findings did not support this hypothesis. This study did not indicate any beneficial impacts for NLS7 when cultivated with *P. chlororaphis*. The MOR and growth of NLS7 were not statistically different in the co-cultures compared to the pure NLS7 culture. Enhanced final cell concentration of *P. chlororaphis* was reported in dialysis membrane experiments. That indicates the dialysis membrane use does not restrict the cell growth of microorganisms. Therefore, in biotechnological applications, the dialysis membrane can be an efficient system to co-cultivate microbial consortia. The use of the dialysis membrane can ease the separation of microbial species, potentially reducing the time and cost of downstream applications such as extraction and separation when highly pure value-added products are desired in small quantities.

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# CHAPTER FOUR

# CONCLUSION

## Project Outcomes

Expanding the knowledge in methanotrophic-heterotrophic interactions is crucial, both from an environmental and a biotechnological perspective. Previous studies have mostly focused on understanding the methanotrophic activity and growth as individual strains. However, in nature, most microbial processes are not achieved by single species but by a microbial consortium with diverse microorganisms.

This study advanced the knowledge on methanotrophic activity and growth in methanotrophic-heterotrophic interactions and provided future questions for successive work. The main goals of the project here were to:

(1) Evaluate methanotrophic activity and growth of pure *Methylocystis sp.* NLS7 in a methane-based fed-batch STR. The methanotrophic activity and growth of only NLS7 culture was investigated based on the specific growth rate, methane-oxidation and $CO_2$-production rates (MOR and CPR), and cell-based MOR and CPR. In addition, the growth of *P. chlororaphis* in the filtered spent medium of NLS7 was investigated. The filtered spent medium was free from NLS7 cells and only contained the by-products of methane oxidation.

(2) Determine the co-cultivation effects of *Methylocystis sp.* NLS7 and *P. chlororaphis* on methanotrophic activity and microbial growth of both organisms. The NLS7 activity and growth were evaluated based on the specific growth rate, MOR, CPR, and cell-

based MOR and CPR values to understand the impact of *P. chlororaphis* presence on NLS7. In addition, the simultaneous growth of *P. chlororaphis* with NLS7 was also evaluated. The co-cultivation experiments were designed by utilizing a dialysis membrane. The dialysis membrane device was used to inoculate *P. chlororaphis* cells by physically separating the two organisms and promoting a high cell density of *P. chlororaphis*.

*Methylocystis sp.* NLS7 Activity and Growth in NLS7-*P. chlororaphis* co-culture

In this study, methanotrophic activity and growth of NLS7 was evaluated by cultivating it with *P. chlororaphis*. Moreover, the growth of *P. chlororaphis* was evaluated in the co-culture of NLS7 and *P. chlororaphis*. The NLS7`s MOR, CPR, the specific growth rate, MOR and CPR on a per cell basis were evaluated by the presence of *P. chlororaphis*. In the co-culture experiments, dialysis membrane device was used to inoculate *P. chlororaphis* and physically separate the species from each other. The co-culture experiments indicated that the presence of *P. chlororaphis* does not have any beneficial or harmful effects to the methanotrophic growth and activity of NLS7. The co-culture experiment results also revealed that *P. chlororaphis* can grow simultaneously with NLS7 in methane-fed STR. Higher final cell concentration of *P. chlororaphis* was observed when co-cultivated with NLS7, indicating a potential carbon source(s) for *P. chlororaphis* to grow in methane-driven system. The findings of this study give us an important insight on the interaction of methanotrophic and heterotrophic bacteria in methane-driven ecosystem. Furthermore, the fed-batch STR with dialysis membrane can be an efficient model to cultivate co-cultures and evaluate interactions and by-products.

## Concluding Remarks

In this study, we hypothesized that the addition of a heterotrophic bacterium would stimulate the growth and/or activity of methanotrophic species. We chose two organisms, NLS7 and *P. chlororaphis* which represented species isolated from the same environment. However, our findings did not support this hypothesis. This study did not indicate any beneficial impacts for NLS7 when cultivated with *P. chlororaphis*. The MOR and growth of NLS7 were not statistically different in the co-cultures compared to the pure NLS7 culture. Even though in this study we did not report any enhanced methanotrophic activity and growth of NLS7, an enhanced final cell concentration of *P. chlororaphis* was reported in dialysis membrane experiments. That indicates the dialysis membrane use does not restrict the cell growth of microorganisms. Therefore, in biotechnological applications, the dialysis membrane can be an efficient system to co-cultivate microbial consortia. The use of the dialysis membrane can ease the separation of microbial species, potentially reducing the time and cost of downstream applications such as extraction and separation when highly pure value-added products are desired in small quantities.

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# CHAPTER FIVE

# FUTURE WORK

## High Value-added Bioproduct Production from Methanotrophic-heterotrophic Co-culture

The co-culture systems have been utilized in various biotechnology applications, such as treatment of wastewater, removal of toxic compounds, and production of foods and value-added compounds (Rosero-Chasoy 2021). Therefore, there is a great interest in understanding the communication mechanisms between species within co-culture to investigate novel biotechnology processes.

Methane has gained significant interest in producing high-value-added products such as methanol (Patel 2018), biopolymer (Zuñiga 2013) , and single-cell protein (Anupama 2000) as a cheap and abundant feedstock. However, the slow enzyme and growth kinetics of methanotrophs limits the use of methanotrophs in biotechnological applications (Guerrero-Cruz 2021). To solve this problem, the utilization of methanotrophic consortia in biotechnological applications can appeal the methane usage as a feedstock. Previous studies reported methanotrophic -heterotrophic consortia with stimulated methanotrophic activity and growth (Petersen 2009, Iguchi 2011, Jeong 2014). The fed-batch STR design with a dialysis membrane can help to better understand methanotrophic-heterotrophic interactions. This system can be tested with different heterotrophic strains to investigate whether these findings unique to *P. chlororaphis*. Investigation of different methanotrophic consortia might result finding cultures with robust methanotrophic activity and growth of NLS7. In addition, future work can focus on methanotrophic-genetically modified heterotrophic consortium cultivation in methane fed STR with dialysis membrane. The selection

of right species is time consuming and require intensive labor. In synthetic biology, the manipulation of cells for desired applications have been intensively studied. The methanotrophic-heterotrophic consortia can benefit from the novel methods in synthetic biology to manipulate *P. chlororaphis* cells to build efficient cultures for methane conversion to high value-added products technologies.

The future work can focus on investigating compound sources and enhancing the capabilities of methanotrophic-heterotrophic consortia. The previous studies showed that methanotrophic and heterotrophic bacteria co-cultivation can benefit methanotrophic activity and growth in different ways (Iguchi 2011, Stock 2013). For example, Iguchi *et al.* (Iguchi 2011) reported that selective heterotrophs were found to deliver essential nutrients to when co-cultivated. The organic compound delivered by the heterotrophic bacterium was identified as cobalamin (Vitamin $B_{12}$). These methanotroph strains showed no growth or very weak growth when cobalamin was not introduced to the culture medium. In addition these methanotrophs did not produce cobalamin by their selves (Iguchi 2011). Therefore, investigation of potential sources in methanotrophic-heterotrophic co-cultures can be beneficial in order to better understand methanotrophic activity and growth and improve strategies to cultivate them in methane fed systems.

The results obtained from this study have been prepared for scientific journal submission. The strategies used in this study can reference for future studies.

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# APPENDICES

# APPENDIX A

TABULATED DATA

Figure 1.Standard curve of NLS7 and *P. chlororaphis* cell concentration over $OD_{600nm}$ readings in microplate reader.

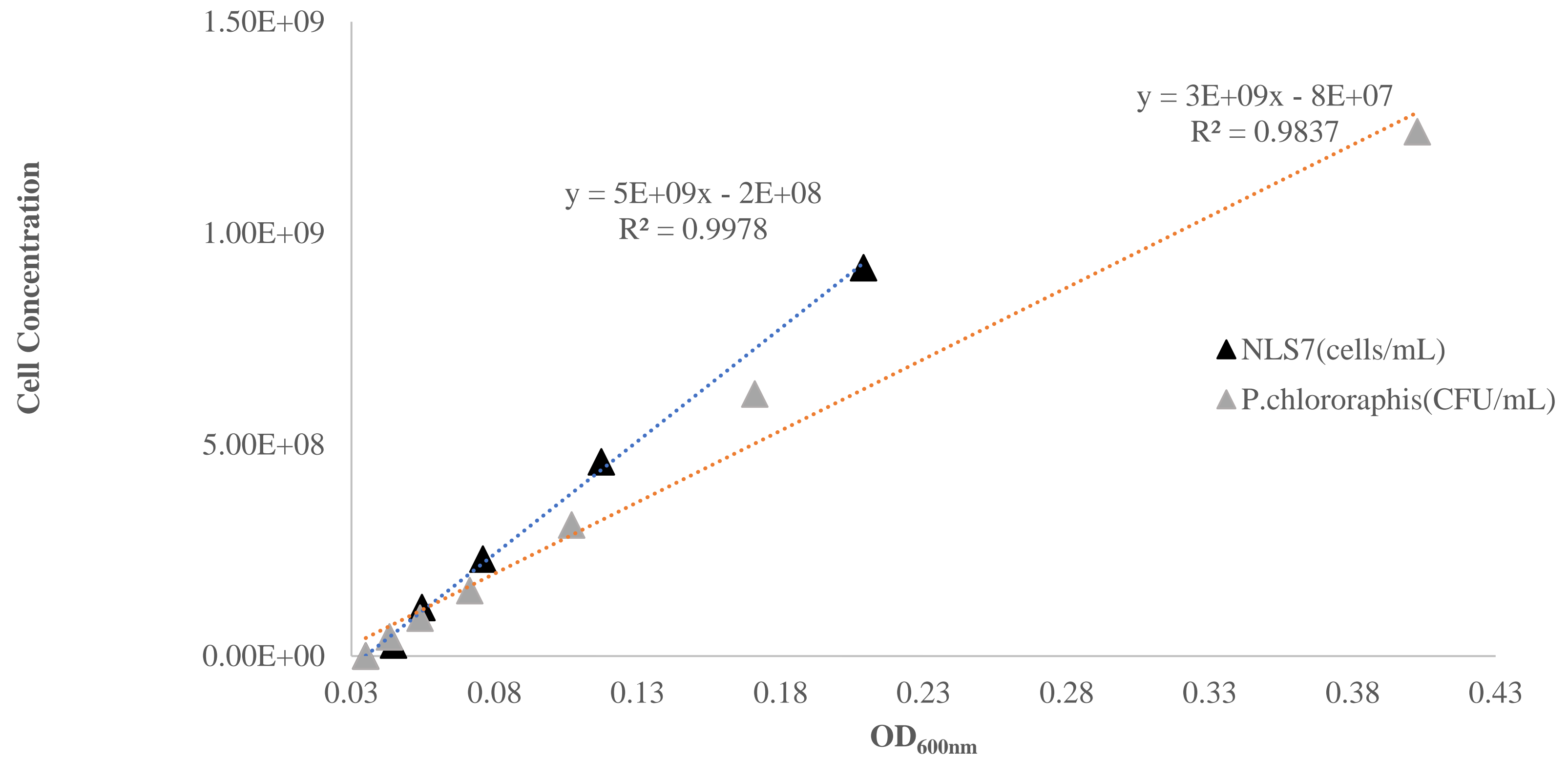

Figure 2. Average NLS7 cell concentration in NLS7- only group with standard deviations over $OD_{600nm}$ readings. The black triangles represent calculated values while gray dots represent actual cell concentration values.

