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Optimizing the Methanotrophic Production of Polyhydroxyalkanoates Using Mixed Microbial Bacteria Cultures

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OPTIMIZING THE METHANOTROPHIC PRODUCTION OF

POLYHYDROXYALKANOATES USING MIXED MICROBIAL CULTURES

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The use of petroleum-based plastics has caused serious economic and environmental problems. It is imperative to develop sustainable ways to produce valuable and environmentally-friendly materials. Methanotrophic bacteria are known for assimilating methane while generating polyhydroxybutyrate (PHB) biopolymers, which are potential substitutes for conventional plastics. Additionally, biologically generated methane and volatile fatty acids (VFAs) are abundant and readily accessible through anaerobic digestion of organic wastes. Thus, optimizing the methanotrophic production will bring significant economic and environmental benefits.

In section one, a feast-famine based cultivation system was adopted to produce PHAs using *Hyphomicrobium,* and *Methylocysits* dominated bacterial culture. The mixed bacterial culture was fed with acetic acid along with methane was subsequently evaluated for the enhancement of PHB production. Throughout the 27 cycles of cultivations, the mixed bacterial culture fed with methane alone produced the PHB in an average of 26.4 ± 3.5 wt%. By comparison, the PHB production was increased up to 34.8 ± 1.8 wt% when acetic acid was added at the concentration of 300 mg/L under pH-controlled conditions. Additionally, the characterization of PHB polymers (e.g., chemical composition and molecular weight) illustrated

that the presence of acetic acid at optimal pH conditions facilitated the elongation of the polymer chain with higher molecular weight.

In section two, the feasibility and suitability for scaling up PHAs production by using inocula from wastewater treatment plants (WWTPs) were explored. Methanotrophic enrichment cultures were cultivated by using various inocula from activated sludge and anaerobic digestion processes. Methanotrophs and PHA-accumulating bacteria were characterized by polymerase chain reaction (PCR) products of the *pmoA* and *phaC* functional gene. After 5 and 15 cycles of enrichment with methane as the sole carbon source, all cultures presented positive results of the *pmoA* and *phaC* gene, which indicate the accumulation of methanotrophs and PHA-accumulating bacteria. The metagenomic sequencing of the 16S rRNA gene from original sludges and enrichment cultures showed that the microbial community of enrichment narrowed down the operational taxonomic unit (OTU) for approximately 96.4%. After the enrichment processes, taxonomy diversity of culture with sludge inoculum from Village Creek Reclamation Facility displayed higher bacterial diversity compared with the cultures from Southside WWTP.

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Chapter 1

INTRODUCTION

The massive production of plastics began in the 1940s, and the inexpensive manufacturing techniques have led to intensive use of plastics in nearly inexhaustible applications with 359 million metric tons of plastic produced globally in 2018 (Europe, 2019). While the social benefits of plastics are far-reaching, the disposal of plastic waste becomes problematic because the durability of plastic makes it highly resistant to degradation in the environment. Current estimates indicate that approximately 60% of all plastics ever produced remain undegraded by 2015 (Cox et al., 2019). In recent years, there have been increasing environmental and health concerns about plastic contamination.

Large plastic items have been reported in the marine environment since the early days of production. They act as vectors for the chemical absorption and transfer of pollutants within the food chain. These pollutants are regarded as persistent organic pollutants (POPs), which are toxic, persistent in the environment, bioaccumulative, hydrophobic, and have long-range transport potential (Zarfl & Matthies, 2010).

Microplastics are small plastic fragments (~20 μm) derived from the breakdown of macroplastics and the direct usage of tiny plastic granules in the cosmetic industry

(Cole et al., 2011; Hartmann et al., 2019). A growing body of evidence has proved that microplastics are ubiquitous in the marine environment and pose a threat to biota owing to their bioavailability (W. C. LI et al., 2016). Microplastics can be ingested by low trophic organisms and accumulate intracellularly, resulting in physical harm such as internal abrasions and blockages (Wright et al., 2013). Toxicity could also arise from leaching constituent contaminants such as monomers and plastic additives, which is capable of causing carcinogenesis and endocrine disruption (Oehlmann et al., 2009; Wright et al., 2013). Most importantly, the microparticles can eventually enter human bodies via diverse pathways. Recent studies demonstrated that biological accumulation of microplastics has the potential to translocate to human tissues and trigger a localized immune response by releasing monomers and toxic additives (Cox et al., 2019).

Though the production of petroleum-based plastics has wide commercial applicability, the manufacturing process still poses threats to global warming and fossil fuel depletion (Harding et al., 2007). Most efforts to solve the problem of plastic pollution have been focused on the source control: the reduced production of petroleum-based plastics and substitution with sustainable and biodegradable materials (Lambert & Wagner, 2018; Wu et al., 2016). Among the alternatives, biopolymers are gaining commercial interest and providing significant environmental benefits compared to traditional polymers in this contemporary world because of their biodegradable characteristics and production from renewable resources instead of fossil fuels.

Polyhydroxyalkanoates (PHAs) are biopolymers synthesized and deposited intracellularly as granules inside microbial cells that serve as a source of carbon, energy, or reducing power by exposing an active culture to excessive carbons while under a nutrient limitation (Strong et al.,

2015). There are different microorganisms with the capability to produce different types of PHAs, and the nature of carbon feedstock affects the type of PHA produced. Over 100 PHA molecules have been identified containing long polyester chains with repeated monomer units. A PHA molecule is typically made up of 600 to 35000 hydroxy monomer units. Each monomer unit possesses a side chain *R* group, which is usually a saturated alkyl group (Tan et al., 2014).

1.1. Methanotrophs

Methylotrophs and autotrophs are regarded as biosynthetic virtuosi of the planet because both organisms are capable of carbon fixation (Anthony, 1982). Autotrophs utilize carbon dioxide as the sole carbon source to synthesize their cell components. By contrast, methylotrophs metabolize reduced one-carbon compounds such as methane, methanol, and methylamine as their sole carbon and energy source. Methanotrophs were firstly identified in 1906 (Söhngen, 1906) and are distinguished from other microorganisms because of their ability to use methane as their sole carbon and energy source. Methanotrophs play a vital role in global carbon cycling, and they have been found to inhabit a wide range of environments, including wetlands, freshwater, rivers, lakes, swamps, marine sediments, sewage sludge, hydrothermal vents, agricultural soils (Hanson & Hanson, 1996). With the growing concerns over climate changes and global warming, increasing attention has been paid to how to cost-effectively reduce anthropogenic emissions of greenhouse gases, particularly methane from landfills and agricultural soils.

Most of the methanotrophs are gram-negative, and the shapes of different genera vary from cocci, bacillus, to vibrio. Due to the significant differences in intracytoplasmic structures and the metabolic pathways, methanotrophs were tentatively classified into two types above the

genus level as Type I and Type II (Colby, 1979). Modern taxonomy involves more aspects than just the morphology. The current phylogenic distribution of known methanotrophic genera includes the 16S rRNA gene sequence, carbon assimilation pathways, the ability to commit full tricarboxylic acid cycle, DNA base ratio, and more, in deciding the category. Aerobic methanotrophs mostly fall into the *Alpha* and *Gamma* subdivisions of *Proteobacteria* phylum, while a few of them are located in the *Verrucomicrobia* phylum. The Type I methanotrophs are found in the family of *Methylococcaceae,* which consists of the genera *Methylococcus*, *Methylomonas, Methylobacter, Methylothermus, Methylocaldum.* Type II methanotrophs, including the genera *Methylosinus, Methylocystis, Methylocella, and Methylocapasa*, are categorized into the family *Methylocystaceae* and *Beijerinckiaceae* (AlSayed, Fergala, & Eldyasti, 2018).

1.2. Structural features of methanotrophic bacteria

All the methanotrophs have different complex internal membrane structures. Specifically, Type I methanotrophic bacteria have intracytoplasmic membrane arranged as bundles of vesicular discs that transverse the width of the cell (Smith et al., 1970), while Type II methanotrophic bacteria have the intracytoplasmic membranes localized around the cell periphery (Weaver & Dugan, 1975). The morphology of intracytoplasmic membranes may change as bacteria enter different growing conditions. For example, under exponential growth conditions, bacterial cells presented a more orderly stacked disc of membranes; while as bacteria entered the stationary phase, these cellular membrane bundles became less numerous and orderly (Hyder et al., 1979). By comparison, the methylotrophs that are capable of growing on methanol, but not methane, do not have such internal membranes. These critical membranes perform

special functions on the oxidation of methane to methanol, which is the very first step of methane catabolism and the growth-limiting step (Murrell, 2010).

1.3. Biochemical and physiological features of methanotrophs

Two major carbon assimilation pathways widely occur among methanotrophs. As indicated in Figure 1.1 (Juan C. López et al., 2019), methane is firstly oxidized to methanol. In this process, methane monooxygenases (MMOs) split the O-O bonds of dioxygen: one of the oxygens is used to form water, and the other is incorporated into methane to form methanol. Two forms of MMOs are found in methanotrophs: soluble MMO (sMMO) and particulate MMO (pMMO). sMMO does not contain a heme cofactor or any other cofactors, while pMMO, also known as membrane-bound MMO, only presents when the copper concentration is high enough to induse activity (Hanson & Hanson, 1996). For both forms of MMOs, the metal copper and its intracellular concentration are known to be a key factor in regulating the expression genes encoding both enzymes. All methanotrophs that have been studied are able to express pMMO when growing in the presence of copper, but the ability to form sMMO has been observed only in some Type II methanotrophs. Bacterial cells that contain pMMO have higher growth yields and show a more significant affinity for methane than bacteria cells that express sMMO, because sMMO oxidation requires the reduced nicotinamide adenine dinucleotide (NADH) as electron donor, whose supply can be a growth-limiting factor (Hanson & Hanson, 1996; Patel et al., 1982). The membrane-associated MMOs found in the methanotrophs are located in the cytoplasmic membrane, while sMMO existing in some methanotrophs is dislocated in the

cytoplasm. Until now, the components of sMMO have been well characterized, while little is known about the molecular properties of the pMMO.

Methanol from both endogenous and exogenous sources is oxidized to formaldehyde by methanol dehydrogenase (MDH). MDH, along with cytochrome, completes the transformation of methanol to formaldehyde (Kalyuzhnaya et al., 2015). Formaldehyde functions as a significant intermediate metabolite which enters different metabolic pathways. Type I methanotrophs typically utilize the ribulose monophosphate (RuMP) pathway for formaldehyde assimilation, while Type II methanotrophs use the serine pathway for formaldehyde assimilation. In the meantime, formaldehyde can be continuously oxidized to formic acid and further be entirely mineralized to carbon dioxide (Trotsenko & Murrell, 2008).

Formaldehyde is assimilated by the Type II methanotrophs into the serine pathway, in which malyl-CoA is generated and readily converted to one molecule of acetyl-CoA and one molecule of glycosylate. When bacteria are living in an environment with balanced nutrients, acetyl-CoA serves as the precursor and enters the TCA cycle to reach the maximum generation of energy. However, when bacteria are living in an environment with imbalanced nutrients such as a deficit of nitrogen or phosphates (Pieja et al., 2011), acetyl-CoA enters the PHB cycle to conserve the carbon source in the form of intracellular inclusions.

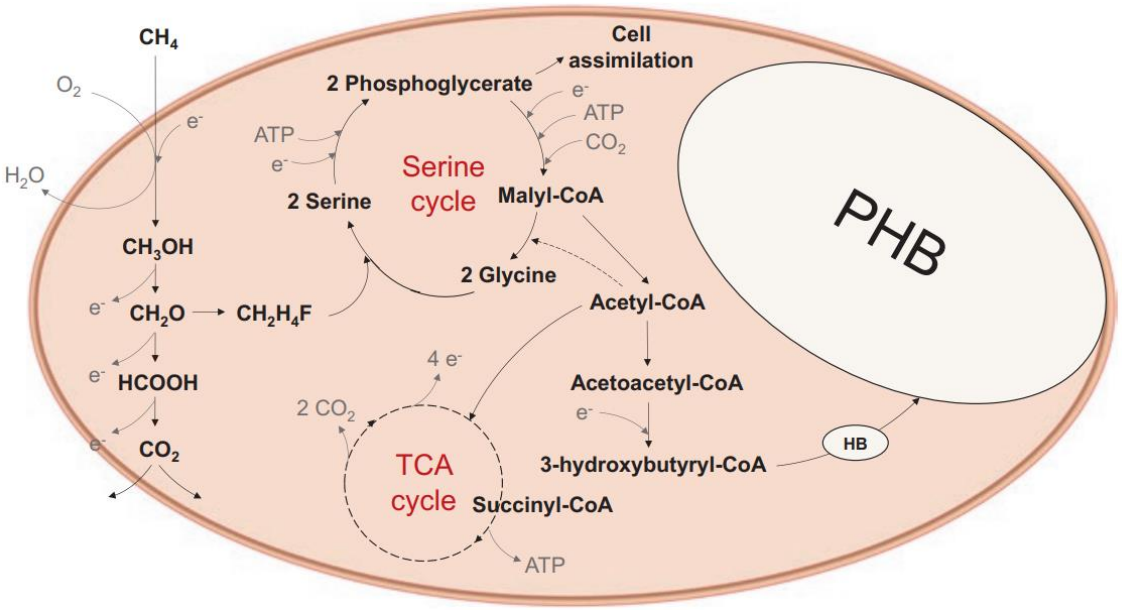

Figure 1. 1 Metabolism of Type II methanotrophs (Juan C. López et al., 2019)

1.4. Production of PHB and its environmental significance

At the end of the life cycle, most petroleum-based plastic products are disposed of and end up in landfills. Plastics generally occupy about 20% of landfills by volume (Harding et al., 2007) and can persist for over centuries due to the recalcitrance to chemical and biological decomposition. Additionally, other concerns related to plastic wastes include in-use leaching of potentially harmful additives, such as bisphenol A and phthalates, and the release of unwanted microplastic particulates (Hernandez et al., 2019). Bio-based PHB is an aliphatic thermoplastic polyester with several similar properties to polypropylene. It has beneficial properties such as biodegradability, biocompatibility, and thermoplasticity (Alkotaini et al., 2018). Figure 1.2 shows the methanotrophic metabolism of storing PHB granules under nutrient-limiting conditions. In this microbial process, methane is assimilated by methanotrophic bacteria into

PHB. Thus, methanotrophic conversion of methane to PHB has the great potential to produce valuable and environmentally-friendly materials and reduce greenhouse at the same time.

The concept of cradle-to-cradle processes for the production of PHB by methanotrophic bacteria has been proposed as a circular economy (Rostkowski, Criddle, & Lepech, 2012). After the production, extraction, and usage of PHB, anaerobic digestion facility units can further convert the discarded PHB products into bio-methane, which is subsequently captured and collected to serve as feedstock for methanotrophic PHB production in a new cycle. Therefore, the opportunities for symbiotic industrial coordination become obvious: a landfill or wastewater treatment plant could be co-located with a PHB production facility, allowing the use of a continuous supply of bio-produced methane.

Anaerobic digestion is a complex process in which organic matter can be eventually recycled as bio-methane and volatile fatty acids (VFAs). The main fermentation product in wastewater treatment facilities and landfills is usually acetic acid (Arsova, 2010; Ghosh & Pohland, 1974; Kleerebezem et al., 2015), which can account for over 55 % of total VFAs. As shown in the previous research (Belova et al., 2011; M Vechersaya, 2001), acetic acid can be selectively incorporated into PHB. In this context, a successful bioconversion of methane and VFA into biopolymers would represent the cornerstone of a new generation of biorefineries supporting a low-cost and environmentally friendly conversion of both methane and the residual organic matter into multiple high-added-value products.

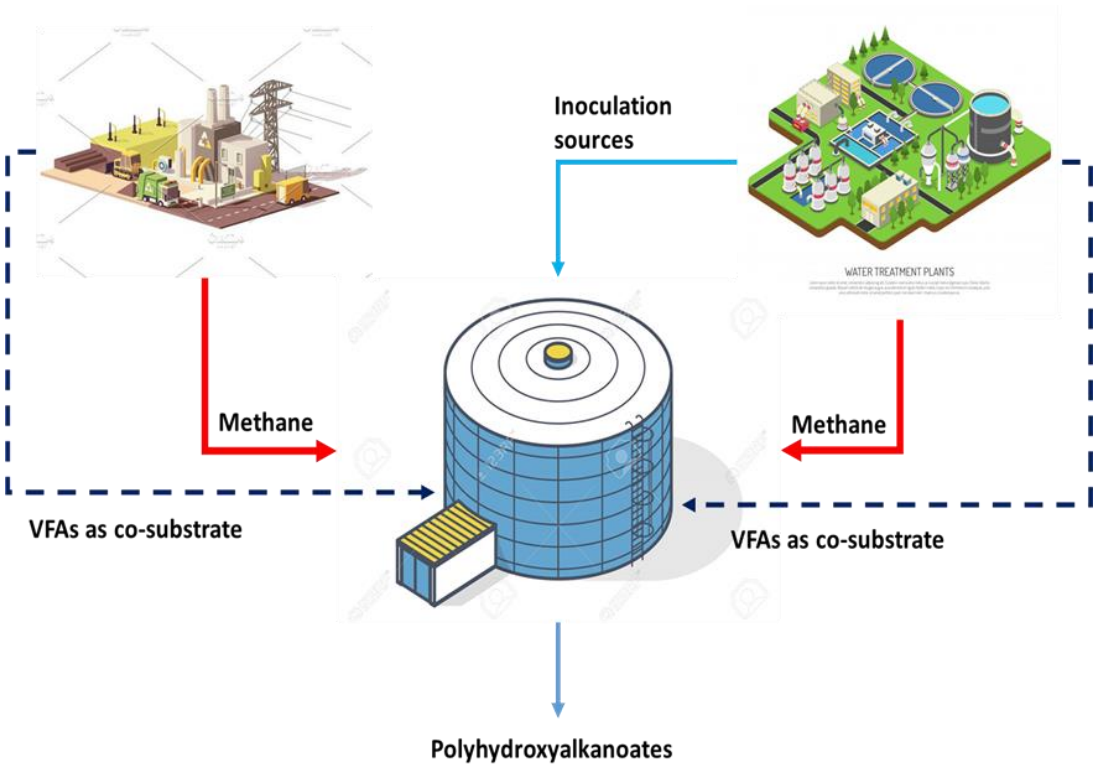

Figure 1. 2 Concept of symbiotic industrial coordination for PHAs generation

1.5. Research Objectives

The overall objectives of this research are to study the impact of acetic acid addition on the intracellular formation of PHB in mixed bacterial culture. For the purpose of scaling up PHB production, inocula from different treatment units in the WWTPs will be enriched to gain methanotroph-dominated microbial cultures for PHB generation.

