Exploring the potential of *Methylomicrobium album BG8* as a platform for the bioconversion of methane and methanol to isoprenoids

by

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Abstract

Methane and methanol are inexpensive and sustainable carbon feedstocks for bioconversion to value-added products. Methane being a greenhouse gas, its conversion has the added benefit of mitigating emissions that affect climate change. Methanotrophic bacteria consume methane and methanol as sole sources of carbon and energy and can convert them into value-added products such as bioplastics, biofuels, and platform chemicals. The methanotroph *Methylomicrobium album* BG8 is notable for its fast and robust growth and rich genetic potential, making it an excellent candidate for bioproduction. The aim of this work was to develop genetic tools and processing methods to expand the range of potential bioproducts synthesized by this bacterium.

Towards this goal, three broad host range plasmids based on the IncP, IncQ and pBBR1 incompatibility families were developed. These vectors were introduced into *M. album* BG8 through conjugation and were successfully propagated. Two expression vectors based on the IncP replicon and IncQ replicon were validated with expression of a luciferase protein under the P_{MMA} (Mxa type methanol dehydrogenase) constitutive promoter. pBBR1 and IncQ plasmids were propagated simultaneously by *M. album* BG8, suggesting plasmids from different incompatibility families can be co-stabilized in this strain. Three different promoters were used in these expression vectors to demonstrate expression levels of heterologous genes. These results open the door towards production of commercially useful products in a modified *M. album* BG8 strain.

To improve strain production, batch, sequential batch and fed-batch strategies were developed for *M. album* BG8 cultures in bioreactors. In the fed-batch operation of *M. album* BG8, maximum optical density reached 16.56, a value comparable to that obtained with more

widely studied methylotrophic strains that grow on methanol. This culturing study further lays the basic framework for advanced bioreactor operations aimed at achieving high density growth of *M. album* BG8 towards an industrial platform for methanol bioconversion.

Isoprenoids are a class of molecules with a broad range of applications, including as precursors for aviation fuel, and thus represent a promising class of value-added products for methane and methanol bioconversion. Genetically modified *M. album* BG8 has the potential to synthesize certain types of isoprenoids, some of which are toxic to microbes. Based on their potential toxicity, a study was conducted with six isoprenoids: α-pinene (+), α-pinene (-), β-pinene (-), D-limonene, sabinene and farnesene. *M. album* BG8 was more tolerant towards pinene isomers than other isoprenoid classes tested. The addition of a top layer of n-dodecane increased LD_{50} of all isoprenoids. Thus, the inclusion of an n-dodecane overlayer during production can facilitate industrial biosynthesis of isoprenoids by *M. album* BG8.

Together, the results from this thesis lay a framework for future isoprenoid production from methane and methanol by a genetically modified strain of *M.album* BG8.

Preface

This thesis is an original work performed by Shibashis Das. Chapters 3, 4, and 5 are in preparation for publication.

Chapter 3 will be submitted for publication as Das S., Sauvageau, D, Stein, L. Y., "Development of genetic tools for heterologous protein expression in *Methylomicrobium album* BG8." As primary author I was responsible for conceptualizing, performing the experiments, analyzing the data and writing the manuscript. Dr. Dominic Sauvageau (supervisor) and Dr. Lisa Stein (supervisor) were the supervisory authors who contributed to all parts of the study including financial support, the experimental design and data interpretation, advice and guidelines and editing of the manuscript.

Chapter 4 will be submitted for publication as Das S., Ansalemi P, Stein, L. Y., Sauvageau, D., "Developing batch and fed-batch strategies for methanol bioconversion by *Methylomicrobium album* BG8." As the primary author I was responsible for analyzing the data and writing the manuscript. Pablo Ansalemi and I conceptualized and performed the experiments. Dr. Dominic Sauvageau (supervisor) and Dr. Lisa Stein (supervisor) were the supervisory authors who contributed to tall parts of the study including financial support, the experimental design and data interpretation, advice and guidelines and editing of the manuscript.