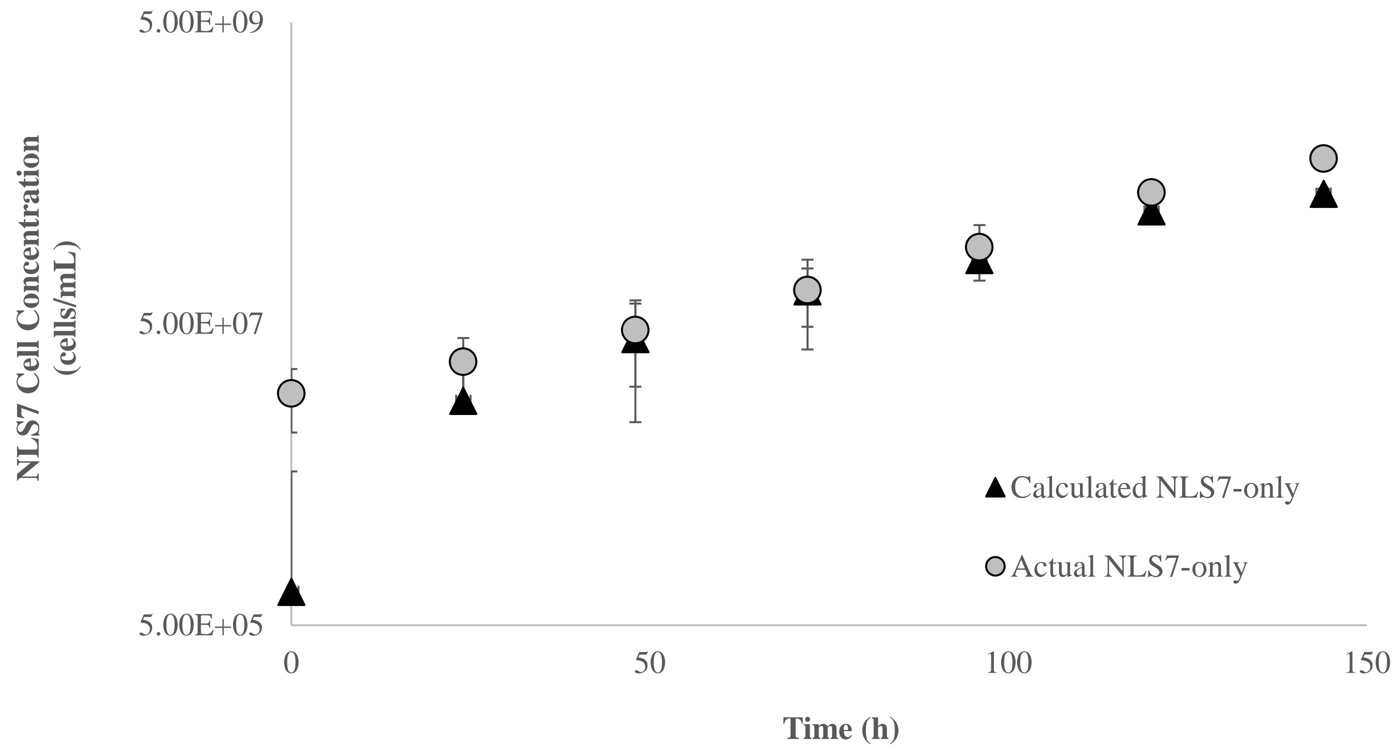

Figure 3. Average NLS7 cell concentration in NLS7- *P. chlororaphis* (in) group with standard deviations over $OD_{600nm}$ readings. The black triangles represent calculated values while gray dots represent actual cell concentration values.

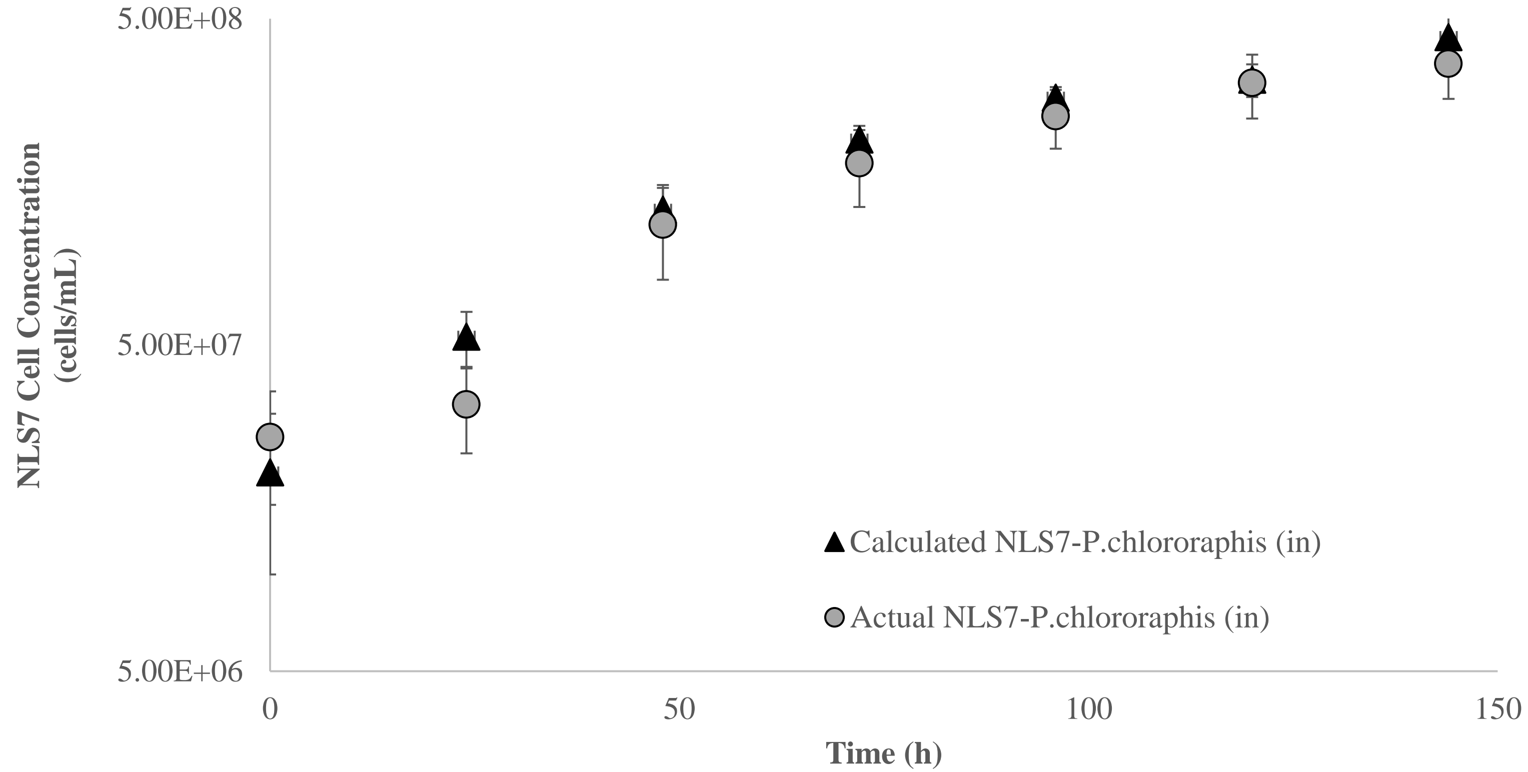

Figure 4. Average NLS7 cell concentration in NLS7- *P. chlororaphis* (out) group with standard deviations over $OD_{600nm}$ results. The black triangles represent calculated values while gray dots represent actual cell concentration values.