The main objectives of this research aim to explore the following questions: First, the influence of acetic acid as co-substrate of methane to mixed bacterial culture; Second, the pH-associated effect on the production of PHB when acetic acid is added at high concentration; Third, the mechanistic role of acetic acid as it participates in the cellular metabolism; Fourth, the

bacterial community shift during enrichment and the characterization of target bacteria using molecular tools.

1.6. Thesis organization

This thesis is organized based on the above research objectives and includes five chapters: an introduction (Chapter 1), materials and analytical methods that apply to all research chapters (Chapter 2), two research chapters (Chapter 3-4), and a summary chapter (Chapter 5).

Chapter 3 explores the production of PHB by a methylotroph-dominated bacterial culture. Acetic acid was added as the co-substrate with methane. Particular attention was given to the role of pH-associated effect as this parameter has been consistently shown to result in the changes of PHB production, microbial activity, and PHB properties.

Chapter 4 evaluates the potentials of sludges sampled from eight different treatment units in two wastewater treatment plants. Sludges were inoculated to start the enrichment bioreactors. In the enrichment phase, molecular assays were performed to detect the change of microbial communities, and the expression of functional genes such as *pmoA* and *phaC*. PHB generation of each culture was combined with the results of the molecular assay to access enrichment evolvement.

Chapter 2

MATERIALS AND METHODS

2.1. Experiment setup

Figure 2.1 illustrates the experimental matrix used for the production of PHB by methanotrophs under nitrogen-limited growth conditions. The methanotrophic production of PHB can be achieved in two step-wise processes: in process 1, cells replicate under conditions of nutrient-sufficient growth (i.e., sufficient methane, oxygen, major nutrients, and minor nutrients for cell division); and in process 2, cells accumulate PHB inclusion granules under conditions of nutrient-limited growth (i.e., sufficient methane and oxygen, but lacking one or more major or minor nutrients needed for cell division).

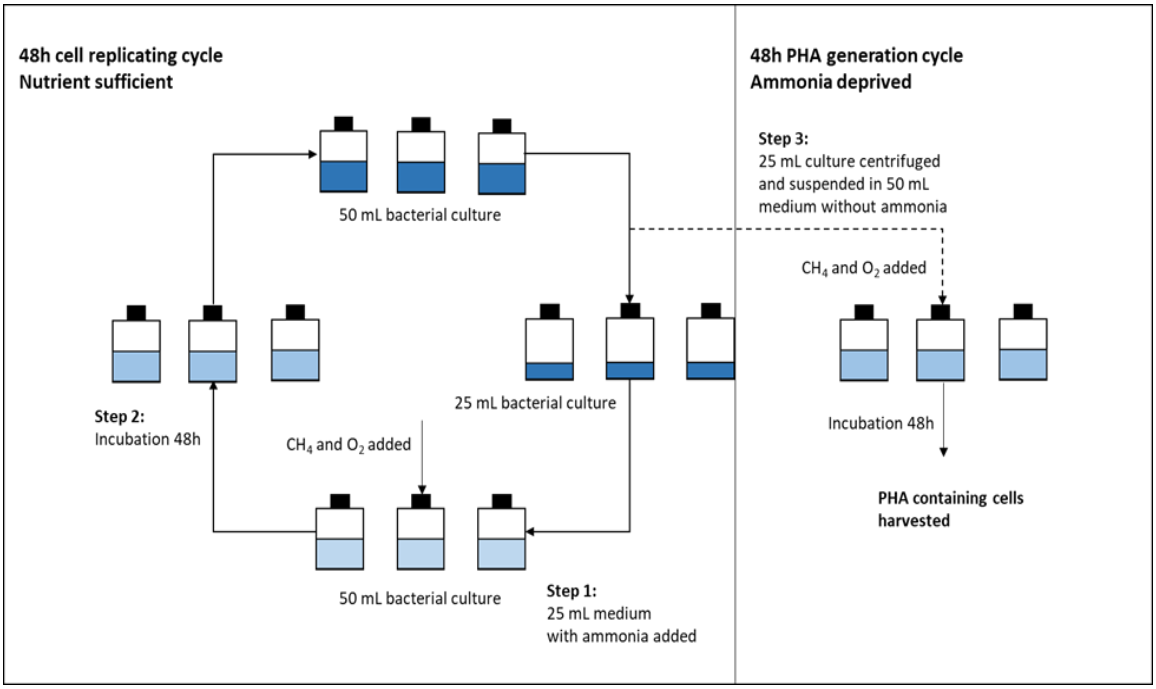

Figure 2. 1 Experiment set-up of the bacterial growth cycle and PHB production cycle (Myung, Galega, et al., 2015)

A volume of 25 mL of bacterial culture was taken from the bottles in a cell replicating cycle, in which nutrients were sufficient. The bacterial culture was centrifuged (Eppendorf 5804, Germany) at 14000 rpm, and the supernatant was removed. Pellets were resuspended in a nutrient-limiting substrate deprived of ammonia. The headspace of each bottle was filled with a CH_4: O_2 gas mixture (Ultra-high purity, Airgas, PA, USA). All cultures were incubated in 170-mL serum bottles capped with butyl rubber stoppers and crimp-sealed with a ratio of CH_4: O_2 as 1:1 in the headspace, in which the liquid volume was 50 mL and the headspace volume was 120 mL. Culture bottles were placed horizontally on an orbital shaker at 150 rpm with a room temperature of 30 °C. After 48 h of incubation, cells were harvested from the triplicate samples

by centrifugation and freeze-dried. Preserved samples were prepared for the analysis of PHB content.

2.2. Analytical methods

2.2.1. Analysis of volatile fatty acids

The concentrations of volatile fatty acids (VFAs) were measured using a 1220 High-Performance Liquid Chromatograph (Agilent, CA, USA) equipped with RID and UV detectors, an automated sampler, and a Hi-plex H column. The following operation parameters were used: flow rate 0.5 mL/min; column 55 °C, UV 210 nm; RID 55 °C. Peak areas of VFAs were compared to standards to quantify the concentration (Myung, Wang, et al., 2015).

2.2.2. Analysis of gases composition

To analyze the concentrations of CH_4, O_2, and CO_2, 0.1 mL gas from the headspace of each enrichment culture was injected into Gas Chromatograph (Agilent 7890B, USA) with carbonxen 1010 column (Supelco, Sigma-Aldrich) and a thermal conductivity detector. The following operation parameters were used: injector 200 °C; column 100 °C; detector 230 °C; and run for 10 min. Peak areas of CH_4, O_2, and CO_2 were compared to standards to quantify the concentration (Myung, Wang, et al., 2015).

2.2.3. PHB extraction

PHB granules were extracted from the cells by suspending 250 mg of freeze-dried cell material in 25-mL Milli-Q water, adding 200 mg of sodium dodecyl sulfate and 180 mg of EDTA, followed by heating to 60 °C for 60 mins to induce cell lysis. The solution was centrifuged (3,000 × g) for 15 mins, and the pellet washed three times with deionized water. To purify the PHB, pellets were washed with a 50-mL sodium hypochlorite (bleach) solution

(Clorox 6.15%), incubated at 30 °C with continuous stirring for 60 mins, and then centrifuged (3,000 × g) for 15 min. Sample pellets were then washed three times with deionized water. The washed PHB pellets were preserved in the freezer under -20 °C and freeze-dried for further analysis (Myung, Wang, et al., 2015).

2.2.4. PHB weight percentage and monomer composition

Between 3 and 6 mg of freeze-dried biomass were weighed and then transferred to a 12-mL glass vial. Each vial was amended with 2 mL of methanol containing sulfuric acid (3%, vol/vol) and benzoic acid (0.25 mg/mL methanol), supplemented with 2 mL of chloroform, and then sealed with a Teflon-lined plastic cap. All vials were shaken and then heated at 95-100 °C for 3.5 h. After cooling to room temperature, 1 mL of deionized water was added to create an aqueous phase separated from the organic chloroform phase. The reaction cocktail was mixed on a vortex mixer for 30 s and then allowed to partition until phase separation was complete. The organic phase was sampled by syringe and analyzed using a GC (Agilent 7890B) equipped with an HP-5 column (containing 5% phenyl- methylpolysiloxane; Agilent Technologies, USA) and a flame ionization detector. The oven temperature program was set up as follows: 50 °C for 3 mins, ramp increase to 60 °C over 9 mins, 60 °C for 3 mins, ramp increase to 260 °C over 9 mins, ramp increase to 300 °C over 6 mins, and finally held at 300 °C for 6 mins. DL-hydroxybutyric acid sodium salt (Sigma-Aldrich, St Louis, MO, USA) and PHBV with 3HV fractions of 5 mol%, 8 mol%, and 12 mol% (Sigma-Aldrich, St Louis, MO, USA) were used to prepare external calibration curves. The PHA content (wt%, wPHA/w CDW) of the samples and 3HV fraction of the PHAs (mol%) was calculated by normalizing to initial dry mass (Myung, Galega, et al., 2015).

2.3. PHB Characterization

Molecular weights of PHAs were determined using a gel permeation chromatography (GPC). Sample pellets dissolved in chloroform at a concentration of 5 mg/mL for 90 mins at 60 °C were filtered through a 0.2-μm PTFE filter and then analyzed with an HPLC system (Agilent 1220, CA, USA) equipped with a RID. The GPC was equipped with a GPC/SEC analytical column (PLgel, 10 μm miniMix-B, Agilent, USA). The temperature of the columns was maintained at 40 °C, and the flow rate of the mobile phase (chloroform) was 1 mL/min. Molecular weights were calibrated with polystyrene standards from Agilent (GPC/SEC Calibration Kit S-M-10, Agilent Technologies, USA) (Myung, Galega, et al., 2015).

Peak melting temperatures (Tm) and onset glass transition temperatures (T^0g) of PHA were evaluated using differential scanning calorimetry (NETZSCH DSC 204 F1, Phoenix). Thermal data were collected under a nitrogen flow of 10 mL/ min. About 5 mg of melt-quenched PHA samples encapsulated in aluminum pans were heated from -40 °C to 200 °C at a rate of 10 °C / min. The peak melting temperatures were determined from the position of the endothermic peaks (Myung, Wang, et al., 2015).

Spectra were recorded with a Thermo Nicolet 6700 FTIR Spectrometer fitted with a liquid N_2-cooled mercury-cadmium-tellurium detector. Absorbance spectra were collected at wavenumber values between 3500 and 300 cm^{-1} with spectral resolution of 8 cm^{-1}, and 10 scans were recorded and averaged.

2.4. The optimization of PHB generation by adding acetate

2.4.1. Culture conditions

All cultures were grown in medium JM2, which is a modified version of ammonium mineral salts (AMS) medium. Medium JM2 contains the following chemicals per L of solution: 2.4 mM $MgSO_4 \cdot 7H_2O$, 0.26 mM $CaCl_2$, 36 mM $NaHCO_3$, 4.8 mM KH_2PO_4, 6.8 mM K_2HPO_4, 10.5 μM $Na_2MoO_4 \cdot 2H_2O$, 7 μM $CuSO_4 \cdot 5H_2O$, 200 μM Fe-EDTA, 530 μM Ca-EDTA, 5 mL trace metal solution, and 20 mL vitamin solution. The trace stock solution is composed of the following chemicals per L of the solution: 500 mg $FeSO_4 \cdot 7H_2O$, 400 mg $ZnSO_4 \cdot 7H_2O$, 20 mg $MnCl_2 \cdot 7H_2O$, 50 mg $CoCl_2 \cdot 6H_2O$, 10 mg $NiCl_2 \cdot 6H_2O$, 15 mg H_3BO_3, and 250 mg EDTA. The vitamin stock solution contained the following chemicals per L of the solution: 2.0 mg biotin, 2.0 mg folic acid, 5.0 mg thiamine·HCl, 5.0 mg calcium pantothenate, 0.1 mg vitamin B12, 5.0 mg riboflavin, and 5.0 mg nicotinamide (Myung, Wang, et al., 2015).

Chapter 3

OPTIMIZING THE METHANOTROHIC PRODUCTION OF POLYHYDROXYALKANOATES (PHAs) USING HIGHLY ENRICHED CULTURE IN THE PRESENCE OF ACETIC ACID

Abstract

The use of petroleum-based plastics has caused serious economic and environmental problems. It is imperative to develop sustainable ways to produce valuable and environmentally-friendly materials. Methanotrophic bacteria are known for assimilating methane while generating polyhydroxyalkanoates (PHAs) biopolymers, which are potential substitutes for conventional plastics.

In this study, a feast-famine based cultivation system was developed to produce PHAs using *Hyphomicrobium,* and *Methylocysits* dominated mixed culture, which was fed with methane with and without acetic acid as co-substrates. Throughout the 27 cycles of cultivations, the mixed bacterial culture fed with methane alone started to produce the PHB consistently and achieved an average of 26.4 ± 3.5 wt%. By comparison, the PHB production significantly increased up to 34.8 ± 1.8 wt% when acetic acid was added at an optimal concentration of 300 mg/L under controlled pH conditions. Additionally, the characterization of PHB polymers (e.g., chemical composition and molecular weight) illustrated that the presence of acetic acid at

optimal pH conditions facilitated the elongation of the polymer chain with higher molecular weight.

3.1. Introduction

Extensive research studies on methanotrophs have disclosed the prospective commercial use in the production of biopolymers, but there are still significant challenges to achieve a sustainable application at an industrial scale. The major problems include the relatively slow growth rate of methanotrophic bacteria, which is attributed to the low mass transfer rate of methane from the gas phase into cells because of the low solubility of methane in the medium, and the obligate nature of most methanotrophs as they are not able to grow on multi-carbon compounds. Considering the strong interconnections between the rates of the mass transfer, cell growth, and biopolymer formation (Myung, Kim, et al., 2016), finding a solution to fill in the gap is needed to enable the use of methanotrophs in both natural and engineered systems for expanding the biotechnological platform for the production of value-added bio-products such as PHAs.

Some strains of methanotrophs possess the essential enzymatic systems to channel acetic acid into the PHB biosynthetic pathway. In the previous study (Myung et al., 2018), acetic acid is produced intracellularly by *Methylocystis spp* via the oxidation of ethanol and assimilated into PHB under nutrient-limiting conditions. We adopted a mixed enrichment culture for methanotrophic production of PHB and investigated the effects of acetic acid as the co-substrate of methane on the PHB generation. This study aims to address the metabolic pathway for acetic acid utilization by mixed enrichment culture and to evaluate the efficiency of methane assimilation co-metabolized with acetic acids at different concentrations under the nutrient-

limiting condition. The joint assimilation of methane and a second substrate represents a variant of co-metabolism by the mixed culture, which could result in more intensive and efficient growth as well as PHB production.

3.2. Materials and methods

The experiment used the same protocols described in section 2.2. The analytical methods for the measurement of PHB weight percentage, gas concentrations have been presented in section 2.2. The PHB polymer extraction from the mixed bacterial cells and the characterization in terms of molecular weight, Fourier-transform infrared spectroscopy (FTIR), polymer melting temperature, and NMR refer to the detailed procedures given in section 2.3. The mixed enrichment culture was developed, maintained, and used for all the experiments (27 cycles). 100-µL samples were collected from the initial mixed enrichment culture and the culture after 27 repeated cycles of cultivation, centrifuged to collect the pellets. Then genomic DNA (gDNA) was extracted from the pellets using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) per the manufacture's protocol. The concentration of DNA was determined using spectrophotometry using A260/A280 (NanoDrop One, Thermo Scientific Inc., WI, USA). To verify the microbial community stability of the mixed enrichment culture during this study, the extracted DNA from the original culture and the culture after 27 cycles were amplified using metagenomic sequencing by targeting the V3-V4 hypervariable regions of 16S rRNA genes with the primer set of bakt_341f (5′- CCTACGGGNGGCWGCAG-3′) and bakt_805r (5′-GACTACHVGGGTATCTAATCC-3′) with an eight-based sequence-unique barcode to each sample. The amplicon sequencing was performed on the Illumina MiSeq platform with the service provided by Macrogen (Seoul, South Korea), and 400-500 bp paired-end reads were

generated. The sequencing libraries were generated according to the 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B using Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2.

3.3. Results and discussions

3.3.1. Microbial community analysis of mixed enrichment culture

At the beginning and the end of the 27th cycle, genomic DNA was extracted from triplicate samples of each culture and sequenced using metagenomic sequencing by targeting the V3-V4 hypervariable regions of 16S rRNA genes. As shown in Figure 3.1, the microbial communities in both samples showed similar compositions but slightly different proportions, which indicated that this bacterial enrichment maintained stable microbial composition without disturbance during the entire experiments. *Hyphomicrobium* dominated the mixed microbial culture, followed by *Methylocystis, Pandoraea,* and other minor genera, including *Achromobacter, Paraburkholderia, Ralstonia, and Stenotrophomonas*. *Hyphomicrobium* accounted for a relative abundance of 32.8% and 35.4% in the initial and end cycles of the culture. *Methylocystis* accounted for the second most proportion for around 15%.