Chapter 5 will be submitted for publication as Das S., Sauvageau, D, Stein, L. Y., "Toxicity of isoprenoid precursor molecules on methanotrophic bacteria." As primary author I was responsible for conceptualizing, performing the experiments, analyzing the data and writing the manuscript. Dr. Dominic Sauvageau (supervisor) and Dr. Lisa Stein (supervisor) were the supervisory authors who contributed to tall parts of the study including financial support, the experimental design and data interpretation, advice and guidelines and editing of the manuscript.

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List of Abbreviations

2,3-BDO:	Butandiol
BHR:	Broad host range plasmid
CJF:	Conventional jet fuel
DMAPP:	Dimethyl allyl pyrophosphate
DNA:	Deoxy ribonucleic acid
FB:	Fed batch
FPP:	Farnesyl pyrophosphate
FPPS:	Farnesyl pyrophosphate synthase
GC:	Gas chromatography
GFP:	Green fluorescent protein
GHG:	Green house gas
GPP:	Geranyl pyrophosphate
GPPS:	Geranyl pyrophosphate synthase
IPP:	Isopentenyl pyrophosphate
LD:	Lethal dose
LD_{20}:	Lethal dose 20

LD_{50}: Lethal dose 50

LD_{80}: Lethal dose 80

LS: Limonene synthase

MDH: Methanol dehydrogenase

MEP: Methylerythritol phosphate

MIC: Minimum inhibitory concentration

MMO: Methane monoxygenase

MOB: Methane oxidizing bacteria

MVA: Mevalonate

MXA: Mxa type methanol dehydrogenase

NA: Not available

NAD: Nictoniamide adenine dinucleotide

NADH: Nictoniamide adenine dinucleotide (reduced)

NB: Nutrient broth

ND: Not determined

NFI: Normalized fluorescence intensity

NHOC: Net heat of combustion

NMS: Nitrate mineral salt

NT: Not toxic

OD_{540} : Optical density at 540 nm

PCN: Plasmid copy number

PCR: Polymerase chain reaction

P_{GAP}: Promoter of glycerladehyde 3-phosphate dehydrogenase

PHA: Polyhydroxyalkanoate

PHB: Polyhydroxybutyrate

P_{MMA}: Promoter of M. album BG8 mxa type methanol dehydrogenase

P_{MMO}: Promoter of particulate methane monoxygenase

P_{PYC}: Promoter of pyruvate carboxylase

PS Pinene synthase

P_{Tet}: Tetracycline inducible promoter

RBS: Ribosomal binding site

RNA: Ribo nucleic acid

SAF: Sustainable aviation fuel

SB: Sequential Batch

SCP: Single cell protein

WT: Wild type

Chapter 1: Introduction

1.1 Context and motivation

Methane and methanol are garnering interest as feedstocks for bioindustry due to their high availability and low cost (Kalyuzhnaya et al. 2015, Cotton et al. 2020). Methane is a major greenhouse gas that must be reduced to mitigate climate change and its conversion can also add value to the bioeconomy (Clomburg et al., 2017). It is a major waste product of the oil and gas, landfill and agriculture industries. Methanol can be derived from natural gas, biomass, and carbon dioxide conversion (Tan et al. 2023) and is a biproduct of blue and turquoise hydrogen production (Banu et al. 2023). Developing methane and methanol as feedstocks for bioconversion has the added advantage of substituting sugar-based feedstocks which can directly compete with food production, such as the case with corn ethanol (Wu et al., 2018).