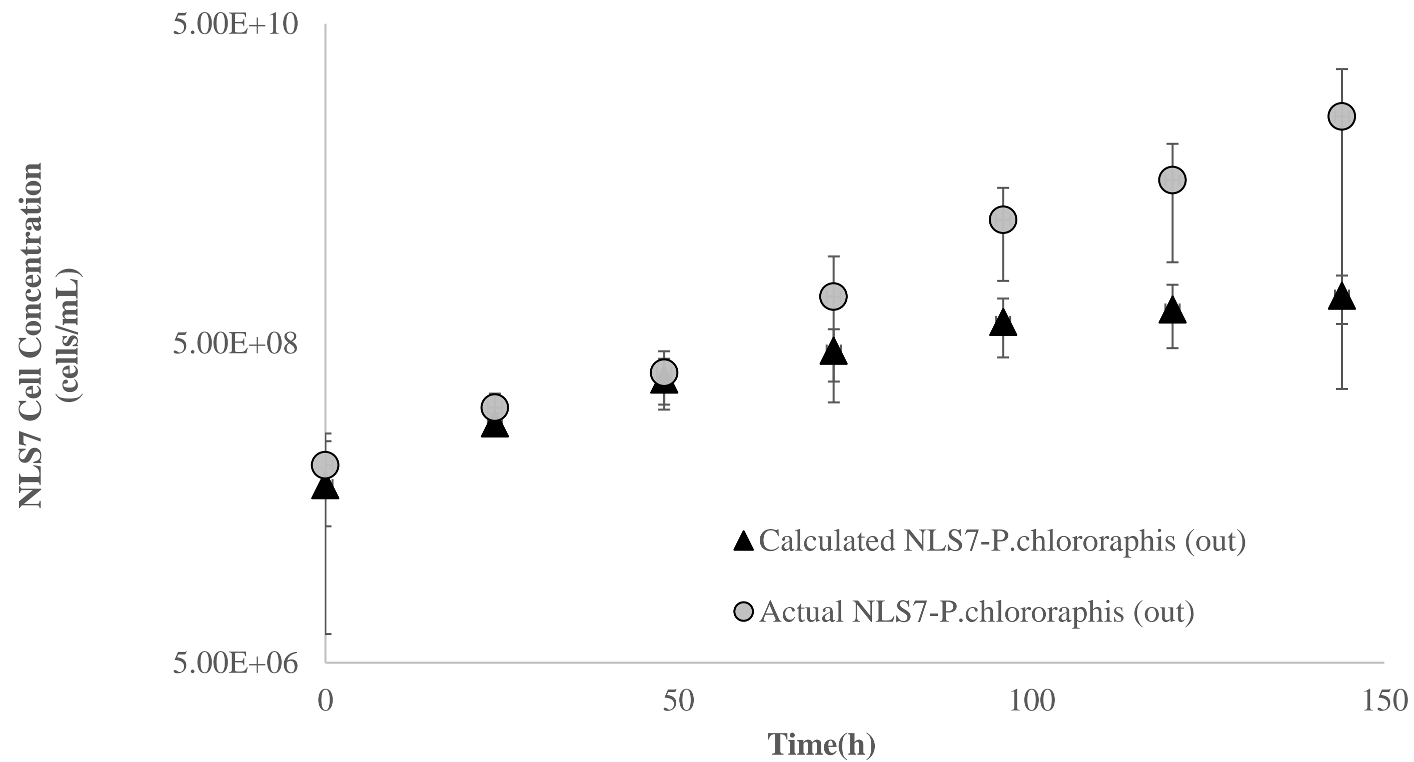

Table 1. Tabulated raw data of NLS7 in NLS7-only (cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7 only-1** | 6.00E+02 | 1.10E+03 | 1.50E+03 | 2.00E+03 | 6.00E+03 | 2.00E+04 | 3.20E+04 |
| | 8.00E+02 | 1.00E+03 | 1.20E+03 | 2.20E+03 | 7.00E+03 | 2.10E+04 | 3.30E+04 |
| | 8.00E+02 | 9.00E+02 | 1.30E+03 | 2.30E+03 | 7.00E+03 | 2.50E+04 | 3.40E+04 |
| | 7.00E+02 | 9.00E+02 | 1.20E+03 | 3.20E+03 | 8.00E+03 | 2.40E+04 | 3.40E+04 |
| | 6.00E+02 | 8.00E+02 | 1.40E+03 | 2.40E+03 | 6.00E+03 | 2.90E+04 | 3.50E+04 |
| | 5.00E+02 | 1.00E+03 | 1.40E+03 | 2.40E+03 | 6.00E+03 | 2.60E+04 | 3.70E+04 |
| | 7.00E+02 | 1.10E+03 | 1.40E+03 | 2.40E+03 | 7.00E+03 | 2.40E+04 | 3.80E+04 |
| | 3.00E+02 | 1.00E+03 | 1.30E+03 | 2.30E+03 | 4.00E+03 | 2.20E+04 | 3.80E+04 |
| | 2.00E+02 | 1.20E+03 | 1.20E+03 | 1.80E+03 | 6.00E+03 | 2.20E+04 | 3.80E+04 |
| | 8.00E+02 | 1.10E+03 | 1.30E+03 | 1.80E+03 | 7.00E+03 | 2.10E+04 | 3.80E+04 |
| **NLS7 only-2** | 5.00E+02 | 1.00E+03 | 1.10E+03 | 1.90E+03 | 8.00E+03 | 2.80E+04 | 3.60E+04 |
| | 4.00E+02 | 8.00E+02 | 1.50E+03 | 2.30E+03 | 7.00E+03 | 2.90E+04 | 3.40E+04 |
| | 4.00E+02 | 7.00E+02 | 1.60E+03 | 2.20E+03 | 6.00E+03 | 2.90E+04 | 3.30E+04 |
| | 4.00E+02 | 8.00E+02 | 1.20E+03 | 2.20E+03 | 5.00E+03 | 2.70E+04 | 3.30E+04 |
| | 6.00E+02 | 8.00E+02 | 1.20E+03 | 2.20E+03 | 6.00E+03 | 2.20E+04 | 3.30E+04 |
| | 8.00E+02 | 9.00E+02 | 1.50E+03 | 2.40E+03 | 7.00E+03 | 2.60E+04 | 3.30E+04 |
| | 8.00E+02 | 1.00E+03 | 1.50E+03 | 2.40E+03 | 4.00E+03 | 2.90E+04 | 3.80E+04 |
| | 9.00E+02 | 1.30E+03 | 1.50E+03 | 2.50E+03 | 4.00E+03 | 2.20E+04 | 3.80E+04 |
| | 7.00E+02 | 1.20E+03 | 1.10E+03 | 2.60E+03 | 6.00E+03 | 2.20E+04 | 3.40E+04 |
| | 5.00E+02 | 1.20E+03 | 1.20E+03 | 2.40E+03 | 4.00E+03 | 2.20E+04 | 3.40E+04 |

Table 1(cont.). Tabulated raw data of NLS7 in NLS7-only (cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7 only-3** | 1.00E+03 | 3.00E+03 | 5.00E+03 | 1.20E+04 | 1.30E+04 | 1.30E+04 | 3.90E+04 |
| | 2.20E+03 | 2.50E+03 | 2.00E+03 | 1.10E+04 | 1.20E+04 | 1.80E+04 | 4.00E+04 |
| | 1.20E+03 | 1.20E+03 | 3.00E+03 | 1.00E+04 | 1.60E+04 | 1.50E+04 | 4.10E+04 |
| | 1.20E+03 | 2.90E+03 | 2.00E+03 | 1.00E+04 | 1.20E+04 | 2.00E+04 | 3.90E+04 |
| | 1.20E+03 | 1.20E+03 | 3.00E+03 | 5.00E+03 | 1.40E+04 | 1.00E+04 | 3.70E+04 |
| | 1.20E+03 | 3.00E+03 | 3.00E+03 | 6.00E+03 | 1.20E+04 | 1.20E+04 | 3.90E+04 |
| | 1.60E+03 | 3.10E+03 | 3.00E+03 | 5.00E+03 | 9.00E+03 | 1.70E+04 | 3.80E+04 |
| | 1.80E+03 | 2.00E+03 | 4.00E+03 | 4.00E+03 | 8.00E+03 | 1.90E+04 | 2.30E+04 |
| | 8.00E+02 | 3.20E+03 | 3.00E+03 | 5.00E+03 | 7.00E+03 | 2.50E+04 | 4.00E+04 |
| | 6.00E+02 | 1.10E+03 | 5.00E+03 | 6.00E+03 | 9.00E+03 | 2.70E+04 | 4.20E+04 |
| **NLS7 only-4** | 1.60E+03 | 3.00E+03 | 2.00E+03 | 1.20E+04 | 1.00E+04 | 2.50E+04 | 3.00E+04 |
| | 1.90E+03 | 1.80E+03 | 4.00E+03 | 1.00E+04 | 1.20E+04 | 2.60E+04 | 3.20E+04 |
| | 1.20E+03 | 2.70E+03 | 5.00E+03 | 3.00E+03 | 1.30E+04 | 2.30E+04 | 3.80E+04 |
| | 1.40E+03 | 2.30E+03 | 6.00E+03 | 3.00E+03 | 1.50E+04 | 1.20E+04 | 3.20E+04 |
| | 1.10E+03 | 2.60E+03 | 5.00E+03 | 9.00E+03 | 1.60E+04 | 1.70E+04 | 3.70E+04 |
| | 8.00E+02 | 2.90E+03 | 4.00E+03 | 6.00E+03 | 1.70E+04 | 1.90E+04 | 3.40E+04 |
| | 1.40E+03 | 2.20E+03 | 3.00E+03 | 7.00E+03 | 1.30E+04 | 1.60E+04 | 3.50E+04 |
| | 1.70E+03 | 1.70E+03 | 4.00E+03 | 7.00E+03 | 1.20E+04 | 1.60E+04 | 3.50E+04 |
| | 1.00E+03 | 6.00E+02 | 2.00E+03 | 7.00E+03 | 1.20E+04 | 1.20E+04 | 3.20E+04 |
| | 2.20E+03 | 5.00E+02 | 8.00E+03 | 5.00E+03 | 1.20E+04 | 1.00E+04 | 2.80E+04 |

Table 2. Tabulated calculated data of NLS7 in NLS7-only (cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7 only-1** | 1.06E+07 | 1.94E+07 | 2.65E+07 | 3.53E+07 | 1.06E+08 | 3.53E+08 | 5.65E+08 |
| | 1.41E+07 | 1.77E+07 | 2.12E+07 | 3.89E+07 | 1.24E+08 | 3.71E+08 | 5.83E+08 |
| | 1.41E+07 | 1.59E+07 | 2.30E+07 | 4.06E+07 | 1.24E+08 | 4.42E+08 | 6.01E+08 |
| | 1.24E+07 | 1.59E+07 | 2.12E+07 | 5.65E+07 | 1.41E+08 | 4.24E+08 | 6.01E+08 |
| | 1.06E+07 | 1.41E+07 | 2.47E+07 | 4.24E+07 | 1.06E+08 | 5.12E+08 | 6.18E+08 |
| | 8.83E+06 | 1.77E+07 | 2.47E+07 | 4.24E+07 | 1.06E+08 | 4.59E+08 | 6.54E+08 |
| | 1.24E+07 | 1.94E+07 | 2.47E+07 | 4.24E+07 | 1.24E+08 | 4.24E+08 | 6.71E+08 |
| | 5.30E+06 | 1.77E+07 | 2.30E+07 | 4.06E+07 | 7.07E+07 | 3.89E+08 | 6.71E+08 |
| | 3.53E+06 | 2.12E+07 | 2.12E+07 | 3.18E+07 | 1.06E+08 | 3.89E+08 | 6.71E+08 |
| | 1.41E+07 | 1.94E+07 | 2.30E+07 | 3.18E+07 | 1.24E+08 | 3.71E+08 | 6.71E+08 |
| **NLS7 only-2** | 8.83E+06 | 1.77E+07 | 1.94E+07 | 3.36E+07 | 1.41E+08 | 4.95E+08 | 6.36E+08 |
| | 7.07E+06 | 1.41E+07 | 2.65E+07 | 4.06E+07 | 1.24E+08 | 5.12E+08 | 6.01E+08 |
| | 7.07E+06 | 1.24E+07 | 2.83E+07 | 3.89E+07 | 1.06E+08 | 5.12E+08 | 5.83E+08 |
| | 7.07E+06 | 1.41E+07 | 2.12E+07 | 3.89E+07 | 8.83E+07 | 4.77E+08 | 5.83E+08 |
| | 1.06E+07 | 1.41E+07 | 2.12E+07 | 3.89E+07 | 1.06E+08 | 3.89E+08 | 5.83E+08 |
| | 1.41E+07 | 1.59E+07 | 2.65E+07 | 4.24E+07 | 1.24E+08 | 4.59E+08 | 5.83E+08 |
| | 1.41E+07 | 1.77E+07 | 2.65E+07 | 4.24E+07 | 7.07E+07 | 5.12E+08 | 6.71E+08 |
| | 1.59E+07 | 2.30E+07 | 2.65E+07 | 4.42E+07 | 7.07E+07 | 3.89E+08 | 6.71E+08 |
| | 1.24E+07 | 2.12E+07 | 1.94E+07 | 4.59E+07 | 1.06E+08 | 3.89E+08 | 6.01E+08 |
| | 8.83E+06 | 2.12E+07 | 2.12E+07 | 4.24E+07 | 7.07E+07 | 3.89E+08 | 6.01E+08 |