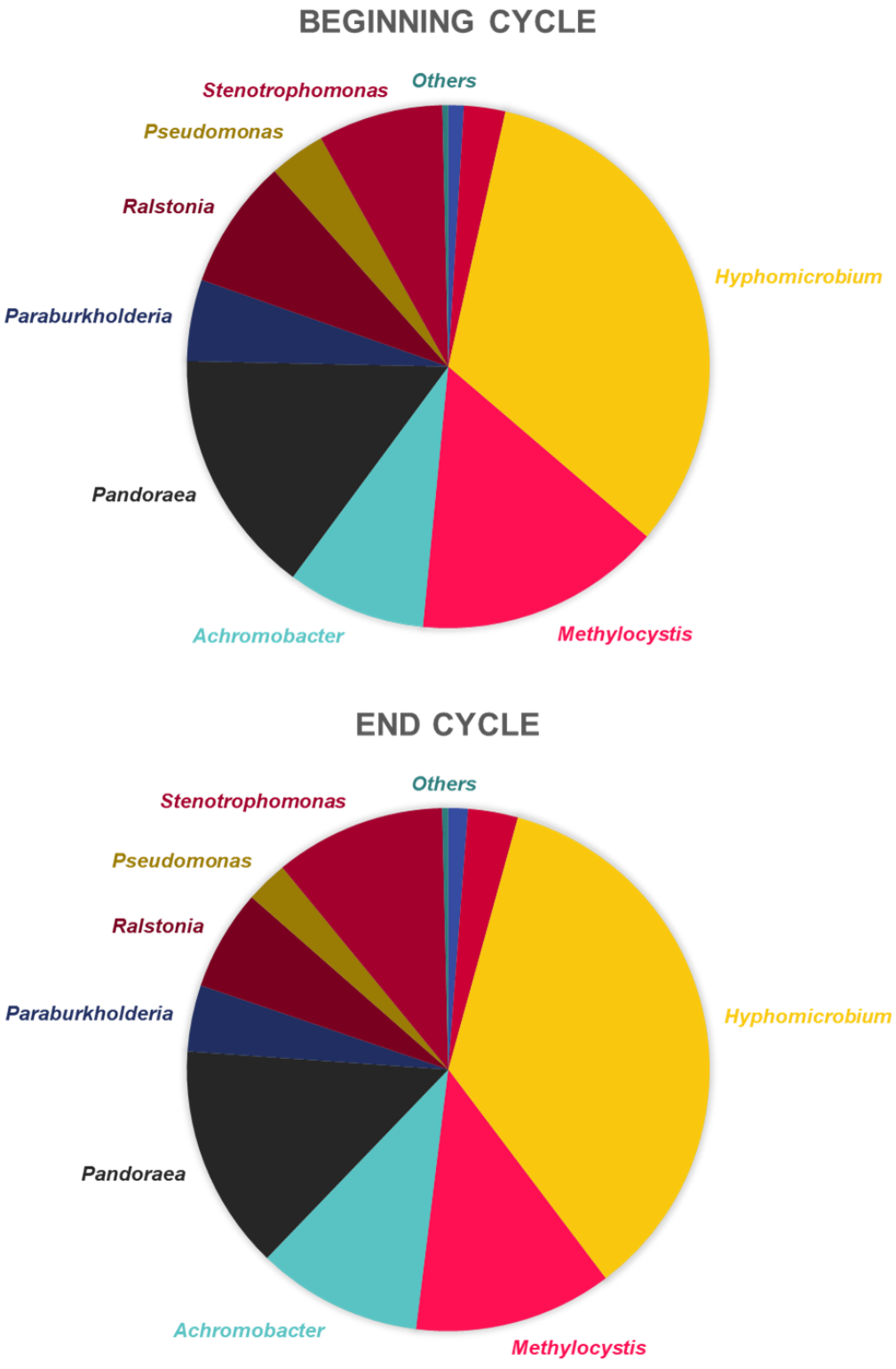

Figure 3. 1 Taxonomy abundance of mixed bacterial culture on species level

Hyphomicrobium is a facultative methylotroph as a non-methane user but relies on methanol produced as a result of methane oxidation by methanotrophs. They are ubiquitous in water and soil and can also be found in sewage treatment plants. Methylotrophic *Hyphomicrobium* often appears to coexist with methanotrophs in methane oxidation processes. Previous studies reported that *Hyphomicrobium* coexisted with methanotrophic bacteria in a groundwater ecosystem (Dedysh et al., 1998; Hutchens et al., 2004). Myung et al. (Myung, Wang, et al., 2015) reported the *Hyphomicrobium* as the second largest proportion in the bacterial culture enriched from wastewater treatment plant inoculation.

Although methane was the sole carbon source in the unique methane-driven system, non-methane users with the ability to accumulate PHA were observed. For example, some strains of *Hyphomicrobium* are known to accumulate intracellular PHB (C. Martineau et al., 2015; Christine Martineau et al., 2013). Research studies also reported that the co-presence of *Hyphomocrobium* serves as the helper of methanotroph *Methylocystis* and increased the methane-oxidation rate, biomass growth, and stability in a fed-batch co-culture (Jeong & Kim, 2019). *Hyphomicrobium* using concurrently existing metabolic pathways, can produce an efficient and stable methane oxidation system, which sometimes can be more efficient than single isolates. Besides, the species of *Pandoraea oxalativorans* is known to be oxalate-utilizing bacteria that use oxalate as the sole carbon source (N. Sahin et al., 2011). Most of oxalotrophic bacteria are facultative methylotrophs and/or facultative hydrogen-oxidizing chemolithoautotrophs (Nurettin Sahin, 2003). De Paula et al. reported the first strain of *Pandoraea MA03* to generate PHA using propionic acid as a substrate (Fabrício Coutinho de Paula et al., 2017). Moreover, *Stenotrophomonas* and *Pandoraea* are phylogenetically related. The strains such as *Ralstonia eutropha*, and *Pandoraea ISTKB* have been reported to generate

PHAs using varied substrates (Fabrício C de Paula et al., 2017; Iqbal et al., 2016; Kumar et al., 2017; Reinecke & Steinbuechel, 2009). The presence of the methylotrophic-heterotrophic bacteria in the microbial consortia have a critical symbiotic relationship with the regulation of acid homeostasis.

3.3.2. PHB generation with acetic acid addition and the impact of pH

Figure 3.2 summarized the PHB generation from mixed microbial culture when acetic acid was added as co-substrate with methane. Three different treatments were conducted to evaluate: 1) PHB production with and without acetic acid addition; 2) PHB production with varying concentrations of acetic acid addition; 3) PHB generation with and without pH control when acetic acid was added. The control treatment without the addition of acetic acid was included for comparison.

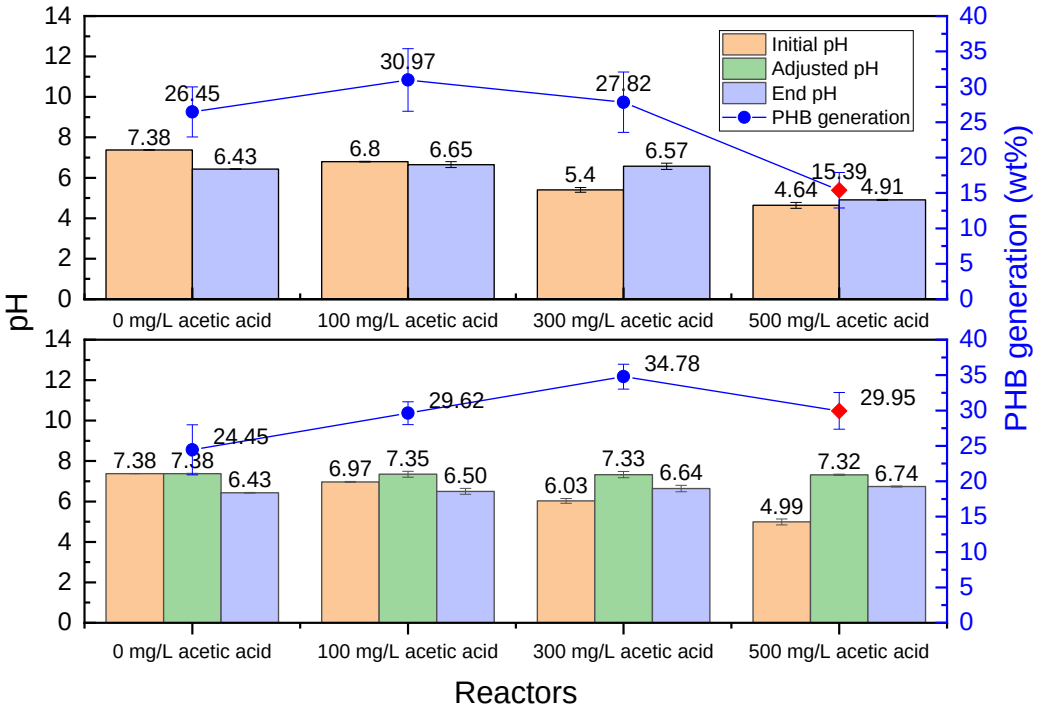

Figure 3. 2 PHB generation with initial acetic acid concentrations at 100 mg/L, 300 mg/L, and 500 mg/L. The top panel presented results of treatments when pH was not controlled; the bottom panel displays the results of treatment when pH was controlled. Error bars represent standard deviation (n = 3)

As shown in Figure 3.2 (top panel), the addition of acetic acid decreased the solution pH with the increase of acetic acid concentrations compared with the control treatment. The initial pH of the mixed bacterial culture in the absence of acetic acid was 7.38 and became 6.43 at the end of the PHB generation cycle. After the addition of acetic acid, the initial solution pH dropped down to 6.80, 5.40, and 4.64 with the acetic acid concentration at 100, 300, and 500 mg/L, with corresponding final solution pH as 6.65, 6.57, and 4.91, respectively. In the presence of acetic

acid, the solution pH increased back to around 6.5 at the end of the PHB generation cycle, except for the 500 mg/L acetic acid treatment.

Different concentrations of acetic acid impacted the PHB generation. The PHB generation yield expressed as the percentage of cell mass in the control treatment without acetic acid was 26.4 ± 3.5 wt%. By comparison, when the addition of acetic acid was 100 mg/L, the PHB generation yield increased to 31.0 ± 4.4 wt%; but with the continuous increase of acetic acid, the PHB generation yield went down to 27.8 ± 4.3 wt% at 300 mg/L, and eventually plummeted to 15.4 ± 2.5 wt% at 500 mg/L. The different PHB yields can be attributed to the varying solution pH with the addition of acetic acid at different concentrations. In the presence of acetic acid at 100 and 300 mg/L, the end pH was able to bounce back to the level of the control treatment. However, in the case of 500 mg/L acetic acid, the solution pH was significantly lower than the optimal pH for methanotrophic bacteria (pH 6.8-7) and inhibited the metabolism and the production of PHB.

To further elucidate the impact of acetic acid on the solution pH and consequent PHB production, the initial solution pH for all treatments after the addition of acetic acid was adjusted to around 7.3 using 0.1 M sodium hydroxide, similar to the control treatment. As presented in Figure 3.2 (bottom panel), the solution pH at the end of the PHB generation cycle remained very close to the range of 6.4-6.7 regardless of the concentrations of acetic acid added. The PHB generation yield showed different patterns in all treatments with the addition of acetic acids. All (in the range of 29.6-34.8 wt%) were higher than 24.4 wt% in the control treatment, suggesting the co-substrate acetic acid enhance the PHB generation under controlled pH conditions. Among these treatments with acetic acid, the maximum PHB generation yield of 34.8 ± 1.7 wt% was observed in the treatment with acetic acid at 300 mg/L. The largest difference of PHB generation

yield was observed in treatments with acetic acid concentration at 500 mg/L. The yields increased from 15.4 ± 2.5 wt% to 29.9 ± 2.6 wt% when pH was regulated. The above results showed that pH was also a crucial parameter for PHB production. Though the addition of acetic acid as co-substrate can enhance PHB, the protons dissociated to the culture may incur inhibition. Chua et al. (Chua et al., 2003) reported that a low PHB content was obtained at a pH of 6.0, while the PHB content increased significantly at a pH of 8.0. Additionally, the PHB generation at an initial pH of 5.5 took a much longer time to attain the maximum PHB contents. Similarly, Liu et al. (Liu et al., 2011) investigated the impact of pH (ranging from 5 to 8) on PHB generation in enrichment culture. The results showed that when pH was below 5.0, the final PHB generation was the lowest at around 2%.

Thus, it is critical to maintaining the solution pH within the optimal range so that the microbial activity is not inhibited. All microorganisms, including methanotrophs, typically have a specific pH range for optimal growth. And most of the known bacteria are neutrophiles. Although microorganisms often grow over a wide range of pH and far from their optima, some limitations/inhibitions do occur as the pH departs the optimum. Methanotrophs from the genus *Methylocystis* have been identified from enrichment culture reactors with initial pH values of 2, 4, or 7, and landfill cover soil under acidic (pH 4.8 and 6.2) and neutral (pH 7.6) conditions (Cébron et al., 2007; Kong, Bai, Su, Yao, & He, 2014; Su, Xia, Tian, Li, & He, 2014). They possess diverse systems of membrane transporters that ensure pH homeostasis (Nguyen et al., 2018). When the concentration of protons is much greater outside of a cell than inside, the protons gradient will drive protons into the cytoplasm and lower the cytoplasmic pH. As a result, drastic variations in the cytoplasmic pH can harm microorganisms by disrupting the membrane or inhibiting the enzymatic activity and membrane transport proteins. Changes in the internal pH

also can alter the ionization of nutrient molecules and thus reduce their availability to the organisms. The responses to the external pH change to maintain the cytoplasmic pH include the exchange of potassium for protons using antiport transport systems and an internal buffering system for pH homeostasis. Moreover, ATPase or other proteins can also engage in pumping excessive protons out of the cells, but these responses involve the energy input and reduce the overall efficiency of energy metabolism. Fleit (Fleit, 1995) indicated that at an unsteady state, the undissociated acetic acid could rapidly diffuse into bacterial cells through the cell membrane, which then dissociated and imposed a proton load on the cytosol and subsequently decreased the intracellular pH.

3.3.3. Metabolic kinetics

Figure 3.3 displays the metabolic kinetics in the control treatment, including the cumulative consumption of substrates CH_4 and O_2 and the cumulative formation of CO_2 and PHB. Methane was used as the sole carbon source with no addition of acetic acid during the nutrient-insufficient growth periods. The dynamics of gaseous concentrations of CH_4 and O_2 in the headspaces were used to evaluate the microbial kinetics of substrate consumption. Both gaseous CH_4 and O_2 consumption occurred rapidly during the first 24 hours of the incubation, and the consumption started to slow down and stabilize from 24 h to 48 h. The total O_2 and CH_4 consumption in two days were 196.1 and 70.4 mg /g TSS. Gaseous CO_2 was measured in the headspace but cannot represent the CO_2 formed from the metabolic reactions because bicarbonate was added in the substrate to support the growth of culture and buffer pH. During the incubation, intracellular PHB accumulation in the culture increased steadily with time, reaching 14.7 wt% in 24 hours and 23.2 wt% in 48 hours.

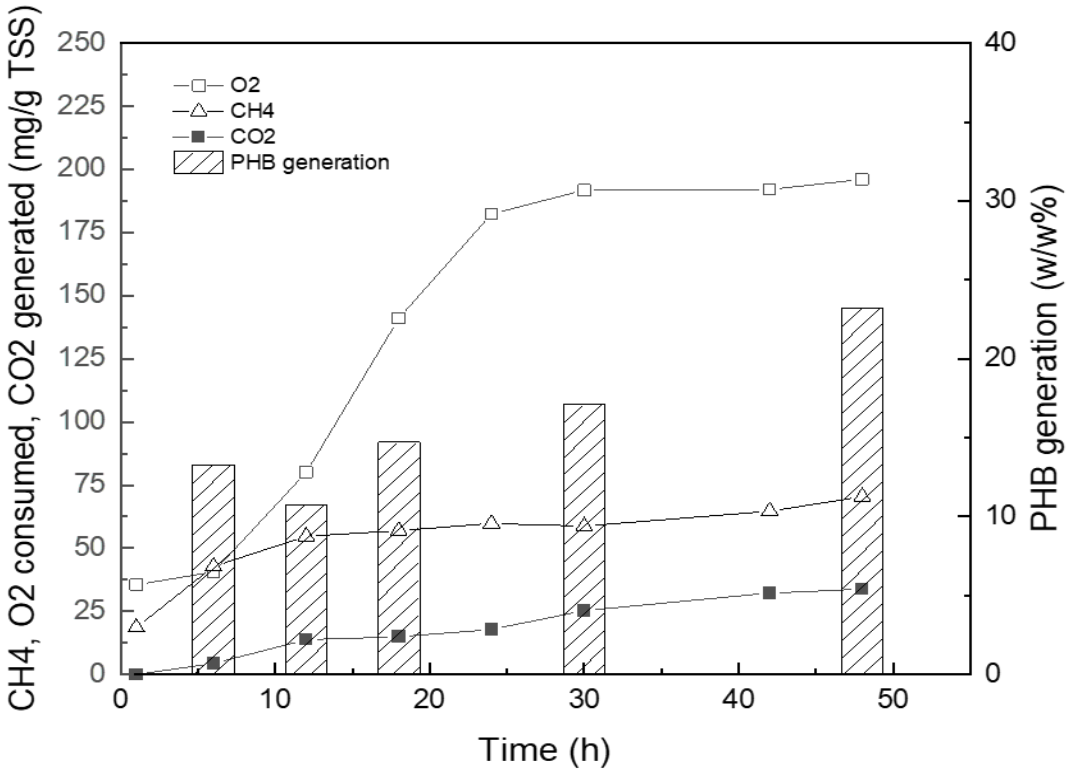

Figure 3. 3 Time course of CH_4 (Δ), O_2 (□), CO_2 (■), and PHB generation during the nutrient-limiting phase in the control treatment

Figure 3.4 displays the kinetics of methanotrophic reactions by mixed enrichment cultures using co-substrates of methane and acetic acid (100 mg/L) without (top panel) and with pH adjustment (bottom panel). Under uncontrolled pH conditions, a higher oxygen consumption of 203.3 mg O_2/g TSS was exhibited compared to 196.1 mg O_2/g TSS in the control treatment; however, the total methane consumption (74.8 mg CH_4/g TSS) was lower than the control treatment (68.2 mg CH_4/g TSS). Acetic acid was fully degraded within 18 h, and the final PHB yield was 28.2 wt%. Compared with the control treatment, the addition of acetic acid increased

the final PHB content by up to 5.1 % but with less methane consumption and higher oxygen consumption. It is reasonable that the oxidation of acetic acid used an extra amount of oxygen, and the incorporation of acetic acid contributed to the PHB generation.

Under controlled pH conditions, 199.8 mg O_2 /g TSS was consumed by the end of the cycle. Noticeably, the presence of acetic acid at 100 mg/L enhanced the PHB generation to 31.8 wt%, which is consistent with the findings in section 3.3.2. The maintenance of pH in the optimal range favored metabolism and PHB production. The lower requirement of oxygen under controlled pH conditions compared with uncontrolled conditions is in alignment with our previous assumption: the responses to the external pH change to maintain the cytoplasmic pH include the energy input. Oxygen is actively engaged in the production of reducing power to drive ATPase or other proteins to pump excessive protons out of the cells.