Aerobic methanotrophs are a group of bacteria that utilize methane and methanol as sole sources of carbon and energy. Hence, they are ideally suited as feedstocks for bioconversion to value-added compounds. Multiple strains of methanotrophs have recently been investigated as potential bioconversion platforms, such as *Methylotuvimicrobium alcaliphilum* 20Z (Nguyen et al. 2020), *Methylotuvimicrobium buryatense* 5GB1 (Puri et al. 2015), and *Methylosinus trichosporium* OB3b (Nguyen et al. 2020). *Methylomicrobium album* BG8 is a gammaproteobacterial methanotroph that shows robust growth under a wide range of conditions (Tays et el. 2018). It can grow reliably on either ammonium or nitrate as the nitrogen source and either methane or methanol as the carbon source (Tays et al. 2018, Sugden et al. 2021). It can be adapted for growth under harsher conditions such as low pH (McDonald, 2019). Its transcriptome and metabolome have been described (Sugden et al. 2021) and a genome scale

metabolic model has been constructed (Villada et al. 2022). These properties make it a strain of interest as a potential bioconversion platform for methane and methanol.

Of the many potential products of bioconversion, the broad class of isoprenoids have garnered particular interest due to their wide range of applications such as precursor of high performance fuel (Woodroffe et al., 2021), food additives, insecticides (Vickers et al., 2014) etc. Microbial bioproduction of these compounds requires genetic engineering of the organisms through the modification or inclusion of a complete isoprenoid biosynthesis pathway. Studies have demonstrated isoprenoid production in multiple strains like *Escherichia coli*, *Saccharomyces cerevisiae*, *Synechocystis* sp., *Yarrowia lipolytica* (Walls & Rios-Solis, 2020). Interestingly, *M. album* BG8 naturally contains the majority of genes required to produce isoprenoid precursors, an important characteristic that opens the door for its genetic modification towards isoprenoid production from methane or methanol as feedstocks. Finding the potential of *M. album* BG8 to produce isoprenoids would lead to utilization of methane and methanol as a feedstock for production of ultraperformance fuel.

1.2. Objective of the thesis

The overarching goal of this thesis is to develop *M. album* BG8 as a potential platform for biological conversion of methane and methanol into isoprenoids.

A robust set of genetic tools is required for industrial development of bacteria that lack essential genes for production of a target product. While tools for generating genetic knockouts have been developed for *M. album* BG8 (Kang-Yun et al., 2022), no tools exist to express heterologous proteins for this organism.

Along with genetic tool development, biomass density and productivity are important factors to increase chemical production and ensure industrial viability. These parameters can be improved through various bioreactor feeding and operation strategies.

Finally, while multiple isoprenoids are potential jet fuel precursors (Walls & Rios-Solis, 2020), they cannot all be produced in *M. album* BG8. Isoprenoids are known for their toxic effect on microbes (Liu et al., 2022), including methanotrophs (Amaral et al., 1998). Hence it is important to determine the toxic effects of various classes of isoprenoids on *M. album* BG8 to best select an appropriate target for production.

Altogether, there are three specific aims in this thesis: 1) to develop robust tools for heterologous gene expression in *M. album* BG8 2) to find a suitable bioreactor operation for generating *M. album* BG8 biomass at high density, and 3) to investigate the toxic effect of select isoprenoids with potential as jet fuel precursors on methanotrophs.

1.3. Thesis structure

Chapter 2 provides a literature review on the importance of methane and methanol as feedstocks, recent bioconversion work in methanotrophs, and approaches and tools developed for genetic engineering in methanotrophs. The physical properties of isoprenoids are discussed in light of their possible application as high-performance fuel precursor molecules. The toxic effect of isoprenoids on the growth of microbial hosts, as well as possible strategies for alleviating those effects, are surveyed. Furthermore, bioreactor strategies applied to other methanotrophs are discussed.

Chapter 3 presents genetic tool development for heterologous gene expression in *M. album* BG8. Results of plasmid transformation and fine-tuned expression of reporter genes under

inducible and constitutive promoters in various expression vectors are discussed. These genetic tools could be used for expression of genes related to isoprenoid production in future work.

Chapter 4 is dedicated to exploring batch, sequential batch and fed-batch operation strategies in *M. album* BG8 using methanol as a carbon source. Batch cultivation is the simplest bioreactor operation. Sequential batch operation can be useful to maintain bioreactor cultures for long periods of time. Fed-batch operation was investigated to obtain high density cultures of *M. album* BG8.