Table 2(cont.). Tabulated calculated data of NLS7 in NLS7-only (cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7 only-3** | 1.77E+07 | 5.30E+07 | 8.83E+07 | 2.12E+08 | 2.30E+08 | 2.30E+08 | 6.89E+08 |
| | 3.89E+07 | 4.42E+07 | 3.53E+07 | 1.94E+08 | 2.12E+08 | 3.18E+08 | 7.07E+08 |
| | 2.12E+07 | 2.12E+07 | 5.30E+07 | 1.77E+08 | 2.83E+08 | 2.65E+08 | 7.24E+08 |
| | 2.12E+07 | 5.12E+07 | 3.53E+07 | 1.77E+08 | 2.12E+08 | 3.53E+08 | 6.89E+08 |
| | 2.12E+07 | 2.12E+07 | 5.30E+07 | 8.83E+07 | 2.47E+08 | 1.77E+08 | 6.54E+08 |
| | 2.12E+07 | 5.30E+07 | 5.30E+07 | 1.06E+08 | 2.12E+08 | 2.12E+08 | 6.89E+08 |
| | 2.83E+07 | 5.48E+07 | 5.30E+07 | 8.83E+07 | 1.59E+08 | 3.00E+08 | 6.71E+08 |
| | 3.18E+07 | 3.53E+07 | 7.07E+07 | 7.07E+07 | 1.41E+08 | 3.36E+08 | 4.06E+08 |
| | 1.41E+07 | 5.65E+07 | 5.30E+07 | 8.83E+07 | 1.24E+08 | 4.42E+08 | 7.07E+08 |
| | 1.06E+07 | 1.94E+07 | 8.83E+07 | 1.06E+08 | 1.59E+08 | 4.77E+08 | 7.42E+08 |
| **NLS7 only-4** | 2.83E+07 | 5.30E+07 | 3.53E+07 | 2.12E+08 | 1.77E+08 | 4.42E+08 | 5.30E+08 |
| | 3.36E+07 | 3.18E+07 | 7.07E+07 | 1.77E+08 | 2.12E+08 | 4.59E+08 | 5.65E+08 |
| | 2.12E+07 | 4.77E+07 | 8.83E+07 | 5.30E+07 | 2.30E+08 | 4.06E+08 | 6.71E+08 |
| | 2.47E+07 | 4.06E+07 | 1.06E+08 | 5.30E+07 | 2.65E+08 | 2.12E+08 | 5.65E+08 |
| | 1.94E+07 | 4.59E+07 | 8.83E+07 | 1.59E+08 | 2.83E+08 | 3.00E+08 | 6.54E+08 |
| | 1.41E+07 | 5.12E+07 | 7.07E+07 | 1.06E+08 | 3.00E+08 | 3.36E+08 | 6.01E+08 |
| | 2.47E+07 | 3.89E+07 | 5.30E+07 | 1.24E+08 | 2.30E+08 | 2.83E+08 | 6.18E+08 |
| | 3.00E+07 | 3.00E+07 | 7.07E+07 | 1.24E+08 | 2.12E+08 | 2.83E+08 | 6.18E+08 |
| | 1.77E+07 | 1.06E+07 | 3.53E+07 | 1.24E+08 | 2.12E+08 | 2.12E+08 | 5.65E+08 |
| | 3.89E+07 | 8.83E+06 | 1.41E+08 | 8.83E+07 | 2.12E+08 | 1.77E+08 | 4.95E+08 |

Table 3. Tabulated raw data of NLS7 in NLS7-*P. chlororaphis* (in) (cells/mL) group via direct cell count, second experiment.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7- *P. chlororaphis* (in)-1** | 2.00E+03 | 2.00E+03 | 5.00E+03 | 1.00E+04 | 1.40E+04 | 2.00E+04 | 2.00E+04 |
| | 1.90E+03 | 2.50E+03 | 5.00E+03 | 9.00E+03 | 1.40E+04 | 1.70E+04 | 1.80E+04 |
| | 2.10E+03 | 2.60E+03 | 5.00E+03 | 1.00E+04 | 1.40E+04 | 1.20E+04 | 1.60E+04 |
| | 2.00E+03 | 2.50E+03 | 6.00E+03 | 1.00E+04 | 1.30E+04 | 1.30E+04 | 1.60E+04 |
| | 2.30E+03 | 2.50E+03 | 2.00E+03 | 1.00E+04 | 1.30E+04 | 2.00E+04 | 1.80E+04 |
| | 2.90E+03 | 2.30E+03 | 7.00E+03 | 6.00E+03 | 1.30E+04 | 2.10E+04 | 1.70E+04 |
| | 2.00E+03 | 2.40E+03 | 7.00E+03 | 7.00E+03 | 1.30E+04 | 2.00E+04 | 1.70E+04 |
| | 2.10E+03 | 2.60E+03 | 8.00E+03 | 8.00E+03 | 1.00E+04 | 2.10E+04 | 1.90E+04 |
| | 3.10E+03 | 2.70E+03 | 9.00E+03 | 1.00E+04 | 1.20E+04 | 1.60E+04 | 1.80E+04 |
| | 3.00E+03 | 2.60E+03 | 1.00E+04 | 9.00E+03 | 1.30E+04 | 1.70E+04 | 2.00E+04 |
| **NLS7 - *P. chlororaphis* (in)-2** | 2.20E+03 | 1.90E+03 | 5.00E+03 | 1.00E+04 | 1.30E+04 | 1.50E+04 | 1.70E+04 |
| | 2.20E+03 | 1.90E+03 | 6.00E+03 | 1.00E+04 | 1.40E+04 | 1.60E+04 | 1.50E+04 |
| | 2.00E+00 | 2.80E+03 | 6.00E+03 | 6.00E+03 | 1.30E+04 | 1.60E+04 | 1.20E+04 |
| | 2.00E+03 | 2.70E+03 | 6.00E+03 | 9.00E+03 | 1.40E+04 | 1.20E+04 | 1.90E+04 |
| | 1.10E+03 | 2.50E+03 | 6.00E+03 | 6.00E+03 | 1.10E+04 | 1.50E+04 | 1.40E+04 |
| | 2.00E+03 | 2.20E+03 | 7.00E+03 | 1.00E+04 | 1.40E+04 | 1.50E+04 | 1.80E+04 |
| | 2.00E+03 | 2.30E+03 | 6.00E+03 | 1.00E+04 | 1.40E+04 | 1.60E+04 | 1.80E+04 |
| | 1.90E+03 | 2.60E+03 | 9.00E+03 | 1.00E+04 | 1.20E+04 | 1.60E+04 | 2.10E+04 |
| | 2.10E+03 | 2.80E+03 | 5.00E+03 | 7.00E+03 | 1.20E+04 | 1.40E+04 | 1.30E+04 |
| | 2.10E+03 | 2.60E+03 | 5.00E+03 | 8.00E+03 | 1.30E+04 | 1.30E+04 | 1.70E+04 |

Table 3. (cont.). Tabulated raw data of NLS7 in NLS7-*P. chlororaphis* (in) (cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7- *P. chlororaphis*(in)-3** | 1.60E+03 | 7.00E+02 | 2.00E+03 | 3.00E+03 | 1.00E+04 | 1.80E+04 | 2.20E+04 |
| | 1.20E+03 | 1.00E+03 | 7.00E+03 | 4.00E+03 | 1.50E+04 | 1.70E+04 | 1.30E+04 |
| | 5.00E+02 | 2.00E+03 | 5.00E+03 | 1.20E+04 | 1.20E+04 | 1.40E+04 | 1.90E+04 |
| | 7.00E+02 | 1.50E+03 | 1.00E+04 | 1.30E+04 | 1.80E+04 | 1.30E+04 | 2.10E+04 |
| | 1.30E+03 | 2.10E+03 | 3.00E+03 | 1.70E+04 | 1.20E+04 | 1.00E+04 | 2.20E+04 |
| | 1.00E+03 | 1.70E+03 | 2.00E+03 | 1.90E+04 | 1.30E+04 | 1.00E+04 | 1.30E+04 |
| | 1.20E+03 | 1.30E+03 | 4.00E+03 | 5.00E+03 | 1.20E+04 | 1.10E+04 | 1.40E+04 |
| | 1.90E+03 | 1.20E+03 | 2.00E+03 | 4.00E+03 | 1.00E+04 | 1.20E+04 | 1.70E+04 |
| | 7.00E+02 | 1.80E+03 | 3.00E+03 | 8.00E+03 | 9.00E+03 | 1.30E+04 | 1.80E+04 |
| | 8.00E+02 | 1.40E+03 | 1.20E+04 | 1.60E+04 | 7.00E+03 | 1.70E+04 | 1.80E+04 |
| **NLS7- *P. chlororaphis*(in)-4** | 1.70E+03 | 2.00E+03 | 7.00E+03 | 2.00E+04 | 1.30E+04 | 2.40E+04 | 2.30E+04 |
| | 1.90E+03 | 1.50E+03 | 4.00E+03 | 1.40E+04 | 2.00E+04 | 2.20E+04 | 3.00E+04 |
| | 1.60E+03 | 2.10E+03 | 3.00E+03 | 2.10E+04 | 2.10E+04 | 2.00E+04 | 3.20E+04 |
| | 1.30E+03 | 2.30E+03 | 9.00E+03 | 1.20E+04 | 1.70E+04 | 1.60E+04 | 2.00E+04 |
| | 1.20E+03 | 1.80E+03 | 3.00E+03 | 1.70E+04 | 1.60E+04 | 1.70E+04 | 1.90E+04 |
| | 1.00E+03 | 1.20E+03 | 4.00E+03 | 1.90E+04 | 2.50E+04 | 1.80E+04 | 2.00E+04 |
| | 9.00E+02 | 1.50E+03 | 2.00E+03 | 1.00E+04 | 2.40E+04 | 2.10E+04 | 2.10E+04 |
| | 7.00E+02 | 1.00E+03 | 5.00E+03 | 1.20E+04 | 2.00E+04 | 2.70E+04 | 2.30E+04 |
| | 4.00E+02 | 7.00E+02 | 6.00E+03 | 1.30E+04 | 1.90E+04 | 2.60E+04 | 3.20E+04 |
| | 8.00E+02 | 5.00E+02 | 1.00E+04 | 1.10E+04 | 1.70E+04 | 1.90E+04 | 3.30E+04 |

Table 3. (cont.). Tabulated raw data of NLS7 in NLS7-*P. chlororaphis* (in) (cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7- *P. chlororaphis*(in)-5** | 5.00E+02 | 1.20E+03 | 1.30E+04 | 1.50E+04 | 1.70E+04 | 2.10E+04 | 2.70E+04 |
| | 3.00E+02 | 1.50E+03 | 1.20E+04 | 1.20E+04 | 1.60E+04 | 2.30E+04 | 2.10E+04 |
| | 9.00E+02 | 1.40E+03 | 1.00E+04 | 1.00E+04 | 1.70E+04 | 2.50E+04 | 2.30E+04 |
| | 6.00E+02 | 9.00E+02 | 7.00E+03 | 7.00E+03 | 1.40E+04 | 1.60E+04 | 2.40E+04 |
| | 8.00E+02 | 1.10E+03 | 5.00E+03 | 9.00E+03 | 1.30E+04 | 1.70E+04 | 1.60E+04 |
| | 1.20E+03 | 1.20E+03 | 8.00E+03 | 5.00E+03 | 1.10E+04 | 2.20E+04 | 1.90E+04 |
| | 1.30E+03 | 1.50E+03 | 1.20E+04 | 4.00E+03 | 2.00E+04 | 2.30E+04 | 3.20E+04 |
| | 1.30E+03 | 1.30E+03 | 1.40E+04 | 1.00E+04 | 1.00E+04 | 2.70E+04 | 3.70E+04 |
| | 1.70E+03 | 2.50E+03 | 1.30E+04 | 7.00E+03 | 1.20E+04 | 3.00E+04 | 3.00E+04 |
| | 1.90E+03 | 2.00E+03 | 9.00E+03 | 6.00E+03 | 1.20E+04 | 2.50E+04 | 2.90E+04 |