As oxygen remained ample at the end of the batch experiment, its concentration can be regarded as a constant because the biological uptake of acetate is independent of oxygen concentration. Pseudo-first order kinetic analysis was performed on the degradation of acetic acid at a concentration of 100 mg/L with and without pH control (Figure. S1 in Appendix I). The pH-controlled condition favored the incorporation of acetic acid, and degradation rate was higher (k_2 = 0.1693) than uncontrolled condition (k_1 = 0.1112).

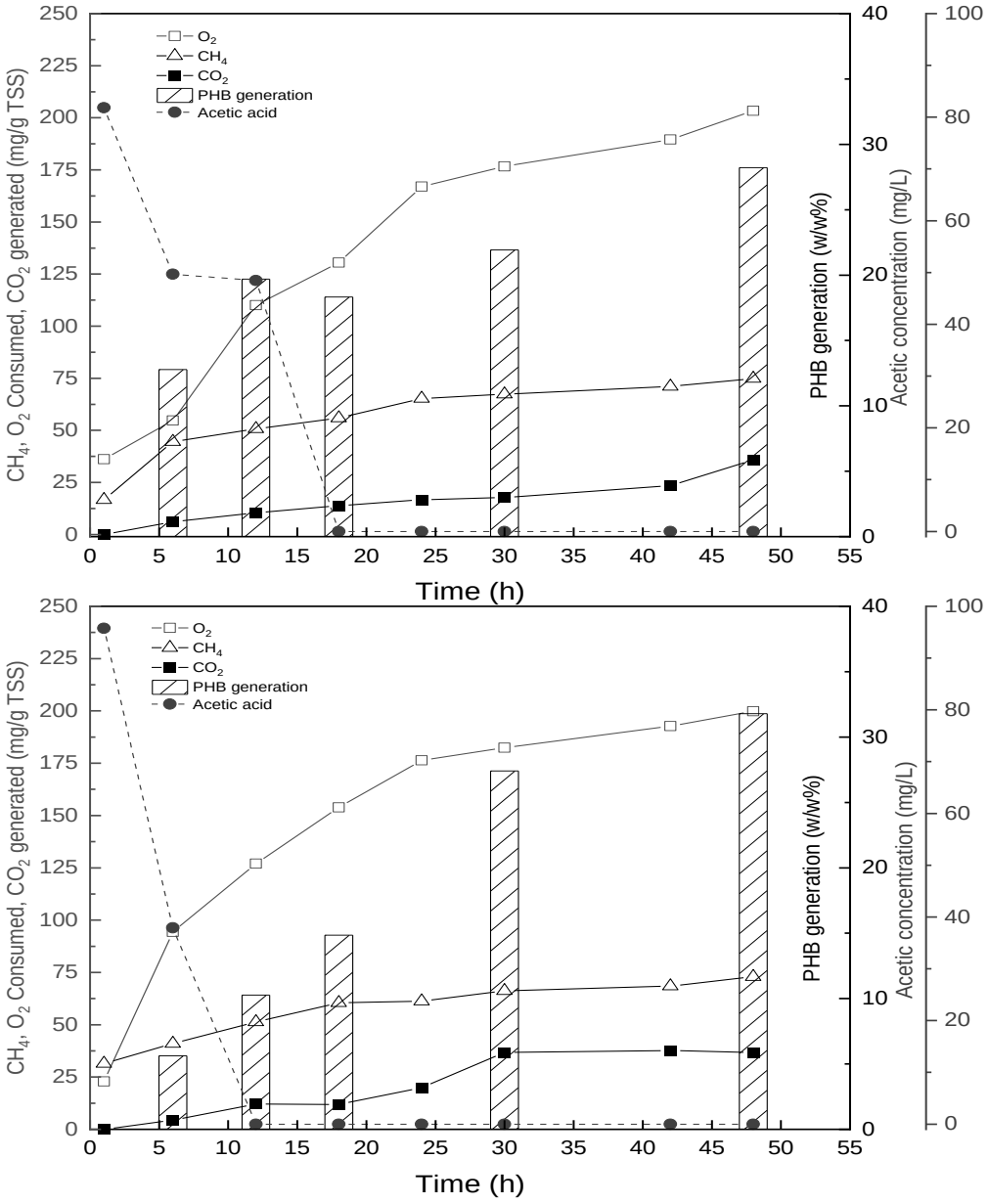

Figure 3. 4 Time course of CH_4 (Δ), O_2 (□), CO_2 (■), acetic acid (●) concentrations, and PHB (column) generation during the nutrient-limiting phase in the treatments with 100 mg/L acetic acid. The top panel had no pH control, and the bottom panel had the pH control.

Figure 3.5 displays the kinetics of methanotrophic reactions using methane and co-substrates of acetic acid at 300 mg/L without (top panel) and with pH adjustment (bottom panel). Under uncontrolled pH conditions, the oxygen consumption of 191.0 mg O_2/g TSS was exhibited, and the methane consumption was 69.3 mg CH_4/g TSS. The PHB generation achieved its maximum of 26.9 wt% in 42 h but reduced down to 22.3 wt% in 48 h. It took the bacterial culture around 42 h to deplete the acetic acid.

Under controlled pH conditions, 202.3 mg O_2 /g TSS was consumed by the end of the cycle. The PHB yield was at 35.1 wt% compared to 26.9 wt% in the uncontrolled treatment. Figure. S2 shows the kinetic analysis when the addition of acetic acid was 300 mg/L. When pH was adjusted to neutral, the consumption of acetic acid was 1.6-folds faster, and it took less than 24 h before the exhaustion of the acetic acid. It should be noted that the maximum generation of PHB was observed at 30 h instead of the end of cycle. The loss of the PHB may result from the consumption when substrate was insufficient to support the bacterial growth. The presence of acetic acid stimulated methane oxidation by improving the consumption from 70.4 mg CH_4/g TSS to 77.2 CH_4 mg/g TSS. Previous research also indicated an improved ability to metabolize methane in the presence of acetate (Im et al., 2011). Quantitative PCR also revealed that *pmoA* mRNA transcripts were enhanced when methanotrophs were grown in presence of both methane and acetate.

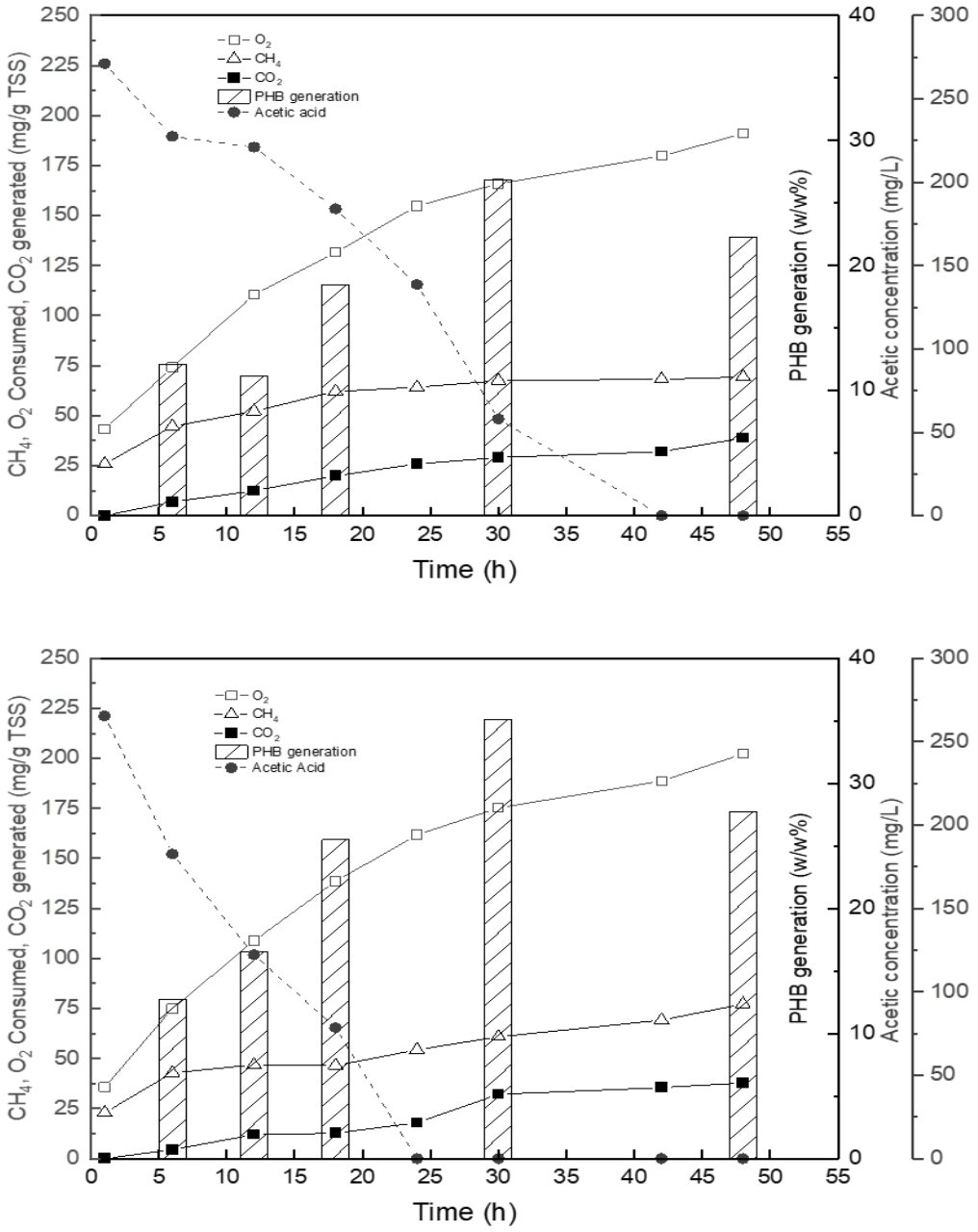

Figure 3. 5 Time course of CH_4 (Δ), O_2 (□), CO_2 (■), acetic acid (●) concentrations, and PHB (column) generation during the nutrient-limiting phase in bottles with 300 mg/L acetic acid. The top panel had no pH control, and the bottom panel had pH control.

Figure 3.6 displays the kinetics of methanotrophic reactions using methane and co-substrates of acetic acid at (500 mg/L) without (top panel) and with pH adjustment (bottom panel)., the oxygen consumption of 234.2 mg/g TSS was exhibited, and it was much higher than other treatments. Methane consumption was only 58.4 mg/g TSS and was 16.6% smaller than the control treatment. When the pH was controlled at 500 mg/L addition, the methane consumption was 74.6 mg/g TSS. A higher addition of acetic acid without pH control resulted in the serious inhibition of methane monooxygenase and further caused a lower rate of methane oxidation. Under uncontrolled pH conditions, acetic acid with a concentration of 500 mg/L was not depleted by the end of the experiment, and the final PHB content was as low as 15.9 wt%. By contrast, when pH was controlled, acetic acid added as co-substrate was completely consumed in 42 h, and the PHB yield was increased back to 32.0 wt%. Similar changes can be observed from other concentrations of addition. The optimal pH is favorable to microbial metabolism.

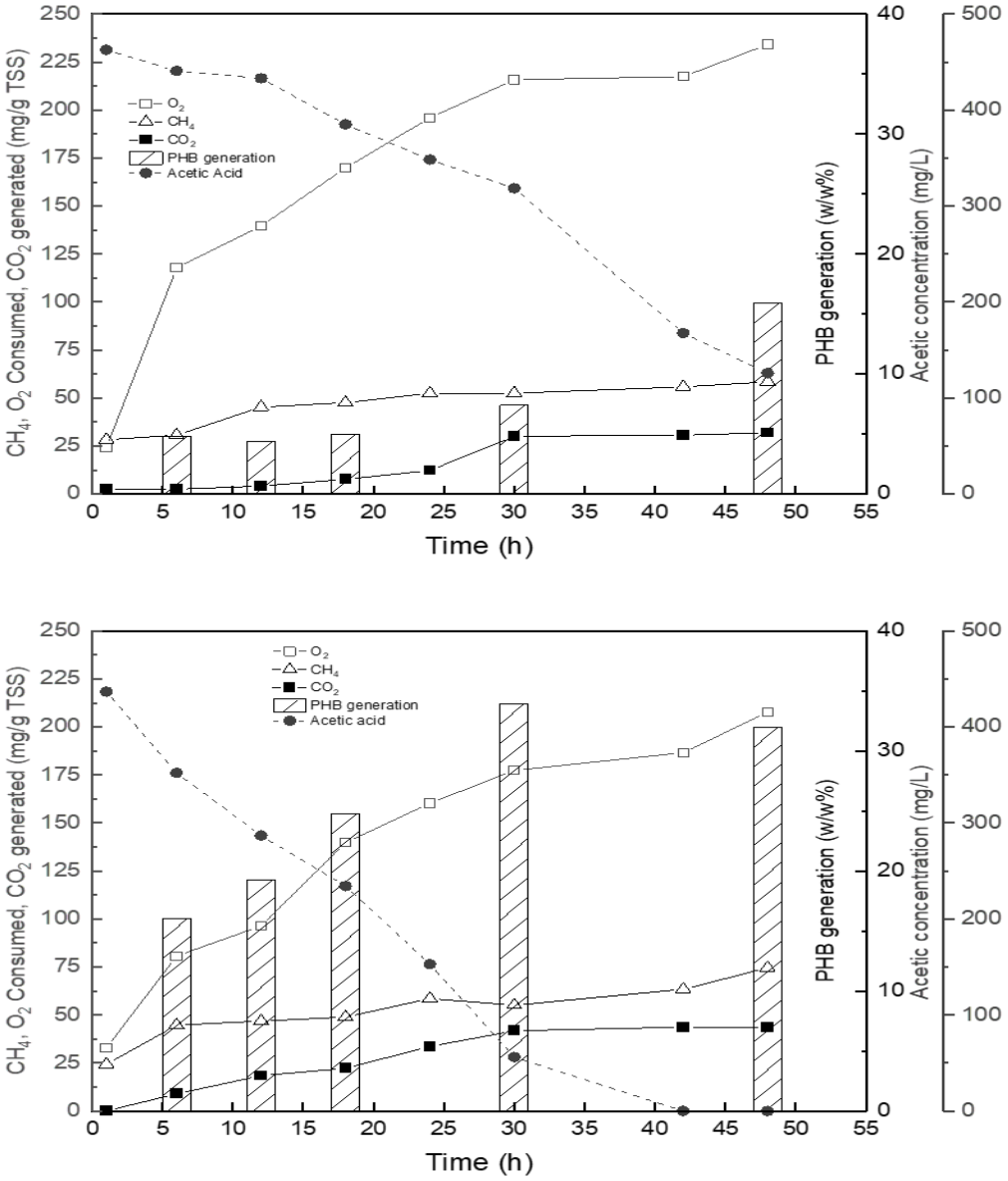

Figure 3. 6 Time course of CH_4 (Δ), O_2 (□), CO_2 (■), acetic acid (●) concentrations, and PHB (column) generation during the nutrient-limiting phase in the treatments with 500 mg/L acetic acid. The top panel had no pH control, and the bottom panel had pH control.

3.3.4. The role of acetic acid in the methanotrophic metabolism

Methane oxidation has been regarded as the limiting step for the growth rate of methanotrophs as evidence suggested from knockout experiments (Costa et al., 2000; Law & Slepecky, 1961). Although the experiment was carried out at relatively high oxygen concentrations with an excess of methane in the headspace, it may still be insufficient to apply non-limiting oxygen and methane conditions to a dense cell suspension due to the gas transfer rate from the gas phase to the liquid phase.

Based on the kinetics data in section 3.3.3, the change of microbial activity can be reflected by the dynamics of gas concentrations and the PHB generation when acetic acid was added. pH is critical to enzymatic activities such as methane monooxygenase. A lower pH directly inhibits methane oxidation, which is the first step of the methane uptake in this methylotrophic dominated bacterial culture. A low consumption of methane consequently decreases the carbon sources available to downstream metabolic reactions. The presence of acetic acid provides extra carbon sources for the heterotrophs existing in the culture. The access to acetic acid reduces their dependence on the intermediate products produced by methane users.

The mechanisms for the energy metabolism of methanotrophic bacteria include the participation of MMO in the acceptance of a major part of electrons of the assimilated substrate. PCR and electrophoresis results from Figure 3.7 showed the absence of sMMO but presence of pMMO in the beginning and end cycle of the mixed microbial culture. Therefore, pMMO serves as the major enzyme that converts methane to methanol in the reactions.

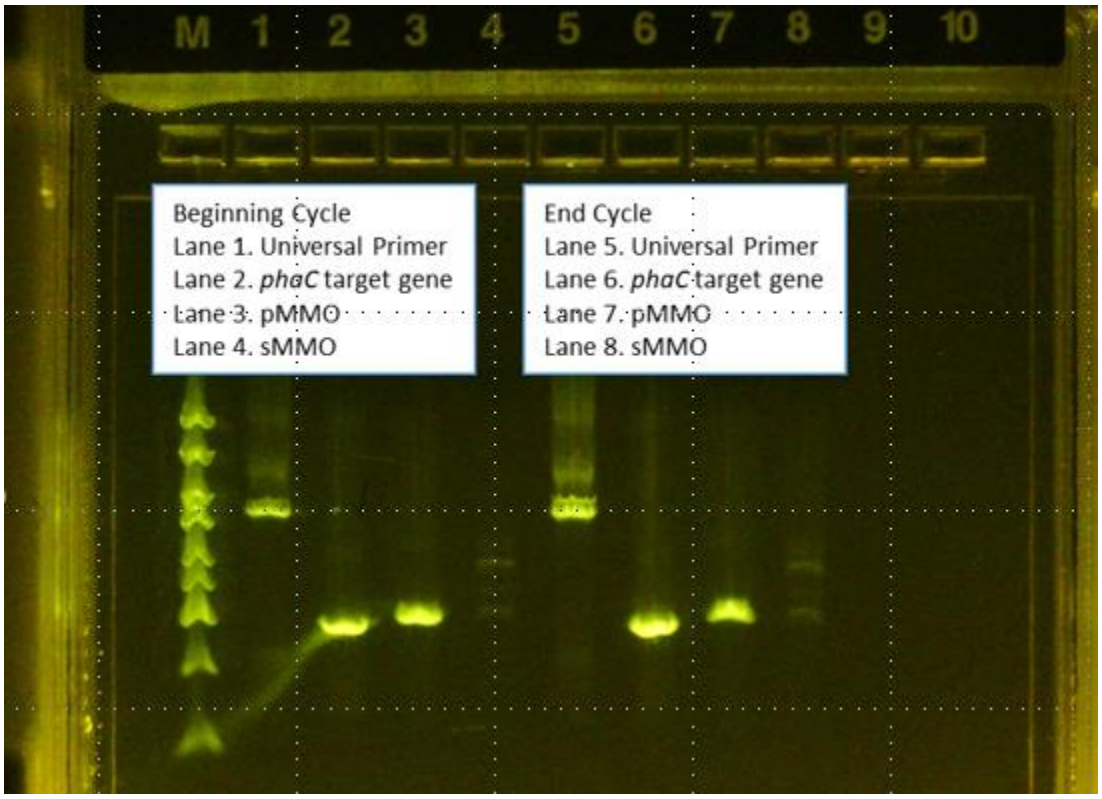

Figure 3. 7 Electrophoresis of different target genes of the mixed culture in the beginning and end cycles

Particulate MMO is composed of three subunits, *α, β, and γ*, also known as *pmoB*, *pmoA*, and *pmoC*, respectively. pMMO has been known as non-specific and capable of oxidizing aliphatic compounds. Substrates and products that can bind to pMMO have been explored computationally, which suggested that hydrocarbons up to five carbons in length accommodate to the pocket active site on the enzyme (Culpepper & Rosenzweig, 2012). It has been found that some species of the genus *Methylocystis* could grow without methane but use acetate as carbon source coincided with the detectable pMMO (Belova et al., 2011; Yoon et al., 2011). Therefore, it is also possible that acetic acid can be converted to methanol by methane monooxygenases given that acetic acid is well dissolved in the substrate and readily available. The joint

assimilation of methane and acetic acid by the mixed bacterial culture provides for the more efficient utilization of the energy and carbon. Apart from being oxidized by pMMO, the incorporation of acetate into PHB was observed with the from being oxidized to Acetyl-CoA by the enzyme of HSCoA(Vecherskaya, 2001). The addition of a co-substrate that has the potential to increase the amount of reducing power available to methanotrophs and strengthens the assimilation of carbons to produce biopolymer.

Figure 3.8 shows the proposed mechanism of acetic acid assimilation in the culture dominated by methylotrophic bacteria. Due to the low solubility of methane in the by oxidase and form acetyl-CoA. The increasing amount of acetyl-CoA generated from different routes readily serves as the precursor of PHB formation under nutrient-limiting conditions.

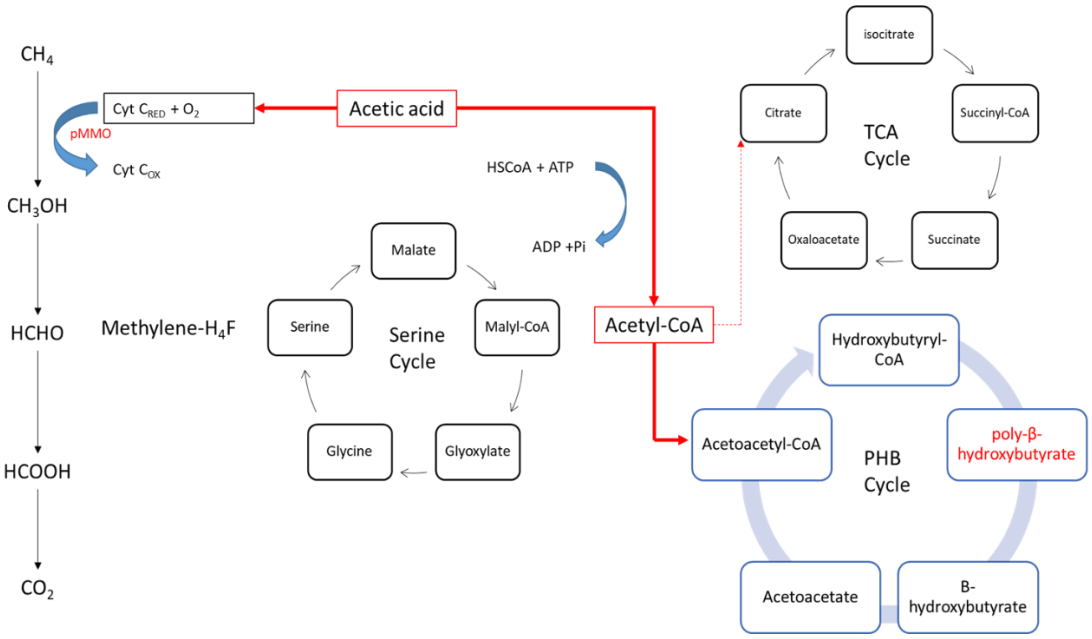

Figure 3. 8 Proposed mechanism of the role of acetic acid in the PHB synthesis

liquid phase, pMMO is not saturated and is available to acetate. The presence of acetate enhanced the utilization of enzyme and energy flow efficiency. Methanol converted from the substrates is transformed into formate and enters the serine cycle, by the end of which Acetyl-CoA is formed. On the other hand, acetate can also be oxidized

Based on the metagenomics analysis, it is suggested in methane-driven consortia, methanotrophs process methane for biomass growth and produce carbon by-products such as methanol. The interactions in the methanotrophic-heterotrophic community have an efficient carbon-cycling system. Many identified bacterial species have indicated capability to accumulate PHB, meaning that the biopolymer was generated by either methylotrophs that utilize methane, methanol, or heterotrophs that feed on byproducts of the methanotrophic activity or products of cell decay. There are five major PHB-producing genera capable of utilizing different carbon substrates for the growth and PHB biosynthesis: *Methylocystis, Hyphomicrobium, Paraburkholderia, Pandoraea, Stenotrophomonas, Ralstonis, and Acrombacter.*

To sum up, acetic acid functions mainly in three ways to enhance the PHB formation in mixed bacterial culture:

(1) The presence of acetate induces the transcription of *pmoA* gene and uses the methane monooxygenase for the conversion to downstream molecules;

(2) The induction of *pmoA* gene can enhance cell response to methane. Apparently, the ability to utilize acetate is a metabolic backup system, allowing them to maintain the methane oxidation machinery in high efficiency;

(3) Acetate, as a sole source of carbon and energy, cannot fulfill all growth requirements of these obligate methanotrophic bacteria, which will lead to the decline of growth. However, when acetate is added as co-substrate along with the methane, and the extra amount of reducing

powers obtained from acetate oxidation allows metabolism to function with sufficient energy source;

(4) The presence of acetic acid provides extra carbon sources for the heterotrophs existing in the culture. The access to acetic acid reduces their dependence on the intermediate products produced by methane users.

3.3.5. The characterization of PHB biopolymer

In order to characterize the PHB biopolymers produced by mixed culture under various conditions, FT-IR spectroscopy was used to evaluate the intermolecular interactions. Usually, the changes in the strength and the position of IR absorption peaks resulting from some characteristic functional groups, such as carbonyl and hydroxyl, can be attributed to the existence of intermolecular interactions. Figure 3.9 and 3.10 show the FT-IR for PHB biopolymers produced by the oxidation of methane alone. There are two main ranges in the infrared spectra that are sensitive to the formation of hydrogen bonds: one is the carbonyl vibration region in 1650–1800 cm^{-1}, and the other is the hydroxyl vibration region in 3000–3600 cm^{-1}. PHB with the chemical structure of carbonyl in the monomeric repeating unit that yields a C=O stretching mode near 1724 cm^{-1}. The functional groups of the extracted PHB polymer were identified as C=O group by FT-IR spectroscopy at 1720 cm^{-1} in all samples, and no major shifts of carbonyl peak were observed, indicating the addition of acetic acid with different concentrations does not change the basic structure and functional group compositions of the monomers. The IR spectroscopic analysis gave further insights into the chemical structure of the polymer and reflected the monomeric units. The results obtained in this study are similar to the findings reported by other researchers (S. D. Li et al., 2003; Xu et al., 2002): 2975, 2978 cm^{-1} (CH, CH_2, CH_3); 2935 cm^{-1} (CH, CH_2, CH_3); 1720 (ester C=O valence); 1136 cm^{-1} (O-C-O).

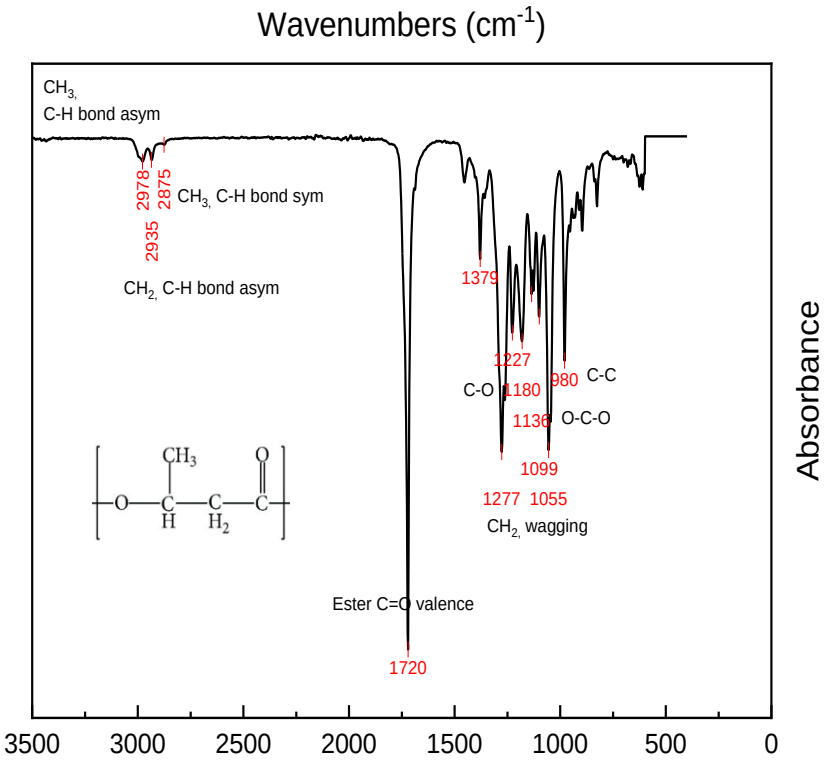

Figure 3. 9 FT-IR spectrum of PHB biopolymer extracted from mixed cultures fed with methane only

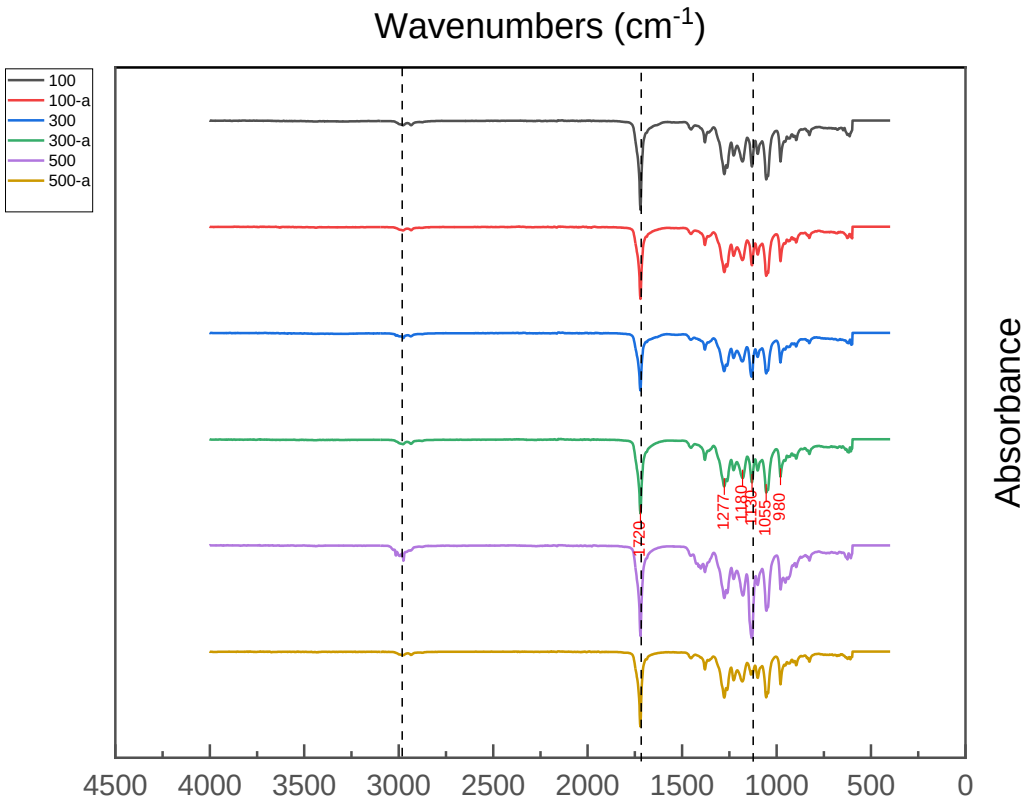

Figure 3. 10 FT-IR spectra of PHB extracted from mixed enrichment cultures with acetic acid additions (100, 300, and 500 represent the treatment with added acetic acid without pH adjustment; 100-a 300-a and 500-a represent the treatment with added acetic acid with pH adjustment).

The DSC analysis was carried out to estimate the glass transition temperature and melting temperature of PHB to evaluate the mobility of the polymer chains. Table 3.1 observed slight variations of melting temperatures for PHB derived from different treatment conditions. In the control treatment using methane as sole substrate, the measured T_m was 171.7 °C, which is lower than 178 °C reported by Myung et al. at very similar conditions (Myung, Flanagan, et al., 2016).

With the addition of acetic acids as co-substrate ranging from 100 to 500 mg/L under pH non-adjusted conditions, the T_m of the PHB biopolymers slightly increased from 164.3, 169.1 to 171 °C with the increase of acetic acid concentrations. By comparison, in the treatments with the addition of acetic acids but under pH adjusted conditions, the T_m of the PHB biopolymers was 170.6, 173.1, and 170.0 °C. It should be noted that the DSC peaks from PHB biopolymers under acid conditions due to the introduction of acetic acid with uncontrolled pH with showed complex patterns, which was not observed from control treatment and the treatments with pH adjustment. The complex peaks with varying glass transition temperatures could be attributed to the crystallinity of the PHB biopolymer, which denotes the arrangement of the polymer chains inside the PHB biopolymer structure. Therefore, it is strongly inferred that the PHB biopolymer derived under optimal pH conditions has better mobility of the polymer chain resulting from the higher melting temperature.

Table 3.1 Melting temperature and peak molecular weight of extracted PHAs

Incubation condition	**pH**	**Melting temperature (°C)**	**Peak molecular weight (Da)**
CH_4 alone	Not controlled	171.7	3.24×10^6
CH_4 + 100 mg/L acetic acid	Not controlled	164.3	2.83×10^6
CH_4 + 100 mg/L acetic acid	Controlled	170.6	3.07×10^6
CH_4 + 300 mg/L acetic acid	Not controlled	169.1	2.79×10^6
CH_4 + 300 mg/L acetic acid	Controlled	173	3.57×10^6
CH_4 + 500 mg/L acetic acid	Not controlled	171	3.19×10^6
CH_4 + 500 mg/L acetic acid	Controlled	171.7	3.21×10^6

3.4 Conclusions

The methanotrophic-heterotrophic community produced in this chapter has shown its capability of the enhanced production of PHB with the addition of acetic acid. With an acetic acid concentration of 300 mg/L and the pH-controlled, the maximum PHB production reached 34.8 ± 1.8 wt% compared to the production of 26.4 ± 3.5 wt% in the control treatment. The joint assimilation of methane and acetic acid by methanotroph and symbionts was due to the coupling of the reactions of methane oxidation with the second substrate, which provides more efficient utilization of the energy and carbon and indicates the contribution of monooxygenase reaction into the energy metabolism.

When acetic acid was added in high concentrations, the dissociation of protons affected the culture pH, this in turn, can lower internal pH and alter the ionization of nutrient molecules and thus reduce their availability to the organisms. The responses to the external pH change to maintain the cytoplasmic pH require the participation of enzymes such as ATPase or other proteins to engage in pumping excessive protons out of the cells. These responses involve energy input and reduce the overall efficiency of energy metabolism.

Chapter 4

MOLECULAR EVIDENCE FOR ENRICHMENT OF METHANOTROPHIC CULTURE FROM WASTEWATER TREATMENT SYSTEM

Abstract

Little is known about the potentials of sludge sources from different treatment processes of WWTPs to enrich methanotrophic cultures in the short term for the production of PHB. Methanotrophic enrichment cultures were cultivated using various inocula from activated sludge and anaerobic digestion processes in wastewater treatment plants, which explored the feasibility and suitability for scaling up PHAs production. Methanotrophs and PHA-accumulating bacteria were characterized by polymerase chain reaction (PCR) of the *pmoA* and *phaC* functional gene to identify the capability of forming particulate methane monooxygenase and PHA synthase. The metagenomic sequencing of16S rRNA gene from original sludges and enrichment cultures showed that the microbial community of enrichment narrowed the operational taxonomic unit (OTU) down for approximately 96.4%. Taxonomy diversity of enrichment culture with sludge inoculum from Village Creek Reclamation Facility observed higher bacterial diversity after the enrichment processes compared with the enrichment cultures from Southside WWTP. After 5 and 15 cycles of enrichment with methane as the sole carbon source, all cultures presented

positive results of the *pmoA* and *phaC* gene, which indicates the enrichment of methanotrophs and PHA-accumulating bacteria.

4.1. Introduction

Microorganisms in the wastewater treatment plants (WWTPs) are essential for treating wastewater and protecting the public and environmental health. The biodiversity of microbes in the sludges from different unit processes of WWTPs are critical for the stable and efficient operation of WWTPs. Methanotrophic bacteria are highly specialized microorganisms that are widespread in nature. The presence of methanotrophic bacteria has been observed in different types of natural environments as well as engineering systems such as biological treatment processes in WWTPs and pipelines (AlSayed, Fergala, Khattab, et al., 2018; Corder et al., 1986; Myung, Galega, et al., 2015; Myung, Wang, et al., 2015).