Table 4. Tabulated calculated data of NLS7 in NLS7-*P. chlororaphis* (in) (cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7-*P. chlororaphis(in)*-1** | 3.53E+07 | 3.53E+07 | 8.83E+07 | 1.77E+08 | 2.47E+08 | 3.53E+08 | 3.53E+08 |
| | 3.36E+07 | 4.42E+07 | 8.83E+07 | 1.59E+08 | 2.47E+08 | 3.00E+08 | 3.18E+08 |
| | 3.71E+07 | 4.59E+07 | 8.83E+07 | 1.77E+08 | 2.47E+08 | 2.12E+08 | 2.83E+08 |
| | 3.53E+07 | 4.42E+07 | 1.06E+08 | 1.77E+08 | 2.30E+08 | 2.30E+08 | 2.83E+08 |
| | 4.06E+07 | 4.42E+07 | 3.53E+07 | 1.77E+08 | 2.30E+08 | 3.53E+08 | 3.18E+08 |
| | 5.12E+07 | 4.06E+07 | 1.24E+08 | 1.06E+08 | 2.30E+08 | 3.71E+08 | 3.00E+08 |
| | 3.53E+07 | 4.24E+07 | 1.24E+08 | 1.24E+08 | 2.30E+08 | 3.53E+08 | 3.00E+08 |
| | 3.71E+07 | 4.59E+07 | 1.41E+08 | 1.41E+08 | 1.77E+08 | 3.71E+08 | 3.36E+08 |
| | 5.48E+07 | 4.77E+07 | 1.59E+08 | 1.77E+08 | 2.12E+08 | 2.83E+08 | 3.18E+08 |
| | 5.30E+07 | 4.59E+07 | 1.77E+08 | 1.59E+08 | 2.30E+08 | 3.00E+08 | 3.53E+08 |
| **NLS7 -*P. chlororaphis*(*in*)-2** | 3.89E+07 | 3.36E+07 | 8.83E+07 | 1.77E+08 | 2.30E+08 | 2.65E+08 | 3.00E+08 |
| | 3.89E+07 | 3.36E+07 | 1.06E+08 | 1.77E+08 | 2.47E+08 | 2.83E+08 | 2.65E+08 |
| | 3.53E+04 | 4.95E+07 | 1.06E+08 | 1.06E+08 | 2.30E+08 | 2.83E+08 | 2.12E+08 |
| | 3.53E+07 | 4.77E+07 | 1.06E+08 | 1.59E+08 | 2.47E+08 | 2.12E+08 | 3.36E+08 |
| | 1.94E+07 | 4.42E+07 | 1.06E+08 | 1.06E+08 | 1.94E+08 | 2.65E+08 | 2.47E+08 |
| | 3.53E+07 | 3.89E+07 | 1.24E+08 | 1.77E+08 | 2.47E+08 | 2.65E+08 | 3.18E+08 |
| | 3.53E+07 | 4.06E+07 | 1.06E+08 | 1.77E+08 | 2.47E+08 | 2.83E+08 | 3.18E+08 |
| | 3.36E+07 | 4.59E+07 | 1.59E+08 | 1.77E+08 | 2.12E+08 | 2.83E+08 | 3.71E+08 |
| | 3.71E+07 | 4.95E+07 | 8.83E+07 | 1.24E+08 | 2.12E+08 | 2.47E+08 | 2.30E+08 |
| | 3.71E+07 | 4.59E+07 | 8.83E+07 | 1.41E+08 | 2.30E+08 | 2.30E+08 | 3.00E+08 |

Table 4 (cont.). Tabulated calculated data of NLS7 in NLS7-*P. chlororaphis* (in)(cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7- *P. chlororaphis*)-3** | 2.83E+07 | 1.24E+07 | 3.53E+07 | 5.30E+07 | 1.77E+08 | 3.18E+08 | 3.89E+08 |
| | 2.12E+07 | 1.77E+07 | 1.24E+08 | 7.07E+07 | 2.65E+08 | 3.00E+08 | 2.30E+08 |
| | 8.83E+06 | 3.53E+07 | 8.83E+07 | 2.12E+08 | 2.12E+08 | 2.47E+08 | 3.36E+08 |
| | 1.24E+07 | 2.65E+07 | 1.77E+08 | 2.30E+08 | 3.18E+08 | 2.30E+08 | 3.71E+08 |
| | 2.30E+07 | 3.71E+07 | 5.30E+07 | 3.00E+08 | 2.12E+08 | 1.77E+08 | 3.89E+08 |
| | 1.77E+07 | 3.00E+07 | 3.53E+07 | 3.36E+08 | 2.30E+08 | 1.77E+08 | 2.30E+08 |
| | 2.12E+07 | 2.30E+07 | 7.07E+07 | 8.83E+07 | 2.12E+08 | 1.94E+08 | 2.47E+08 |
| | 3.36E+07 | 2.12E+07 | 3.53E+07 | 7.07E+07 | 1.77E+08 | 2.12E+08 | 3.00E+08 |
| | 1.24E+07 | 3.18E+07 | 5.30E+07 | 1.41E+08 | 1.59E+08 | 2.30E+08 | 3.18E+08 |
| | 1.41E+07 | 2.47E+07 | 2.12E+08 | 2.83E+08 | 1.24E+08 | 3.00E+08 | 3.18E+08 |
| **NLS7- *P. chlororaphis* -4** | 3.00E+07 | 3.53E+07 | 1.24E+08 | 3.53E+08 | 2.30E+08 | 4.24E+08 | 4.06E+08 |
| | 3.36E+07 | 2.65E+07 | 7.07E+07 | 2.47E+08 | 3.53E+08 | 3.89E+08 | 5.30E+08 |
| | 2.83E+07 | 3.71E+07 | 5.30E+07 | 3.71E+08 | 3.71E+08 | 3.53E+08 | 5.65E+08 |
| | 2.30E+07 | 4.06E+07 | 1.59E+08 | 2.12E+08 | 3.00E+08 | 2.83E+08 | 3.53E+08 |
| | 2.12E+07 | 3.18E+07 | 5.30E+07 | 3.00E+08 | 2.83E+08 | 3.00E+08 | 3.36E+08 |
| | 1.77E+07 | 2.12E+07 | 7.07E+07 | 3.36E+08 | 4.42E+08 | 3.18E+08 | 3.53E+08 |
| | 1.59E+07 | 2.65E+07 | 3.53E+07 | 1.77E+08 | 4.24E+08 | 3.71E+08 | 3.71E+08 |
| | 1.24E+07 | 1.77E+07 | 8.83E+07 | 2.12E+08 | 3.53E+08 | 4.77E+08 | 4.06E+08 |
| | 7.07E+06 | 1.24E+07 | 1.06E+08 | 2.30E+08 | 3.36E+08 | 4.59E+08 | 5.65E+08 |
| | 1.41E+07 | 8.83E+06 | 1.77E+08 | 1.94E+08 | 3.00E+08 | 3.36E+08 | 5.83E+08 |
| **NLS7 -*P. chlororaphis*(*in*)-5** | 8.83E+06 | 2.12E+07 | 2.30E+08 | 2.65E+08 | 3.00E+08 | 3.71E+08 | 4.77E+08 |
| | 5.30E+06 | 2.65E+07 | 2.12E+08 | 2.12E+08 | 2.83E+08 | 4.06E+08 | 3.71E+08 |
| | 1.59E+07 | 2.47E+07 | 1.77E+08 | 1.77E+08 | 3.00E+08 | 4.42E+08 | 4.06E+08 |
| | 1.06E+07 | 1.59E+07 | 1.24E+08 | 1.24E+08 | 2.47E+08 | 2.83E+08 | 4.24E+08 |
| | 1.41E+07 | 1.94E+07 | 8.83E+07 | 1.59E+08 | 2.30E+08 | 3.00E+08 | 2.83E+08 |
| | 2.12E+07 | 2.12E+07 | 1.41E+08 | 8.83E+07 | 1.94E+08 | 3.89E+08 | 3.36E+08 |
| | 2.30E+07 | 2.65E+07 | 2.12E+08 | 7.07E+07 | 3.53E+08 | 4.06E+08 | 5.65E+08 |

Table 4 (cont.). Tabulated calculated data of NLS7 in NLS7-*P. chlororaphis* (in)(cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7 -*P. chlororaphis(in)*-5** | 2.30E+07 | 2.30E+07 | 2.47E+08 | 1.77E+08 | 1.77E+08 | 4.77E+08 | 6.54E+08 |
| | 3.00E+07 | 4.42E+07 | 2.30E+08 | 1.24E+08 | 2.12E+08 | 5.30E+08 | 5.30E+08 |
| | 3.36E+07 | 3.53E+07 | 1.59E+08 | 1.06E+08 | 2.12E+08 | 4.42E+08 | 5.12E+08 |

Table 5. Tabulated raw data of NLS7 in NLS7-*P. chlororaphis* (out)(cells/mL) group via direct cell count.

| **Time(h)** | 0 | 24 | 48 | 72 | 96 | 120 | 144 |
|---|---|---|---|---|---|---|---|
| **NLS7 -*P. chlororaphis* (out)-1** | 2.00E+03 | 1.30E+04 | 3.00E+04 | 1.00E+05 | 3.00E+05 | 5.00E+05 | 1.20E+06 |
| | 2.40E+03 | 1.00E+04 | 2.60E+04 | 1.20E+05 | 2.90E+05 | 5.00E+05 | 1.70E+06 |
| | 2.20E+03 | 1.20E+04 | 2.80E+04 | 1.40E+05 | 2.80E+05 | 5.00E+05 | 1.60E+06 |
| | 2.20E+03 | 1.50E+04 | 2.80E+04 | 1.30E+05 | 2.70E+05 | 6.00E+05 | 1.60E+06 |
| | 2.30E+03 | 1.60E+04 | 2.80E+04 | 1.30E+05 | 2.60E+05 | 6.00E+05 | 1.50E+06 |
| | 2.10E+03 | 1.60E+04 | 2.60E+04 | 1.20E+05 | 3.00E+05 | 5.00E+05 | 1.20E+06 |
| | 1.70E+03 | 1.20E+04 | 2.90E+04 | 1.00E+05 | 3.00E+05 | 5.00E+05 | 1.30E+06 |
| | 2.10E+03 | 1.00E+04 | 3.00E+04 | 1.50E+05 | 2.30E+05 | 6.00E+05 | 1.10E+06 |
| | 1.80E+03 | 1.20E+04 | 2.10E+04 | 7.00E+04 | 2.60E+05 | 7.00E+05 | 1.80E+06 |
| | 2.20E+03 | 1.20E+04 | 2.80E+04 | 1.00E+04 | 2.80E+05 | 7.00E+05 | 1.80E+06 |
| **NLS7 -*P. chlororaphis* (out)-2** | 1.50E+03 | 1.00E+04 | 2.00E+04 | 1.20E+05 | 2.70E+05 | 5.00E+05 | 1.80E+06 |
| | 1.80E+03 | 1.50E+04 | 1.90E+04 | 1.30E+05 | 2.60E+05 | 5.00E+05 | 1.60E+06 |
| | 2.00E+03 | 1.60E+04 | 2.40E+04 | 6.00E+04 | 2.20E+05 | 6.00E+05 | 1.60E+06 |
| | 1.90E+03 | 1.60E+04 | 2.50E+04 | 6.00E+04 | 2.60E+05 | 4.00E+05 | 1.70E+06 |
| | 1.80E+03 | 1.60E+04 | 2.60E+04 | 7.00E+04 | 2.80E+05 | 3.00E+05 | 1.80E+06 |
| | 1.60E+03 | 1.70E+04 | 2.40E+04 | 9.00E+04 | 3.00E+05 | 7.00E+05 | 1.40E+06 |
| | 1.50E+03 | 1.50E+04 | 2.50E+04 | 1.00E+05 | 2.90E+05 | 3.00E+05 | 1.40E+06 |
| | 1.80E+03 | 1.50E+04 | 2.50E+04 | 1.10E+05 | 2.70E+05 | 3.00E+05 | 1.40E+06 |
| | 1.70E+03 | 1.40E+04 | 2.70E+04 | 1.30E+05 | 2.10E+05 | 5.00E+05 | 1.60E+06 |
| | 2.00E+03 | 1.30E+04 | 2.50E+04 | 1.20E+05 | 2.40E+05 | 5.00E+05 | 1.70E+06 |