However, little attention was given to their role in WWTPs facilities, and to our knowledge, only a few studies have yet described the diversity and abundance of methanotrophs from various unit processes in WWTPs. Hence, in this chapter, we aim to 1):develop methane-oxidizing enrichment cultures from activated and anaerobic digesters sludges using the same cultivation approach described in chapter 3; 2):determine the diversity and abundance of methane-oxidizing bacterial communities as well as functional genes in these enrichment cultures; and (3):determine the potentials of these enrichment cultures for the generation of PHB.

4.2. Materials and methods

4.2.1. Cultivation of enrichment cultures

Wastewater sludges were sampled from multiple unit processes of Southside WWTP in the City of Dallas and Village Creek Water Reclamation Facility in the City of Fort Worth that represent the different treatment units in domestic wastewater treatment plants. The sludge samples collected include waste activated sludge (WAS), returned activated sludge (RAS), mixed sludge (MS), digested sludge (DS) from anaerobic digesters, and co-digested sludge (CDS) containing FOG (fat, oil, and grease). Figure 4.1 shows the treatment processes and the specific sludge sampling units in each WWTP, and Table 4.1 summarizes the characteristics of all the sludge samples. All sludge samples were stored in the 4˚C cold room before incubation.

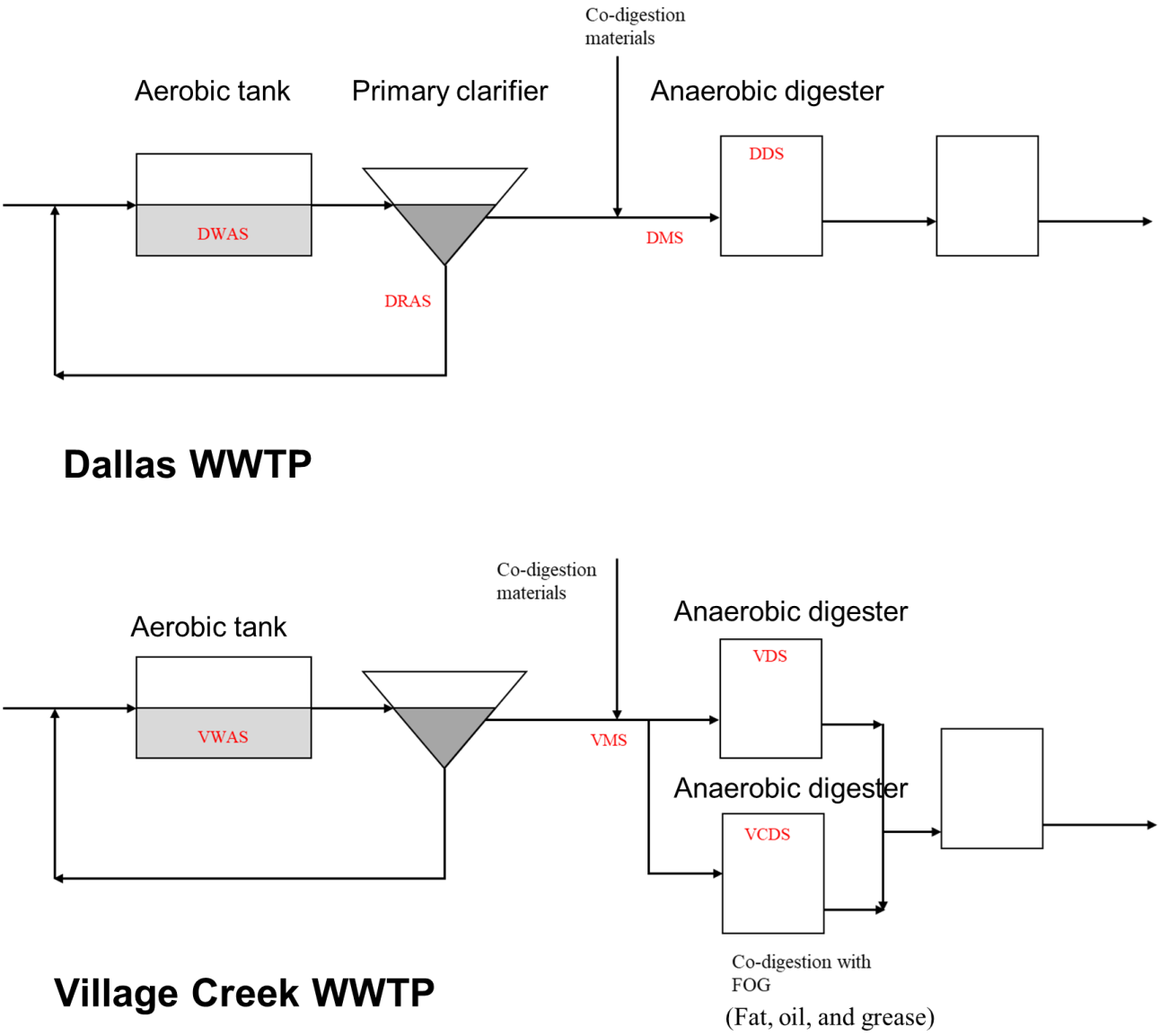

Figure 4. 1 The diagrams of treatment processes in Southside WWTP in City of Dallas (sample prefix "D") and Village Creek Water Reclamation Facility in the City of Fort Worth (sample prefix "V").

Table 4. 1 The characteristics WWTP sludge

	Sample	Location	Description	pH
Dallas Southside WWTP	DWAS	From aerobic tank	Aerobic environment	6.9
	DRAS	Returned sludge from the aerobic tank	Oxic environment	7.0
	DMS	After pretreatment	Mixture with co-digestion material	5.8
	DADS	Primary anaerobic digester	Anaerobic environment	7.8
Village Creek Reclamation Facility	VWAS	Before pretreatment	A mixture of primary sludge and waste activated sludge	5.9
	VMS	After pretreatment	Mixture with co-digestion material	5.9
	VADS	Primary anaerobic digester	Anaerobic environment	7.1
	VCDS	Anaerobic co-digester	Contains FOG	7.2

The original sludge samples were centrifuged (3,000 × g) for 15 min to create a pellet, which was added to enrichment medium with the inoculum at a total suspended solids (TSS) concentration of 1000 mg/L. The pellet was resuspended in 50 mL medium JM2 and shaken to obtain a dispersed cell suspension. Triplicate batch incubations were performed in 170-ml serum bottles capped with autoclaved blue butyl rubber stoppers under the methane/oxygen of 1:1 by volume in the headspace on a shaker (150 rpm) in the dark at 30°C. Headspace gases of methane and oxygen were replenished every two days. After the confirmation of enrichment completion, the headspace of each bottle was flushed with a CH_4: O_2 mixture (volume ratio of 1:1) every 24 h. Enrichment cultures at cycle 0, cycle 5, and cycle 15 were sampled and preserved for at -20°C until further molecular microbiology analysis.

4.2.2. DNA extraction

After establishing a repeated cycle of cultivation, 100-µL samples were removed from each enrichment culture and centrifuged to pellets, and then genomic DNA (gDNA) was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) per the manufacture's protocol. The concentration of DNA was determined using spectrophotometry using A260/A280 (NanoDrop One, Thermo Scientific Inc., WI, USA).

4.2.3. PCR amplification and gel electrophoresis

All primers shown in Table 4.2 were custom ordered from Integrated DNA Technologies, Inc. (Coralville, CA).

Table 4. 2 PCR primers used for the 16S rRNA genes of bacteria and functional genes of methanotrophs

Targeted gene		Sequence (5'-3')	Reference
16s rRNA	8F	AGAGTTTGATCCTGGCTCAG	(Myung, Wang, et al., 2015)
	1492R	GGWTACCTTGTTACGACTT	
pmoA	A189F	GGNGACTGGGACTTCTGG	(Shao et al., 2019)
	mb661R	CCGGMGCAACGTCYTTACC	
mmoX	mmox 945F	TGGGGCGCAATCTGGAT	
	mmox 1401R	TGGCACTCRTARCGGTC	
phaC	CF1	ATCAACAARTWCTACRTCYTSGACCT	(Sheu, 2000)
	CR4	AGGTAGTTGTYGACSMMRTAGKTCCA	

As for PCR of 16s rRNA primers for universal bacteria, reactions were carried out in a 25-µL PCR mixture as follows: consisting of 2.5 µL 10×PCR buffer II (including dNTPs), 0.5 U

high fidelity AccuPrime Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA), 10 μM of both forward and reverse target only primers, 100 ng sample DNA. The PCR operations are described using the following program: denaturation at 94 °C for 1 min, and 10 cycles of 94 °C for 20 s, 53 °C for 25 s, and 68 °C for 45 s, with a final extension at 68 °C for 10 min. The triplicate products of each sample from the first round PCR were combined, purified with GeneJET PCR Purification Kit (Thermo Scientific, Waltham, MA, USA), eluted in 25 μL water, and transferred into a new PCR tube (Myung, Wang, et al., 2015).

As for PCR of 16s rRNA primers for pMOA and mmoX, each 25-μL PCR mixture contained 100 ng DNA, 400 nM forward/reverse primers set, 0.5 U Taq DNA polymerase, and 2.5 μL 10×PCR buffer II (including dNTPs). The PCR amplification reactions were conducted in an automated thermal cycler (SimpliAmp Thermal Cycler, Thermo Fisher Scientific, MA, USA). The PCR operations are described using the following program: 95 °C for 15 min; 35 cycles of 95 °C for 1 min, 55 °C for 45 sec, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The presence of PCR amplicons was determined using gel electrophoresis. The expected amplicon size is 473 and 457 bp (Shao et al., 2019).

As for PCR of 16S rRNA primers for *phaC* polymerase chain reaction (PCR) per 25-μL reaction: 100 ng DNA, 0.75 μM each primer, and 0.5 U Taq DNA polymerase, and 2.5 μL 10×PCR buffer II (including dNTPs). The PCR operations are described using the following program: 94°C for 10 min, 51°C for 2 min, 72°C for 2 min; followed by 35 cycles of denaturation (94°C for 20 s), annealing (57°C for 45 s), and elongation (72°C for 1 min); and a final extension at 72°C for 10 min. The presence of PCR amplicons was determined using gel electrophoresis. The expected amplicon size is 481 bp, and observed bands of this length were stored positive for *phaC* (Sheu, 2000).

Positive PCR reactions were sequenced to verify the identity of the products. Gel electrophoresis was performed to verify the presence of PCR amplicons. Gel cassettes (E-Gel EX 1% agarose, Thermo Fisher Scientific, MA, USA) were operated on E-GE Power Snap Electrophoresis Device (Thermo Fisher Scientific, MA, USA).

4.3. Results and discussions

4.3.1. Bacterial community diversity and abundance

An operational taxonomic unit (OTU) is commonly used to classify groups of microorganisms closely related to each other. Figure 4.2 showed the changes of OTUs detected from original sludges and enrichment culture after 15 cycles of cultivation. In addition, two samples of the highly enriched cultures from chapter 3 were used as the positive control. The 16S rRNA sequences spanning the hypervariable regions V4 and V5 clustered into 614-937 OTUs from the original sludge samples before they were cultivated for enrichment. The high values of OTUs obtained from the original sludge samples taken from the WWTPs indicated the large number of bacterial species. The numbers of OTUs in the sludge samples from aerobic-oxic unit processes in WWTPs such as activated sludges and returned activated sludges were higher than anaerobic digester sludge samples. After the enrichment, the abundance of the bacterial OTUs significantly decreased to the range of 34-99 after the 15 cycles of enrichment processes, especially for the sludges (OTUs 34-56) from Dallas Southside WWTPs. The OTU numbers were close to the OTU numbers of 21/23 from a highly enriched microbial culture, suggesting a successful enrichment approach. For example, there are 34 OTUs detected from DWAS enrichment culture, compared with the 937 OTUs in the original DWAS. Approximately 96.4% of the bacteria species have been screened out during the enrichment process selecting

methanotrophic bacteria. As for the cross-comparison between two WWTPs, the cultures from Southside WWTP had a much lower number of OTUs than cultures enriched from the Village Creek Reclamation facility.

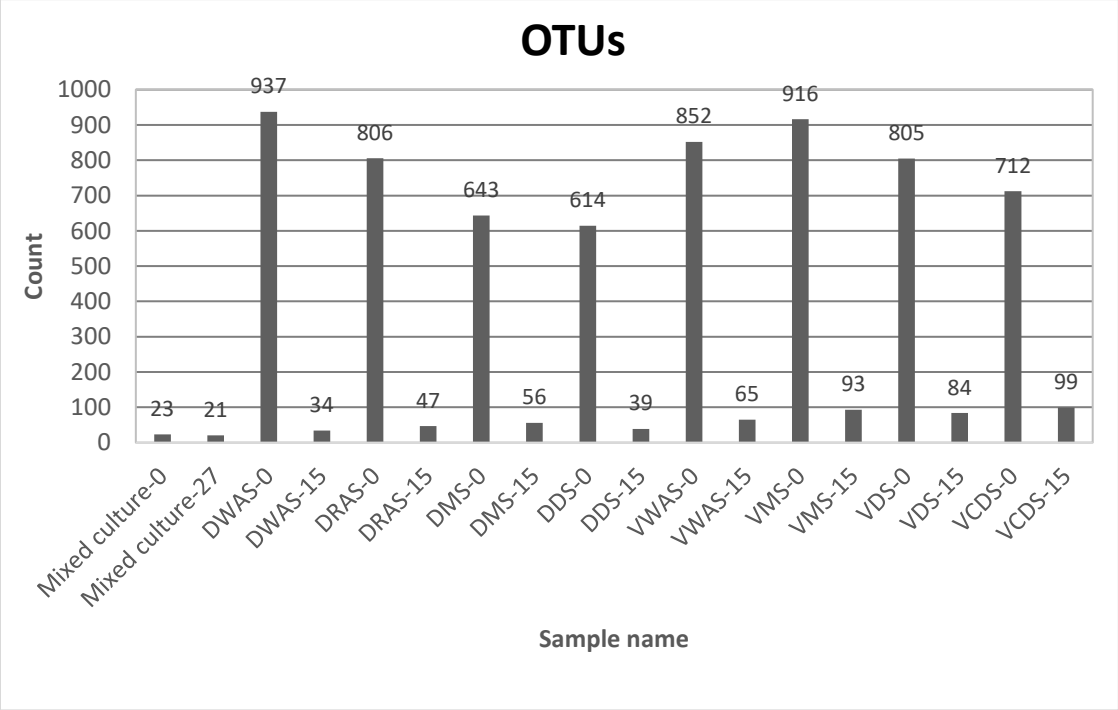

Figure 4. 2 The OTUs detected from original sludges and enrichment culture after 15 cycles of cultivation

Unweighted Pair Group Method with Arithmetic mean (UPGMA) is a type of hierarchical clustering method using average linkage and can be used to interpret the distance matrix from different microbial communities. As shown in Figure 4.3, highly enriched microbial culture (mixed culture-0 and mix culture-27) used in chapter 3 were compared as the control group to calculate the cluster distance of the original sludge samples and the enrichment cultures.

After 15 cycles of enrichment, all the cultures became close to the mixed culture, especially the shortest distance from VWAS-15. DDS-15 and DMS-15 clusters were also close to each other as the distance between them was small at0.025. Enrichment cultures from Southside WWTP have closer cluster hierarchies. It should be noted that, in the original cultures, DDS and VCDS had the closest distance, indicating the general bacterial communities were close to each in anaerobic digesters.

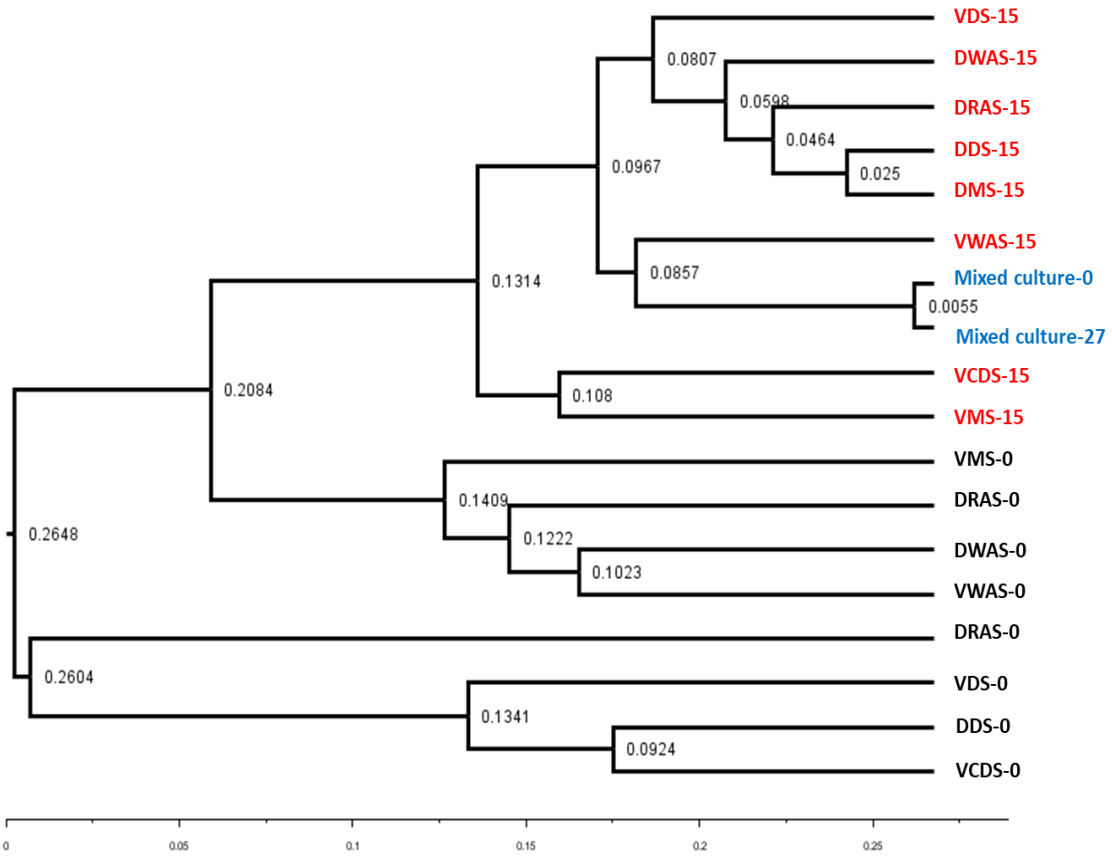

Figure 4. 3 Unweighted Pair Group Method with Arithmetic (UPGMA) dendrogram constructed from similarity matching data.

4.3.2. 16S rRNA gene sequencing

The 16S rRNA sequences were analyzed to understand the composition of bacterial communities. Figure 4.4 showed the bacterial diversity of cultures sampled from Southside WWTP after being enriched for 15 cycles. All enrichments had a common dominant specie as *Dyella agri*. It accounted for 85.2% of the total in DMS and 79.2% in DDS. By contrast, the abundance of the *Dyella agri* was rather low from all original sludge inoculum. The enrichment condition with methane as the sole carbon source favored the growth of this strain. *Paraburkhoderia* was detected as the second-largest genus with 34.8 % in DWAS, 3.83% in DMS, and 8.88% in DDS, respectively. Many species of *Paraburkhoderia*, such as *Paraburkholderia xenovorans* LB400 (Sanhueza et al., 2020; Urtuvia et al., 2018), *Paraburkholderia sacchari* IPT 101 (Oliveira-Filho et al., 2019) have been reported for their capabilities of PHA synthesis. In DRAS, *Methylocystis* was the third-largest bacteria with a percentage of 9.18 %, followed by *Paludibacterium*. DDS, DWAS enrichment cultures have also detected *Methylocystis.* Other genera such as *Stenotrophomonas (Iqbal et al., 2016; Munir et al., 2015),* and many strains from *Rhodanobacter (Juan C López et al., 2014; Zhao et al., 2019)* also contributed to the accumulation of PHAs. Except for the large amount of *Dyella* present in all enrichment cultures, the percentages of the remaining genera were similar to the previously reported enrichment cultures (Cha et al., 2016; Inoue et al., 2016; Myung, Galega, et al., 2015). The remaining bacterial species consisted of *Hydrotalea*, *Hyphomicrobium*, and other minor genera, including *Burkholderia*, *Rhodopseudomonas*, *Castellaniella*, and *Sphingomonas*. There is limited information about *Hydrotalea* species, but they have the reported ability to assimilate formate and can use carbohydrates as growth substrates. Methanotrophic cultures frequently coexist with methylotrophs or heterotrophs that survive on methanotrophic by-products such as

methanol. Thus, a distinctive feature of the PHA-synthesizing community was the predominant groups of genera. The presence of methanotrophs and multiple different genera of bacteria capable of PHA synthesis made the enrichment cultures available for PHA generation.

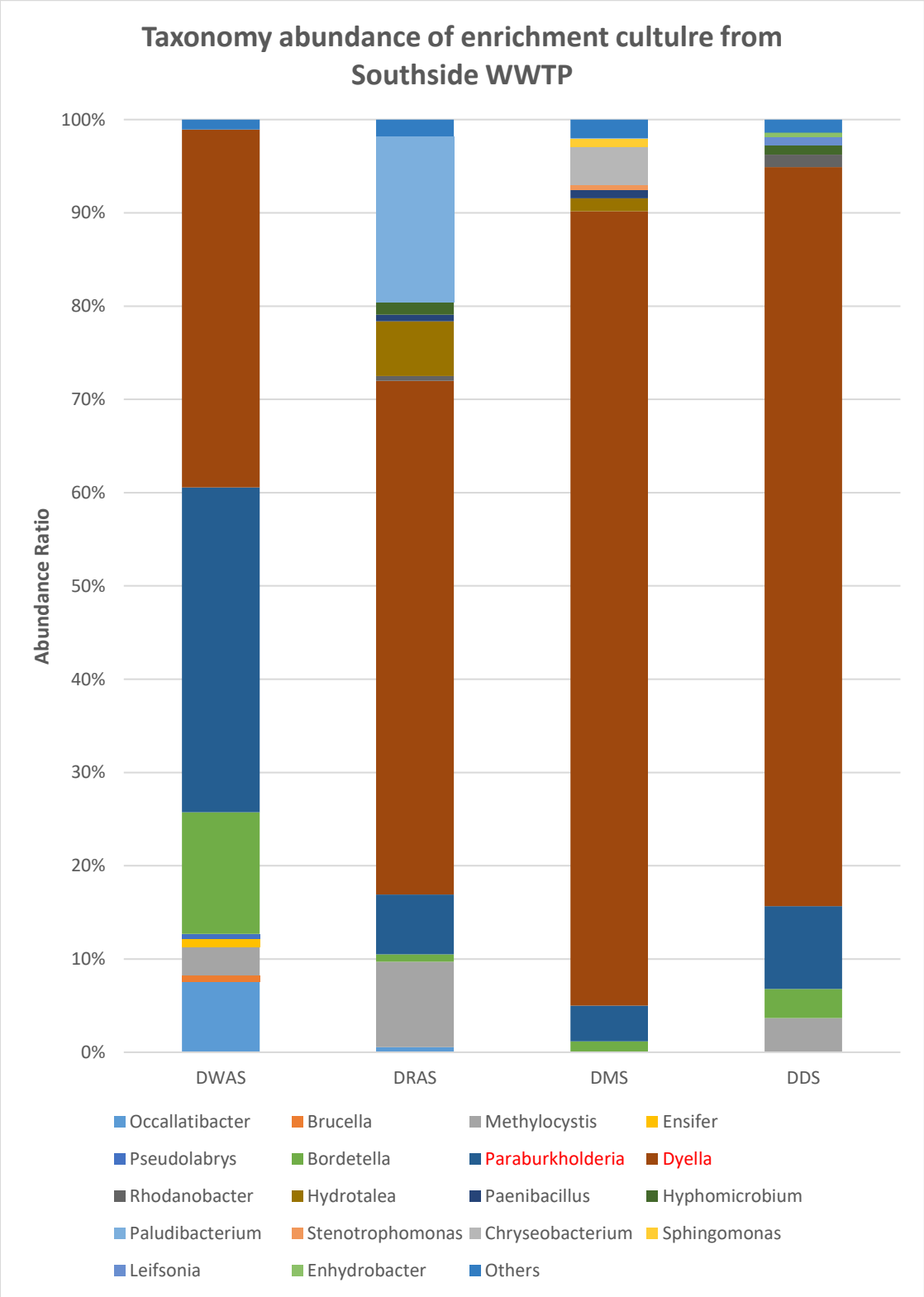

Figure 4. 4 Taxonomy abundance of enrichment culture from sludge samples in Southside WWTP

Figure 4.5 shows the taxonomy diversity of enrichment culture with sludge inocula from Village Creek Reclamation Facility. Compared with the enrichment cultures from Southside WWTP, a higher bacterial diversity was observed after the enrichment processes. *Dyella* was still the dominant genus in VWAS. It is under the family of *Rhodanobacteraceae* that is mostly isolated from soil, rock, and environmental sediment samples (Anandham et al., 2011; Xie & Yokota, 2005). The methanotroph *Methylocystis* was detected as the dominant genus (28.4%) in the VCDS enrichment culture. VMS and VWAS also had 25.7%, and 9.9% of *Methylocystis* detected, respectively. Facultative methylotroph *Hyphomicrobium* has also been accumulated from enrichment cultures in the range of 7-11% in VWAS, VMS, and VCDS. *Pseudomonas* that accounted for 16.3%, 17.8%, and 6.2% of the total bacterial abundance in VMS, VDS, and VCDS has been frequently reported to contribute to PHA synthesis with the presence of *phaC* gene (Huijberts et al., 1992; Langenbach et al., 1997; Salvachúa et al., 2020; Ward et al., 2005). Although methane was supplied as the sole carbon source in the enrichment cultures, many strains detected from the enrichment cultures are heterotrophs. Thus, it is possible that the intermediate metabolites from methane users served as the carbon sources to support heterotroph growth.

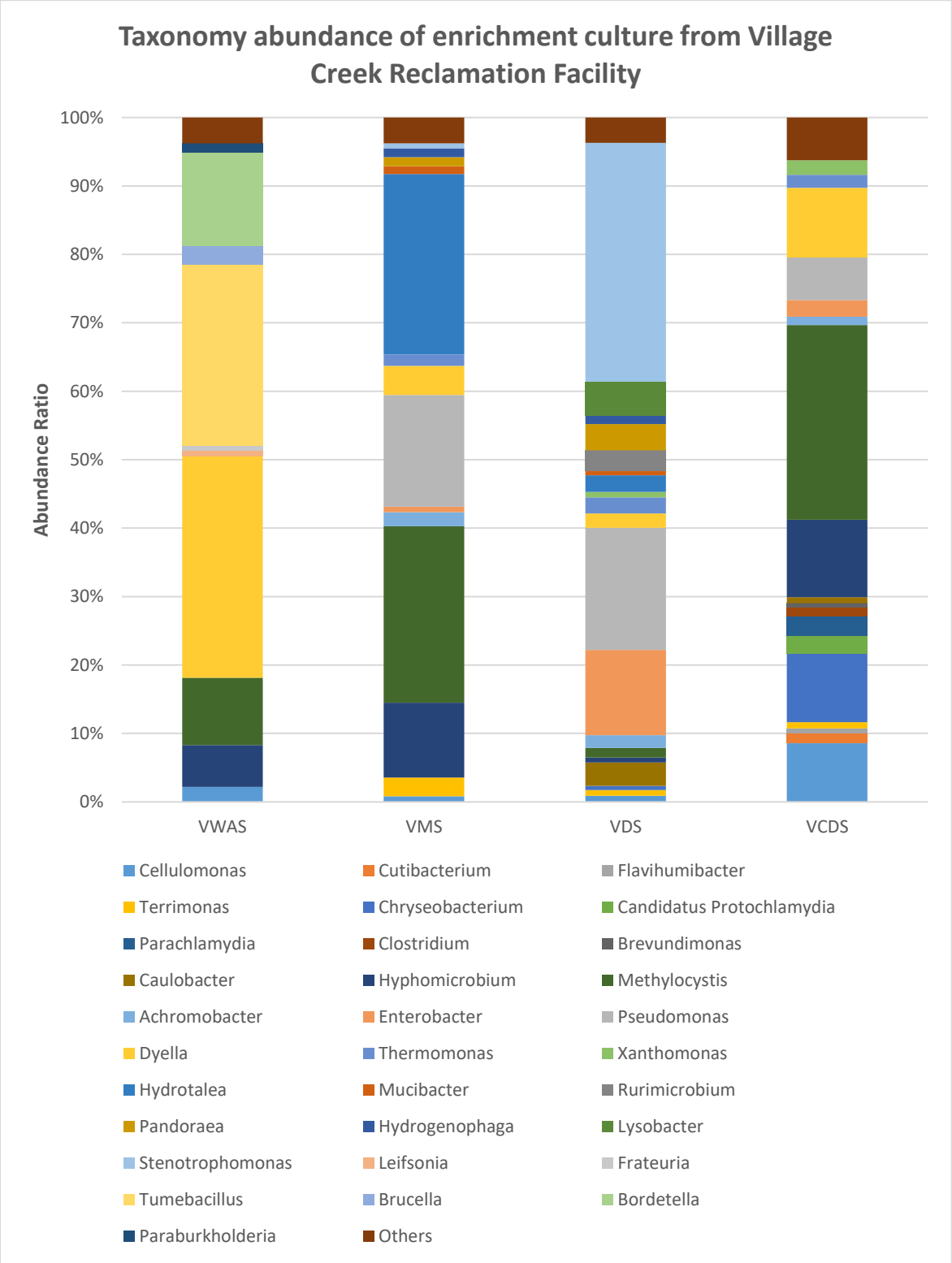

Figure 4. 5 Taxonomy abundance of enrichment culture from sludge samples in Village Creek Reclamation Facility

4.3.3. The detection of functional genes

PHAs function as carbon and energy reserves in prokaryotic cells. They are accumulated by a wide range of bacteria when a carbon source is provided in excess and one essential growth nutrient is limited. The synthesis of PHAs requires the presence of enzymatic PHA synthase (encoded by *phaC*), which uses β-hydroxyacyl-coenzyme A substrates for polymerization. The production of such substrates can occur through a variety of pathways, including using the enzymes β-ketothiolase (encoded by *phaA*) and acetoacetyl-CoA reductase (encoded by *phaB*), and β-oxidation. To examine the presence of PHA-accumulating bacteria in our enrichment culture, the PCR approach with specifically degenerate primers, phaCF1, and phaCR4, was performed on cycles 0, 5, and 15 of all cultures. The expected PCR products amplified from bacterial genomic DNA should have a fragment length of 496 bp.

Figure 4.6 shows the results of PCR amplification with the primer pair of phaCF1 and phaCR4. All the original sludge samples except DRAS and VMS (very weak band) are negative because of the absence of bands. The results suggested that bacteria with the capability to accumulate PHA were deficient before the enrichment processes. DRAS and VAS were sludge samples taken from unit processes where sludges were recycled. Bacteria were exposed to an anaerobic-oxic environment with limited nutrients, which might be favorable for some PHA-accumulating bacteria to survive in the sludges. With the progress of the enrichment process up to cycle 5, all the cultures exhibited the presence of *phaC* gene, which indicated the proliferation of PHA-accumulating bacteria. The band of *phaC* on electrophoresis was even brighter after cycle 15. The selection pressure imposed by methane as the sole carbon source under nutrient limiting condition successfully shifted the bacterial communities to the direction of methane-

utilizing and the cohabitants of methane users with the potential for PHA accumulation.

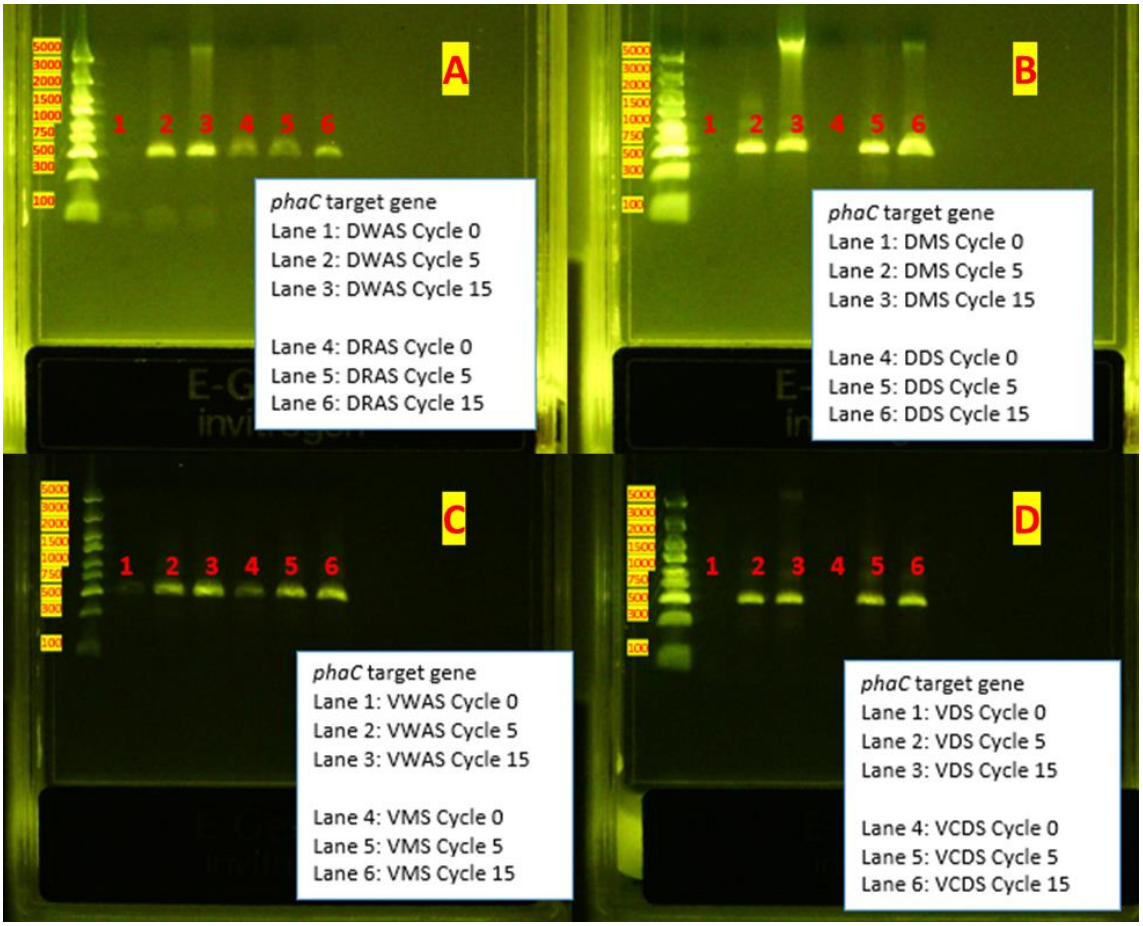

Figure 4. 6 Gel electrophoresis of PCR products using primers for phaC gene: A) Lane 1-3 are results of DWAS from cycle 0, 5, and 15; Lane 4-6 are the results of DRAS from cycle 0, 5, and 15; B) Lane 1-3 are results of DMS from cycle 0, 5, and 15; Lane 4-6 are the results of DDS from cycle 0, 5, and 15; C) Lane 1-3 are results of VWAS from cycle 0, 5, and 15; Lane 4-6 are the results of VMS from cycle 0, 5, and 15; D) Lane 1-3 are results of VDS from cycle 0, 5, and 15; Lane 4-6 are the results of VCDS from cycle 0, 5, and 15; The PCR amplification should have fragment length of 496 bp.

Aerobic proteobacterial methanotrophs possess the particulate methane monooxygenase, the key enzyme in methane oxidation. The *pmoA* gene encodes the β subunit of the enzyme protein and is present in a vast number of methanotrophs, making it a suitable marker for the

enrichment studies. To capture aerobic methanotroph diversity, a *pmoA*-based analysis was performed with the A189f-mb661r primer pair. The *pmoA* gene is present in virtually all methanotrophs, allowing wider coverage of the methanotroph (Ho et al., 2013). PCR primer pair of A189f-mb661r that amplify a fragment of *pmoA* has been designed to detect the presence of methanotroph, methane users, and their activities among the bacteria communities.

Figure 4.7 shows the *pmoA* PCR products amplified from genomic DNA obtained from all sludges and enrichment cultures. Samples taken from DWAS and VWAS showed very vague bands at cycle 0, while the samples taken from other WWTP units showed negative results. The aerobic environment in DWAS and VWAS are conducive to the growth of methanotrophic bacteria because of the participation of oxygen in methane oxidation. After 5 and 15 cycles of enrichment with methane as the sole carbon source, amplification was observed with all cultures obtained during the enrichment process. The positive bands demonstrated the presence of target *pmoA* genes from methane-utilizing bacteria.