Table 5(cont.). Tabulated raw data of NLS7 in NLS7-*P. chlororaphis* (out)(cells/mL) group via direct cell count.

| **Time(h)** | 0 | 24 | 48 | 72 | 96 | 120 | 144 |
|---|---|---|---|---|---|---|---|
| **NLS7 - *P. chlororaphis* (out)-3** | 5.00E+03 | 3.00E+03 | 1.70E+04 | 2.00E+04 | 5.00E+04 | 1.30E+05 | 3.00E+04 |
| | 4.00E+03 | 7.00E+03 | 1.80E+04 | 2.10E+04 | 4.00E+04 | 1.20E+05 | 4.00E+04 |
| | 7.00E+03 | 9.00E+03 | 1.50E+04 | 3.20E+04 | 9.00E+04 | 7.00E+04 | 5.00E+04 |
| | 1.00E+04 | 1.00E+04 | 1.40E+04 | 3.30E+04 | 7.00E+04 | 2.40E+05 | 2.00E+05 |
| | 9.00E+03 | 1.10E+04 | 1.60E+04 | 3.50E+04 | 8.00E+04 | 2.00E+05 | 2.10E+05 |
| | 1.20E+04 | 1.10E+04 | 1.30E+04 | 3.00E+04 | 6.00E+04 | 9.00E+04 | 2.70E+05 |
| | 1.10E+04 | 1.00E+04 | 1.20E+04 | 2.90E+04 | 1.00E+05 | 7.00E+04 | 2.80E+05 |
| | 6.00E+03 | 9.00E+03 | 1.00E+04 | 1.00E+04 | 1.20E+05 | 1.20E+05 | 1.90E+05 |
| | 8.00E+03 | 7.00E+03 | 9.00E+03 | 1.50E+04 | 2.00E+04 | 1.00E+05 | 2.10E+05 |
| | 3.00E+03 | 1.30E+04 | 2.00E+03 | 2.00E+04 | 3.00E+04 | 2.50E+05 | 2.70E+05 |
| **NLS7 - *P. chlororaphis* (out)-4** | 6.00E+03 | 2.00E+03 | 9.00E+03 | 1.20E+04 | 2.00E+04 | 2.00E+04 | 1.20E+05 |
| | 2.00E+03 | 3.00E+03 | 1.70E+04 | 1.30E+04 | 5.00E+04 | 3.00E+04 | 1.30E+05 |
| | 7.00E+03 | 1.10E+04 | 1.50E+04 | 1.14E+05 | 7.00E+04 | 1.00E+05 | 1.40E+05 |
| | 8.00E+03 | 1.50E+04 | 1.70E+04 | 9.00E+03 | 9.00E+04 | 1.10E+05 | 2.00E+05 |
| | 3.00E+03 | 1.20E+04 | 1.60E+04 | 7.00E+03 | 1.20E+05 | 1.30E+05 | 2.10E+05 |
| | 4.00E+03 | 1.40E+04 | 1.80E+04 | 6.00E+03 | 1.30E+05 | 1.50E+05 | 2.70E+05 |
| | 5.00E+03 | 1.20E+04 | 2.00E+04 | 1.70E+04 | 9.00E+04 | 1.60E+05 | 1.90E+05 |
| | 9.00E+03 | 1.00E+04 | 2.10E+04 | 1.90E+04 | 1.20E+05 | 1.70E+05 | 1.70E+05 |
| | 7.00E+03 | 7.00E+03 | 5.00E+03 | 2.00E+04 | 7.00E+04 | 1.80E+05 | 1.60E+05 |
| | 2.00E+03 | 4.00E+03 | 6.00E+03 | 2.20E+04 | 6.00E+04 | 1.20E+05 | 1.50E+05 |
| **NLS7 - *P. chlororaphis* (out)-5** | 4.00E+03 | 1.20E+04 | 1.00E+04 | 3.00E+04 | 1.20E+05 | 2.10E+05 | 3.00E+05 |
| | 5.00E+03 | 1.00E+04 | 1.20E+04 | 3.10E+04 | 1.30E+05 | 2.30E+05 | 2.40E+05 |
| | 2.00E+03 | 7.00E+03 | 1.30E+04 | 2.90E+04 | 1.60E+05 | 2.60E+05 | 2.90E+05 |
| | 9.00E+03 | 6.00E+03 | 1.40E+04 | 3.20E+04 | 1.70E+05 | 2.30E+05 | 2.70E+05 |
| | 9.00E+03 | 4.00E+03 | 1.70E+04 | 5.00E+03 | 1.80E+05 | 2.40E+05 | 4.00E+05 |
| | 1.00E+04 | 1.00E+04 | 1.90E+04 | 1.20E+04 | 1.00E+05 | 1.90E+05 | 3.90E+05 |
| | 9.00E+03 | 1.10E+04 | 1.80E+04 | 1.70E+04 | 1.90E+05 | 3.00E+05 | 3.60E+05 |

Table 5(cont.). Tabulated raw data of NLS7 in NLS7-*P. chlororaphis* (out)(cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7 - *P. chlororaphis* (out)-5** | 6.00E+03 | 1.30E+04 | 1.50E+04 | 3.00E+04 | 2.00E+05 | 1.20E+05 | 2.70E+05 |
| | 1.20E+04 | 1.50E+04 | 1.20E+04 | 2.70E+04 | 2.30E+05 | 1.00E+05 | 2.40E+05 |
| | 1.10E+04 | 1.70E+04 | 1.10E+04 | 2.20E+04 | 7.00E+04 | 1.10E+05 | 1.90E+05 |

Table 6. Tabulated calculated data of NLS7 in NLS7-*P. chlororaphis* (out)(cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| **NLS7 - *P. chlororaphis* (out)-1** | 3.53E+07 | 2.30E+08 | 5.30E+08 | 1.77E+09 | 5.30E+09 | 8.83E+09 | 2.12E+10 |
| | 4.24E+07 | 1.77E+08 | 4.59E+08 | 2.12E+09 | 5.12E+09 | 8.83E+09 | 3.00E+10 |
| | 3.89E+07 | 2.12E+08 | 4.95E+08 | 2.47E+09 | 4.95E+09 | 8.83E+09 | 2.83E+10 |
| | 3.89E+07 | 2.65E+08 | 4.95E+08 | 2.30E+09 | 4.77E+09 | 1.06E+10 | 2.83E+10 |
| | 4.06E+07 | 2.83E+08 | 4.95E+08 | 2.30E+09 | 4.59E+09 | 1.06E+10 | 2.65E+10 |
| | 3.71E+07 | 2.83E+08 | 4.59E+08 | 2.12E+09 | 5.30E+09 | 8.83E+09 | 2.12E+10 |
| | 3.00E+07 | 2.12E+08 | 5.12E+08 | 1.77E+09 | 5.30E+09 | 8.83E+09 | 2.30E+10 |
| | 3.71E+07 | 1.77E+08 | 5.30E+08 | 2.65E+09 | 4.06E+09 | 1.06E+10 | 1.94E+10 |
| | 3.18E+07 | 2.12E+08 | 3.71E+08 | 1.24E+09 | 4.59E+09 | 1.24E+10 | 3.18E+10 |
| | 3.89E+07 | 2.12E+08 | 4.95E+08 | 1.77E+08 | 4.95E+09 | 1.24E+10 | 3.18E+10 |
| **NLS7 - *P. chlororaphis* (out)-2** | 2.65E+07 | 1.77E+08 | 3.53E+08 | 2.12E+09 | 4.77E+09 | 8.83E+09 | 3.18E+10 |
| | 3.18E+07 | 2.65E+08 | 3.36E+08 | 2.30E+09 | 4.59E+09 | 8.83E+09 | 2.83E+10 |
| | 3.53E+07 | 2.83E+08 | 4.24E+08 | 1.06E+09 | 3.89E+09 | 1.06E+10 | 2.83E+10 |
| | 3.36E+07 | 2.83E+08 | 4.42E+08 | 1.06E+09 | 4.59E+09 | 7.07E+09 | 3.00E+10 |
| | 3.18E+07 | 2.83E+08 | 4.59E+08 | 1.24E+09 | 4.95E+09 | 5.30E+09 | 3.18E+10 |
| | 2.83E+07 | 3.00E+08 | 4.24E+08 | 1.59E+09 | 5.30E+09 | 1.24E+10 | 2.47E+10 |
| | 2.65E+07 | 2.65E+08 | 4.42E+08 | 1.77E+09 | 5.12E+09 | 5.30E+09 | 2.47E+10 |
| | 3.18E+07 | 2.65E+08 | 4.42E+08 | 1.94E+09 | 4.77E+09 | 5.30E+09 | 2.47E+10 |
| | 3.00E+07 | 2.47E+08 | 4.77E+08 | 2.30E+09 | 3.71E+09 | 8.83E+09 | 2.83E+10 |
| | 3.53E+07 | 2.30E+08 | 4.42E+08 | 2.12E+09 | 4.24E+09 | 8.83E+09 | 3.00E+10 |

Table 6 (cont.). Tabulated calculated data of NLS7 in NLS7-*P. chlororaphis* (out)(cells/mL) group via direct cell count.