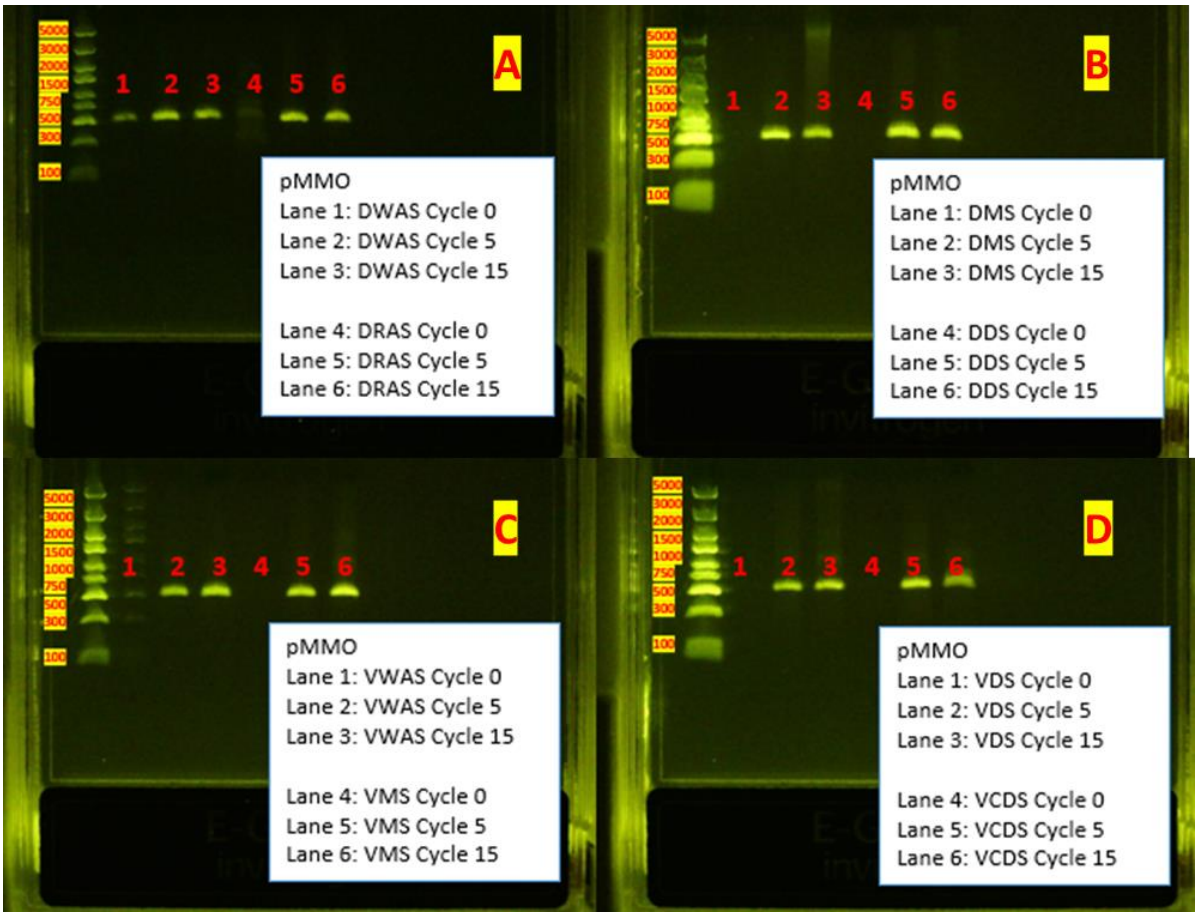

Figure 4. 7 Gel electrophoresis of PCR products using primers for pmoA gene: A) Lane 1-3 are results of DWAS from cycle 0, 5, and 15; Lane 4-6 are the results of DRAS from cycle 0, 5, and 15; B) Lane 1-3 are results of DMS from cycle 0, 5, and 15; Lane 4-6 are the results of DDS from cycle 0, 5, and 15; C) Lane 1-3 are results of VWAS from cycle 0, 5, and 15; Lane 4-6 are the results of VMS from cycle 0, 5, and 15; D) Lane 1-3 are results of VDS from cycle 0, 5, and 15; Lane 4-6 are the results of VCDS from cycle 0, 5, and 15; Amplified fragment should have a length of 525 bp.

4.4 Conclusions

In this chapter, we conclude that a repeating cycle of methanotroph cultivation can yield a stable community capable of reproducibly producing PHA. The OTUs in the enrichment culture was greatly reduced with bacteria capable of producing particulate methane monooxygenase and PHA synthase confirmed through the detection of functional genes. The metagenomic sequencing of enrichment cultures after 15 cycles showed a wide diversity of

methanotrophic bacteria and the bacteria capable of PHA formation. The units in WWTPs such as the unit for returned sludge, and the anaerobic digesters with rich lipid content as co-substrates demonstrated the superior potential for enriching methanotrophic bacteria and PHA synthesis.

Chapter 5

CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

5.1. Conclusions

The core of the research objectives in this thesis is to develop a strategy for enhanced methanotrophic production of PHB using methane and acetic acid and offer a promising opportunity for practical application. The significant outcomes of this research in response to the research questions proposed in Chapter 1 are summarized.

First, the addition of acetic acid as co-substrate of methane to mixed methanotrophic culture and the pH-associated effect on the production of PHB were discussed in **Chapter 3**. An increased PHB yield was achieved with the addition of 300 mg/L acetic acid under pH-controlled conditions. When pH was not adjusted at high acetic concentration, changes in the intracellular pH can alter the ionization of nutrient molecules and thus reduce their availability to the organisms. The joint assimilation of methane and acetic acid by methanotroph and symbionts was due to the coupling of the reactions of methane oxidation with the second substrate, which provides more efficient utilization of the energy and carbon and indicates the contribution of monooxygenase reaction into the energy metabolism.

Second, the mechanistic role of acetic acid as it participates in cellular

metabolism was proposed by the experimental results in **Chapter 3**. The addition of a co-substrate promotes the metabolism and presents a great possibility to increase the amount of reducing power available to methanotrophs; thus, the assimilation of carbons intracellularly to produce biopolymer. The presence of acetate induces the transcription of the *pmoA* gene and uses the methane monooxygenase for the conversion to downstream molecules, which provides an extra amount of reducing power obtained from acetate oxidation to provide pMMO function with sufficient energy source.

Third, the potentials of sludge sources from different treatment processes of WWTPs to enrich methanotrophic cultures in the short term were discussed in **Chapter 4**. Taxonomy diversity of enrichment culture with sludge inocula from Village Creek Reclamation Facility exhibited higher bacterial diversity after the enrichment compared with the enrichment cultures from Southside WWTP. The aerobic-oxic units in WWTP, such as a unit for returned sludge and anaerobic digesters with rich lipid content as co-substrates demonstrated the superior potential for methanotroph dominated cultures.

5.2. Recommendations for future work

Overall, this study has highlighted the potential of mixed microbial cultures for the methanotrophic production of PHB using methane as the sole substrate or co-substrate of methane and acetic acid. However, more research is urgently needed to achieve higher rates and yields, maximize gas-liquid mass transfer, and determine appropriate strategies to control microbial diversity or metabolic activity for enhanced production of PHB. In addition, the balance between co-substrates types and cost and the values of altered polymer composition remains an important issue to be solved.

APPENDIX I

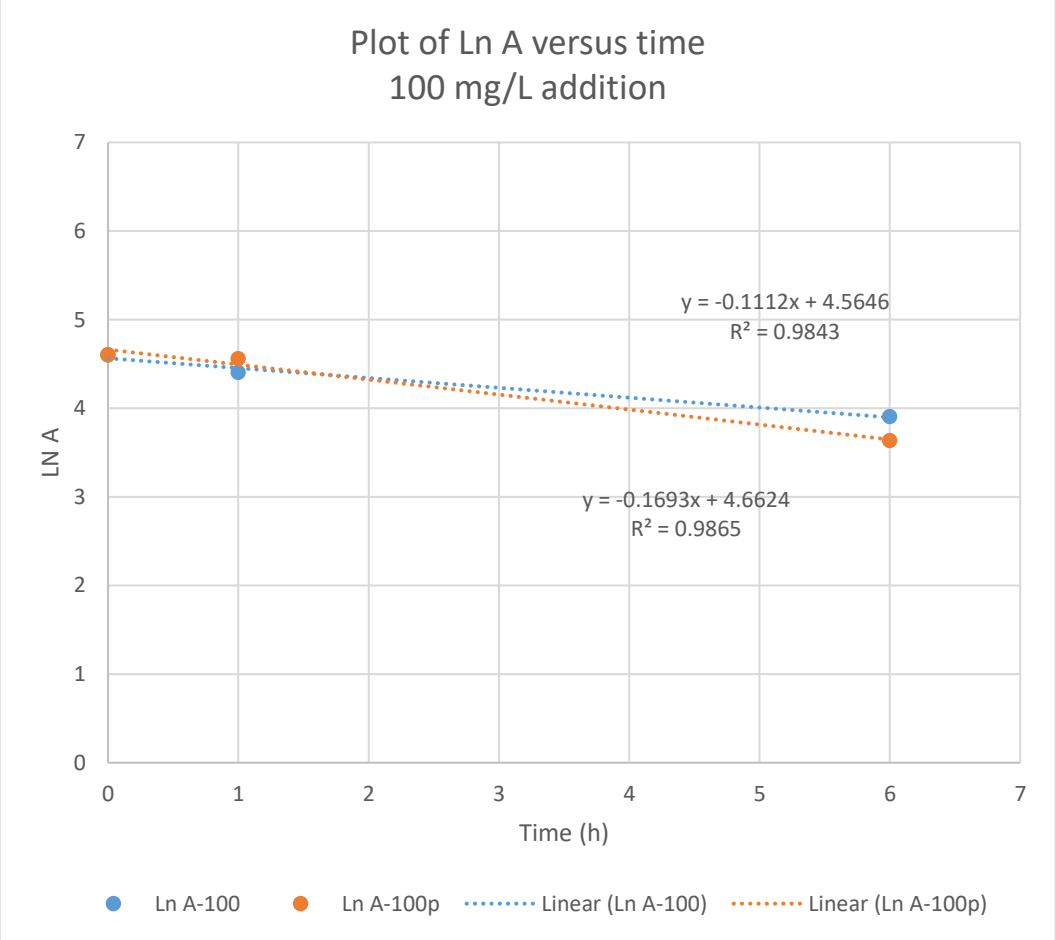

Figure S. 1 Kinetic analysis of acetic consumption at the concentration of 100 mg/L

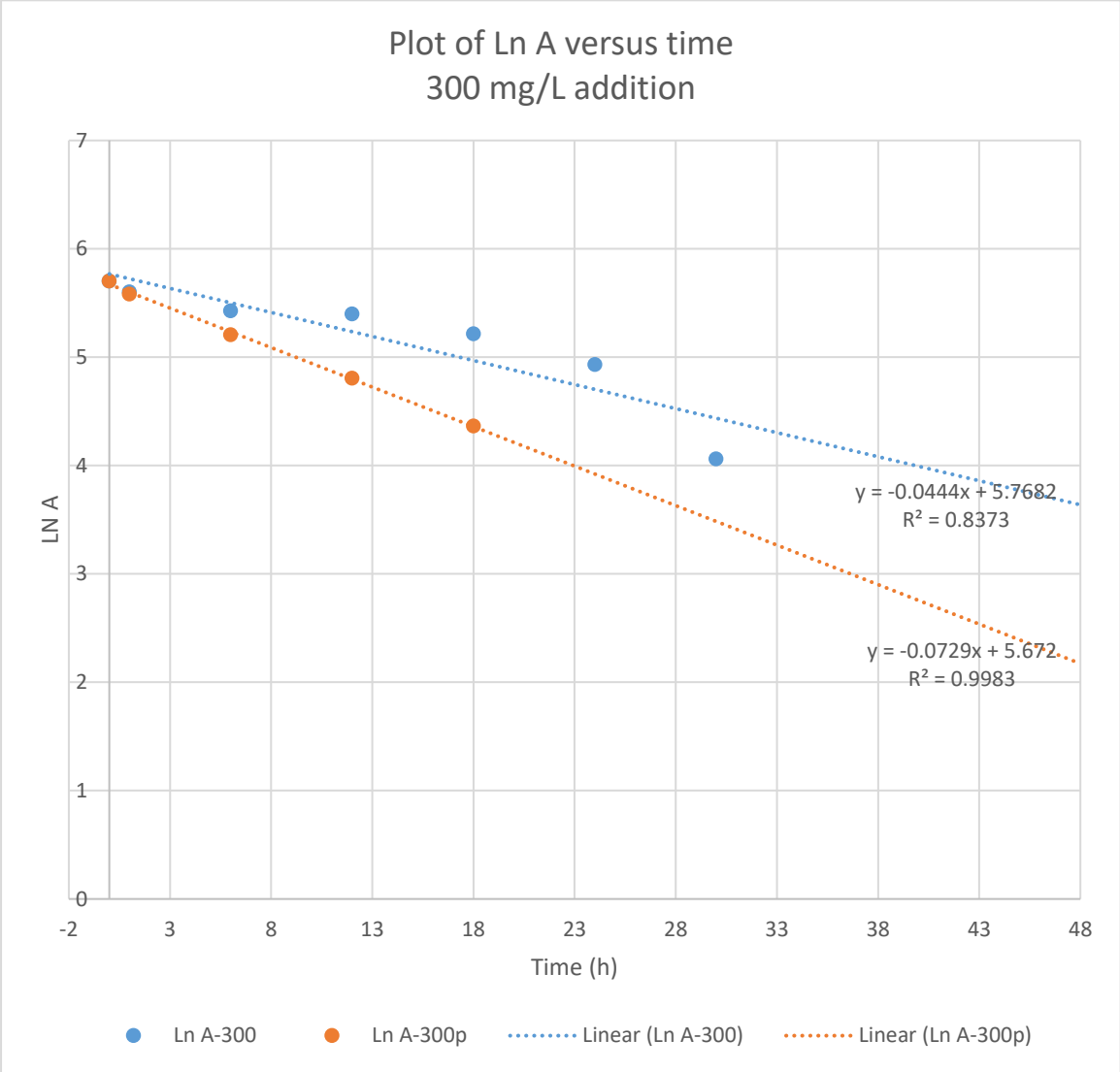

Figure S. 2 Kinetic analysis of acetic consumption at the concentration of 300 mg/L

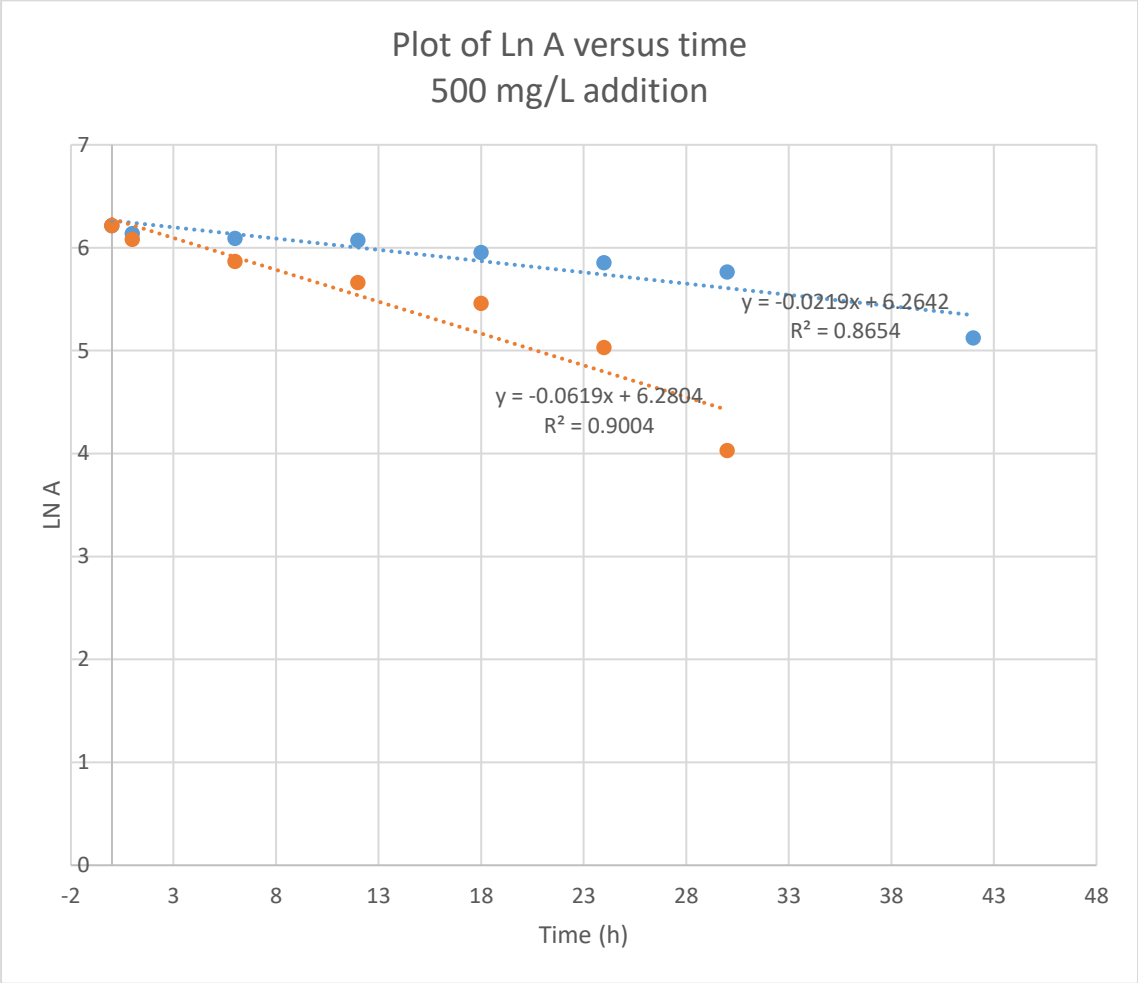

Figure S. 3 Kinetic analysis of acetic consumption at the concentration of 500 mg/L

APPENDIX II

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