| **Time(h)** | 0 | 24 | 48 | 72 | 96 | 120 | 144 |
|---|---|---|---|---|---|---|---|
| **NLS7 - *P. chlororaphis* (out)-3** | 8.83E+07 | 5.30E+07 | 3.00E+08 | 3.53E+08 | 8.83E+08 | 2.30E+09 | 5.30E+08 |
| | 7.07E+07 | 1.24E+08 | 3.18E+08 | 3.71E+08 | 7.07E+08 | 2.12E+09 | 7.07E+08 |
| | 1.24E+08 | 1.59E+08 | 2.65E+08 | 5.65E+08 | 1.59E+09 | 1.24E+09 | 8.83E+08 |
| | 1.77E+08 | 1.77E+08 | 2.47E+08 | 5.83E+08 | 1.24E+09 | 4.24E+09 | 3.53E+09 |
| | 1.59E+08 | 1.94E+08 | 2.83E+08 | 6.18E+08 | 1.41E+09 | 3.53E+09 | 3.71E+09 |
| | 2.12E+08 | 1.94E+08 | 2.30E+08 | 5.30E+08 | 1.06E+09 | 1.59E+09 | 4.77E+09 |
| | 1.94E+08 | 1.77E+08 | 2.12E+08 | 5.12E+08 | 1.77E+09 | 1.24E+09 | 4.95E+09 |
| | 1.06E+08 | 1.59E+08 | 1.77E+08 | 1.77E+08 | 2.12E+09 | 2.12E+09 | 3.36E+09 |
| | 1.41E+08 | 1.24E+08 | 1.59E+08 | 2.65E+08 | 3.53E+08 | 1.77E+09 | 3.71E+09 |
| | 5.30E+07 | 2.30E+08 | 3.53E+07 | 3.53E+08 | 5.30E+08 | 4.42E+09 | 4.77E+09 |
| **NLS7 - *P. chlororaphis* (out)-4** | 1.06E+08 | 3.53E+07 | 1.59E+08 | 2.12E+08 | 3.53E+08 | 3.53E+08 | 2.12E+09 |
| | 3.53E+07 | 5.30E+07 | 3.00E+08 | 2.30E+08 | 8.83E+08 | 5.30E+08 | 2.30E+09 |
| | 1.24E+08 | 1.94E+08 | 2.65E+08 | 2.01E+09 | 1.24E+09 | 1.77E+09 | 2.47E+09 |
| | 1.41E+08 | 2.65E+08 | 3.00E+08 | 1.59E+08 | 1.59E+09 | 1.94E+09 | 3.53E+09 |
| | 5.30E+07 | 2.12E+08 | 2.83E+08 | 1.24E+08 | 2.12E+09 | 2.30E+09 | 3.71E+09 |
| | 7.07E+07 | 2.47E+08 | 3.18E+08 | 1.06E+08 | 2.30E+09 | 2.65E+09 | 4.77E+09 |
| | 8.83E+07 | 2.12E+08 | 3.53E+08 | 3.00E+08 | 1.59E+09 | 2.83E+09 | 3.36E+09 |
| | 1.59E+08 | 1.77E+08 | 3.71E+08 | 3.36E+08 | 2.12E+09 | 3.00E+09 | 3.00E+09 |
| | 1.24E+08 | 1.24E+08 | 8.83E+07 | 3.53E+08 | 1.24E+09 | 3.18E+09 | 2.83E+09 |
| | 3.53E+07 | 7.07E+07 | 1.06E+08 | 3.89E+08 | 1.06E+09 | 2.12E+09 | 2.65E+09 |
| **NLS7 - *P. chlororaphis* (out)-5** | 7.07E+07 | 2.12E+08 | 1.77E+08 | 5.30E+08 | 2.12E+09 | 3.71E+09 | 5.30E+09 |
| | 8.83E+07 | 1.77E+08 | 2.12E+08 | 5.48E+08 | 2.30E+09 | 4.06E+09 | 4.24E+09 |
| | 3.53E+07 | 1.24E+08 | 2.30E+08 | 5.12E+08 | 2.83E+09 | 4.59E+09 | 5.12E+09 |
| | 1.59E+08 | 1.06E+08 | 2.47E+08 | 5.65E+08 | 3.00E+09 | 4.06E+09 | 4.77E+09 |
| | 1.59E+08 | 7.07E+07 | 3.00E+08 | 8.83E+07 | 3.18E+09 | 4.24E+09 | 7.07E+09 |
| | 1.77E+08 | 1.77E+08 | 3.36E+08 | 2.12E+08 | 1.77E+09 | 3.36E+09 | 6.89E+09 |
| | 1.59E+08 | 1.94E+08 | 3.18E+08 | 3.00E+08 | 3.36E+09 | 5.30E+09 | 6.36E+09 |

Table 6 (cont.). Tabulated calculated data of NLS7 in NLS7-*P. chlororaphis* (out)(cells/mL) group via direct cell count.

| **Time(h)** | **0** | **24** | **48** | **72** | **96** | **120** | **144** |
|---|---|---|---|---|---|---|---|
| | 1.06E+08 | 2.30E+08 | 2.65E+08 | 5.30E+08 | 3.53E+09 | 2.12E+09 | 4.77E+09 |
| | 2.12E+08 | 2.65E+08 | 2.12E+08 | 4.77E+08 | 4.06E+09 | 1.77E+09 | 4.24E+09 |
| | 1.94E+08 | 3.00E+08 | 1.94E+08 | 3.89E+08 | 1.24E+09 | 1.94E+09 | 3.36E+09 |

Table 7. Tabulated data of NLS7 average $OD_{600nm}$ measurement, calculated and actual cell concentrations with standard deviations in NLS7-only culture.

| **Time (h)** | **$OD_{600nm}$ Avg.** | **$OD_{600nm}$ Std Dev** | **Calculated Cell Concentration Avg.** | **Calculated Cell Concentration Std Dev** | **Actual Cell Concentration Avg.** | **Actual Cell Concentration Std Dev.** |
|---|---|---|---|---|---|---|
| **0** | 0.040 | 0.001 | 8.33E+05 | 4.41E+06 | 1.73E+07 | 7.77E+06 |
| **24** | 0.043 | 0.003 | 1.54E+07 | 1.81E+07 | 2.80E+07 | 1.23E+07 |
| **48** | 0.048 | 0.005 | 3.96E+07 | 2.85E+07 | 4.53E+07 | 2.62E+07 |
| **72** | 0.056 | 0.006 | 8.21E+07 | 3.43E+07 | 8.34E+07 | 4.96E+07 |
| **96** | 0.066 | 0.004 | 1.32E+08 | 2.20E+07 | 1.61E+08 | 6.45E+07 |
| **120** | 0.096 | 0.004 | 2.78E+08 | 2.50E+07 | 3.72E+08 | 7.21E+07 |
| **144** | 0.113 | 0.005 | 3.65E+08 | 2.93E+07 | 6.24E+08 | 3.37E+07 |

Table 8. Tabulated data of NLS7 average $OD_{600nm}$ measurement, calculated and actual cell concentrations with standard deviations in NLS7-*P chlororaphis* (in).

| **Time (h)** | **$OD_{600nm}$ Avg.** | **$OD_{600nm}$ Std Dev** | **Calculated Cell Concentration Avg.** | **Calculated Cell Concentration Std Dev** | **Actual Cell Concentration Avg.** | **Actual Cell Concentration Std Dev.** |
|---|---|---|---|---|---|---|
| **0** | 0.044 | 0.002 | 2.03E+07 | 1.04E+07 | 2.61E+07 | 9.93E+06 |
| **24** | 0.051 | 0.002 | 5.30E+07 | 1.02E+07 | 3.28E+07 | 9.55E+06 |
| **48** | 0.066 | 0.004 | 1.30E+08 | 2.17E+07 | 1.17E+08 | 3.77E+07 |
| **72** | 0.083 | 0.004 | 2.13E+08 | 2.17E+07 | 1.80E+08 | 4.78E+07 |
| **96** | 0.097 | 0.004 | 2.86E+08 | 2.23E+07 | 2.51E+08 | 5.14E+07 |
| **120** | 0.105 | 0.007 | 3.25E+08 | 3.74E+07 | 3.18E+08 | 7.04E+07 |
| **144** | 0.128 | 0.015 | 4.39E+08 | 8.18E+07 | 3.64E+08 | 8.02E+07 |

Table 9. Tabulated data of NLS7 average $OD_{600nm}$ measurement, calculated and actual cell concentrations with standard deviations in NLS7-*P chlororaphis* (out).

| **Time (h)** | **$OD_{600nm}$ Avg.** | **$OD_{600nm}$ Std Dev** | **Calculated Cell Concentration Avg.** | **Calculated Cell Concentration Std Dev** | **Actual Cell Concentration Avg.** | **Actual Cell Concentration Std Dev.** |
|---|---|---|---|---|---|---|
| **0** | 0.053 | 0.010 | 6.47E+07 | 5.71E+07 | 8.59E+07 | 5.02E+07 |
| **24** | 0.072 | 0.004 | 1.58E+08 | 2.44E+07 | 1.98E+08 | 4.42E+07 |
| **48** | 0.099 | 0.019 | 2.96E+08 | 1.04E+08 | 3.26E+08 | 1.19E+08 |
| **72** | 0.130 | 0.029 | 4.50E+08 | 1.62E+08 | 9.80E+08 | 7.67E+08 |
| **96** | 0.176 | 0.049 | 6.80E+08 | 2.72E+08 | 2.96E+09 | 1.73E+09 |
| **120** | 0.203 | 0.062 | 8.14E+08 | 3.48E+08 | 5.23E+09 | 3.63E+09 |
| **144** | 0.239 | 0.060 | 9.93E+08 | 3.33E+08 | 1.31E+10 | 1.29E+10 |

Table 10. Methane (µmol) changes in NLS7-only, NLS7-*P. chlororaphis* (in) and NLS7-*P. chlororaphis* (out) from batch test

| **Time (h)** | **NLS7-Only Average** | **NLS7-only Std Dev** | **NLS7-*P. chlororaphis* (in) Average** | **NLS7-*P. chlororaphis* (in) Std Dev** | **NLS7-*P. chlororaphis* (out) Average** | **NLS7-*P. chlororaphis* (out) Std Dev** |
|---|---|---|---|---|---|---|
| **0** | 232.85 | 7.47 | 257.77 | 6.71 | 249.19 | 18.45 |
| **3** | 231.26 | 7.02 | 235.85 | 14.40 | 240.99 | 15.58 |
| **6** | 225.01 | 8.27 | 220.32 | 14.60 | 233.09 | 12.47 |
| **9** | 215.70 | 6.68 | 204.75 | 28.62 | 223.67 | 8.90 |
| **12** | 199.22 | 14.01 | 189.98 | 17.32 | 207.29 | 7.84 |

Table 11. $CO_2$ (µmol) changes in NLS7-only, NLS7-*P. chlororaphis* (in) and NLS7-*P. chlororaphis* (out) from batch test

| **Time (h)** | **NLS7-Only Average** | **NLS7-only Std Dev** | **NLS7-*P. chlororaphis* (in) Average** | **NLS7-*P. chlororaphis* (in) Std Dev** | **NLS7-*P. chlororaphis* (out) Average** | **NLS7-*P. chlororaphis* (out) Std Dev** |
|---|---|---|---|---|---|---|
| **0** | 1.24 | 0.27 | 1.86 | 0.32 | 1.21 | 0.39 |
| **3** | 2.43 | 0.51 | 6.67 | 2.27 | 3.75 | 1.05 |
| **6** | 4.44 | 1.69 | 9.07 | 3.32 | 4.52 | 1.60 |
| **9** | 5.76 | 1.27 | 10.48 | 5.04 | 5.96 | 1.38 |
| **12** | 6.56 | 1.39 | 11.60 | 4.90 | 7.07 | 1.31 |

Table 12. Tabulated data of actual viable *P. chlororaphis* cell count in NLS7-*P. chlororaphis* (in) group.

| | **OD600nm Avg** | **OD600nm Std Dev** | **Calculated cell concentration Avg** | **Calculated cell concentration Std Dev** | **Actual cell concentration Avg** | **Actual cell concentration Std Dev** |
|---|---|---|---|---|---|---|
| ***P. chlororaphis* initial inoculum** | 0.044 | 0.003 | 5.30E+07 | 9.90E+06 | 3.68E+06 | 4.28E+06 |
| **NLS7-*P. chlororaphis*(in)-1** | 0.130 | 0.003 | 3.11E+08 | 7.55E+06 | 6.70E+08 | 1.20E+08 |
| **NLS7-*P. chlororaphis*(in)-2** | 0.130 | 0.001 | 3.10E+08 | 3.00E+06 | 5.60E+08 | 1.19E+08 |
| **NLS7-*P. chlororaphis*(in)-3** | 0.112 | 0.002 | 2.56E+08 | 5.20E+06 | 6.60E+08 | 1.67E+08 |
| **NLS7-*P. chlororaphis*(in)-4** | 0.139 | 0.002 | 3.37E+08 | 5.20E+06 | 6.10E+08 | 9.62E+07 |
| **NLS7-*P. chlororaphis*(in)-5** | 0.232 | 0.003 | 6.16E+08 | 7.94E+06 | 7.00E+09 | 7.91E+08 |
| **NLS7-*P. chlororaphis*(in)-6** | 0.211 | 0.001 | 5.52E+08 | 3.46E+06 | 7.40E+09 | 9.62E+08 |
| **NLS7-*P. chlororaphis*(in)-7** | 0.131 | 0.001 | 3.14E+08 | 1.73E+06 | 6.40E+07 | 1.19E+07 |
| **NLS7-*P. chlororaphis*(in)-8** | 0.197 | 0.011 | 5.11E+08 | 3.17E+07 | 3.00E+08 | 0.00E+00 |
| **NLS7-*P. chlororaphis*(in)-9** | 0.182 | 0.002 | 4.67E+08 | 6.24E+06 | 4.40E+08 | 6.52E+07 |
| **NLS7-*P. chlororaphis*(in)-10** | 0.172 | 0.000 | 4.36E+08 | 0.00E+00 | 3.40E+08 | 1.56E+08 |

Table 13. Tabulated data of actual viable *P. chlororaphis* cell count in NLS7-*P. chlororaphis* (out) group.

| **Time(h)** | **NLS7-*P. chlororaphis*(out) Average (CFU/mL)** | **NLS7-*P. chlororaphis*(out) Std Dev (CFU/mL)** | **Only *P. chlororaphis* Control (CFU/mL)** |
|---|---|---|---|
| **0** | 1.38E+05 | 1.14E+05 | 1.60E+04 |
| **24** | 1.40E+05 | 1.17E+05 | 2.40E+04 |
| **48** | 8.94E+05 | 1.09E+06 | 2.50E+04 |
| **72** | 1.31E+06 | 1.39E+06 | 3.20E+04 |
| **96** | 4.99E+06 | 7.42E+06 | 2.30E+04 |
| **120** | 1.25E+07 | 9.28E+06 | 1.90E+04 |
| **144** | 2.21E+07 | 1.41E+07 | 1.50E+04 |

Table 14. Tabulated data of MOR and CPR on a per cell basis in NLS7-only, NLS7-*P. chlororaphis* (in) and NLS7-*P. chlororaphis* (out) groups for each experimental replicate.

| **Group Name** | **$10^{-12}$ µmol $CH_4$ cell $^{-1}$ h $^{-1}$** | **$10^{-12}$ µmol $CO_2$ cell $^{-1}$ h $^{-1}$** |
|---|---|---|
| **NLS7 only-1** | 6.05E+00 | 9.84E-01 |
| **NLS7 only-2** | 6.79E+00 | 1.43E+00 |
| **NLS7 only-3** | 8.70E+00 | 1.94E+00 |
| **NLS7 only-4** | 8.55E+00 | 1.97E+00 |
| **NLS7- *P. chlororaphis* (in)-1** | 1.17E+01 | 1.80E+00 |
| **NLS7- *P. chlororaphis* (in)-2** | 8.70E+00 | 1.63E+00 |
| **NLS7- *P. chlororaphis* (in)-3** | 1.14E+01 | 5.27E+00 |
| **NLS7- *P. chlororaphis* (in)-4** | 1.04E+01 | 1.75E+00 |
| **NLS7- *P. chlororaphis* (in)-5** | 2.24E+01 | 1.36E+00 |
| **NLS7- *P. chlororaphis* (out)-1** | 1.01E+01 | 9.39E-01 |
| **NLS7- *P. chlororaphis* (out)-2** | 6.09E+00 | 8.25E-01 |
| **NLS7- *P. chlororaphis* (out)-3** | 1.22E+00 | 4.27E-01 |
| **NLS7- *P. chlororaphis* (out)-4** | 3.01E+00 | 4.43E-01 |
| **NLS7-*P. chlororaphis*(out)-5** | 1.56E+00 | 6.32E-01 |

Table 15. Tabulated raw data of the standard curve for NLS7 and *P. chlororaphis* cell concentration over $OD_{600nm}$.

| ***P. chlororaphis*** **($OD_{600nm}$)** | ***P. chlororaphis*** **(CFU/mL)** | ***Methylocystis sp.*** **NLS7 ($OD_{600nm}$)** | ***Methylocystis sp.*** **NLS7 (cells/mL)** |
|---|---|---|---|
| 0.403 | 1.24E+09 | 0.209 | 9.18E+08 |
| 0.171 | 6.20E+08 | 0.117 | 4.59E+08 |
| 0.107 | 3.10E+08 | 0.076 | 2.30E+08 |
| 0.071 | 1.55E+08 | 0.054 | 1.15E+08 |
| 0.054 | 9.00E+07 | 0.044 | 2.65E+07 |
| 0.043 | 4.50E+07 | - | - |

# APPENDIX B

## STATISTICAL ANALYSIS IN ANOVA

Figure 5. Tabulated data of statistical analysis in ANOVA, Tukey pairwise comparison test in all three groups for MOR.

Tukey Pairwise Comparisons: Group

**Grouping Information Using the Tukey Method and 95% Confidence**

| **Group** | **N** | **Mean** | **Grouping** |
|---|---|---|---|
| (2) NLS7-P.chlororaphis(in) | 5 | 6.91354 | A |
| (3)NLS7-P.chlororaphis(out) | 5 | 4.29022 | A |
| (1) NLS7-only | 4 | 3.45082 | A |

*Means that do not share a letter are significantly different.*

**Tukey Simultaneous Tests for Differences of Means**

| **Difference of Group Levels** | **Difference of Means** | **SE of Difference** | **DF** | **Simultaneous 95% CI** | **T-Value** | **Adjusted P-Value** |
|---|---|---|---|---|---|---|
| 2 - 1 | 3.46 | 1.59 | 3.20738 | (-3.21, 10.14) | 2.18 | 0.222 |
| 3 - 1 | 0.84 | 1.59 | 3.20738 | (-5.83, 7.51) | 0.53 | 0.864 |
| 3 - 2 | -2.62 | 1.54 | 2.64487 | (-9.09, 3.84) | -1.70 | 0.339 |

*Individual confidence level = 97.50%*

Figure 6. Tabulated data of statistical analysis in ANOVA, Tukey pairwise comparison test in all three groups for CPR.

Tukey Pairwise Comparisons: Group

**Grouping Information Using the Tukey Method and 95% Confidence**

| Group | N | Mean | Grouping |
|---|---|---|---|
| (2) NLS7-P.chlororaphis(in) | 5 | 0.970828 | A |
| (1) NLS7-only | 4 | 0.581890 | A |
| (3)NLS7-P.chlororaphis(out) | 5 | 0.579894 | A |

*Means that do not share a letter are significantly different.*

**Tukey Simultaneous Tests for Differences of Means**

| Difference of Group Levels | Difference of Means | SE of Difference | DF | Simultaneous 95% CI | T-Value | Adjusted P-Value |
|---|---|---|---|---|---|---|
| 2 - 1 | 0.389 | 0.218 | 11 | (-0.199, 0.977) | 1.79 | 0.219 |
| 3 - 1 | -0.002 | 0.218 | 11 | (-0.590, 0.586) | -0.01 | 1.000 |
| 3 - 2 | -0.391 | 0.205 | 11 | (-0.945, 0.164) | -1.90 | 0.183 |

*Individual confidence level = 97.94%*

Figure 7. Tabulated data of statistical analysis in ANOVA, Tukey pairwise comparison test in all three groups for the specific growth rate.

Tukey Pairwise Comparisons: Group

**Grouping Information Using the Tukey Method and 95% Confidence**

| Group | N | Mean | Grouping |
|---|---|---|---|
| (1) NLS7-only | 4 | 0.0292270 | A |
| (2) NLS7-P.chlororaphis(in) | 5 | 0.0259085 | A |
| (3)NLS7-P.chlororaphis(out) | 5 | 0.0162470 | A |

*Means that do not share a letter are significantly different.*

**Tukey Simultaneous Tests for Differences of Means**

| Difference of Group Levels | Difference of Means | SE of Difference | DF | Simultaneous 95% CI | T-Value | Adjusted P-Value |
|---|---|---|---|---|---|---|
| 2 - 1 | -0.00332 | 0.00458 | 2.99927 | (-0.02264, 0.01600) | -0.72 | 0.768 |
| 3 - 1 | -0.01298 | 0.00458 | 2.99927 | (-0.03230, 0.00634) | -2.83 | 0.130 |
| 3 - 2 | -0.00966 | 0.00454 | 2.87750 | (-0.02880, 0.00948) | -2.13 | 0.233 |

*Individual confidence level = 97.50%*

Figure 8. Tabulated data of statistical analysis in ANOVA, Tukey pairwise comparison test in all three groups for MOR on a per cell basis.

Tukey Pairwise Comparisons: Group

**Grouping Information Using the Tukey Method and 95% Confidence**

| Group | N | Mean | Grouping |
|---|---|---|---|
| (2) NLS7-P.chlororaphis(in) | 5 | 12.7056 | A |
| (1) NLS7-only | 4 | 7.5218 | A |
| (3)NLS7-P.chlororaphis(out) | 5 | 4.7179 | A |

*Means that do not share a letter are significantly different.*

**Tukey Simultaneous Tests for Differences of Means**

| Difference of Group Levels | Difference of Means | SE of Difference | DF | Simultaneous 95% CI | T-Value | Adjusted P-Value |
|---|---|---|---|---|---|---|
| 2 - 1 | 5.18 | 3.38 | 3.19288 | (-8.75, 19.12) | 1.53 | 0.392 |
| 3 - 1 | -2.80 | 3.38 | 3.19288 | (-16.74, 11.13) | -0.83 | 0.713 |
| 3 - 2 | -7.99 | 3.31 | 2.84513 | (-21.62, 5.64) | -2.42 | 0.178 |

*Individual confidence level = 97.52%*

Figure 9. Tabulated data of statistical analysis in ANOVA, Tukey pairwise comparison test in all three groups for CPR on a per cell basis.

Tukey Pairwise Comparisons: Group

**Grouping Information Using the Tukey Method and 95% Confidence**

| Group | N | Mean | Grouping |
|---|---|---|---|
| (2) NLS7-P.chlororaphis(in) | 5 | 2.36169 | A |
| (1) NLS7-only | 4 | 1.58226 | A |
| (3)NLS7-P.chlororaphis(out) | 5 | 0.65295 | A |

Means that do not share a letter are significantly different.

**Tukey Simultaneous Tests for Differences of Means**

| Difference of Group Levels | Difference of Means | SE of Difference | DF | Simultaneous 95% CI | T-Value | Adjusted P-Value |
|---|---|---|---|---|---|---|
| 2 - 1 | 0.779 | 0.688 | 11 | (-1.078, 2.637) | 1.13 | 0.514 |
| 3 - 1 | -0.929 | 0.688 | 11 | (-2.786, 0.928) | -1.35 | 0.398 |
| 3 - 2 | -1.709 | 0.648 | 11 | (-3.460, 0.042) | -2.64 | 0.056 |

Individual confidence level = 97.94%

# APPENDIX C

## CULTIVATION SYSTEM PHOTOS

Figure 10. Methane fed batch stirred tank reactor (STR).

Figure 11. Cultures during the batch test.