Chapter 5 is the study of the toxicity of isoprenoids on *M. album BG8* and three other methanotrophic strains of industrial importance: *M. trichosporium* OB3B, *M. alcaliphilum* 20Z and *Methylocystis* sp. WRRC1. The study includes a determination of the lethal isoprenoid dose on each of these strains and of the impact of an n-dodecane overlay on the viability of methanotrophic strains exposed to isoprenoids. The results from this study help identify the most suitable isoprenoid for production by the studied methanotroph strains.

Chapter 6 is a general conclusion from the thesis where the main results and important findings are highlighted. The final conclusion makes recommendations on the suitability of *M. album* BG8 as a bioconversion platform of methane and methanol into isoprenoids. Future research aimed at the industrialization of *M. album* BG8 are also discussed.

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Chapter 2: Literature review

2.1. Methane and methanol as carbon feedstocks

The objective to reach net zero emissions by the middle of this century is an important goal towards keeping the maximum warming within 1.5 ° C, as outlined by COP26 UN climate change summit (https://ukcop26.org/). Fossil fuel is the major source of carbon emission (Global Carbon Project (2022)). Biomanufacturing using processes like microbial fermentation has the potential to replace fossil fuel use in sectors such as production of polymer, industrial solvents, and aviation fuel (Scown, 2022). Glucose derived from biomass is a commonly used feedstock in microbial fermentation; but in this case, biomass generation directly competes with food production (Zhang et al., 2022), and agrochemical use leads to contamination of freshwater resources (Nordborg et al., 2014). Hence other feedstocks are needed. Methane and methanol are inexpensive carbon feedstocks that do not directly compete with food production and are being investigated for their potential to drive the bioeconomy (Kalyuzhnaya et al. 2015, Cotton et al. 2020).

Methane is a major greenhouse gas, second only to CO_2 in terms of contribution to global warming. In addition, its global warming potential is 86 times greater than CO_2 over a 20-year time horizon (The Global Methane Budget 2000-2017). Anthropogenic sources of methane, which accounts for approximately 60% of emissions, include flaring from oil and gas, agriculture, and landfill sites. Methane emissions must be reduced to mitigate climate change. This creates an opportunity as methane conversion can also add value to the bioeconomy (Shindell et al. 2012). In fact, waste methane has the potential to meet global demand of essential organic compounds: for example, the carbon from natural gas vented or flared worldwide in

2014 would be enough to meet demand of several essential building-block organic chemicals like ethylene, propylene, butadiene, xylene, toluene (Clomburg et al., 2017). Methane can also be produced from electrochemically derived hydrogen and CO_2 (Götz et al., 2016).

Methanol is another single-carbon feedstock with high conversion potential into value-added products (Pfeifenschneider et al., 2017). Unlike methane, methanol is highly miscible in water and its liquid nature allows for easier storage and transportation, thus making it more suitable as a feedstock as compared to methane (Cotton et al., 2020). While methanol is currently mostly produced from fossil fuel (Bertau et al., 2014), it can also be produced sustainably through conversion from biogas (Rosha et al., 2021) or combining electrochemically derived hydrogen and CO_2. (Szima & Cormos, 2018). Recently a CO_2 -to-methanol plant has been set up in China with a capacity to produce 110,000 tonnes of methanol annually (https://www.carbonrecycling.is/news-media/worlds-largest-co2-to-methanol-plant-starts-production).

Methane and methanol are thus emerging as important feedstocks and reusable waste products for establishing a circular carbon economy (Cotton et al. 2020).

2.2. Methanotrophs

Methanotrophs are defined as a unique group of microorganisms that can utilize methane as a sole source of carbon and energy (Murrell, 2010). These organisms can be aerobic or anaerobic in their methane oxidation process (Guerrero-Cruz et al., 2021). In the current thesis only aerobic methanotrophs will be discussed. Aerobic methanotrophs belongs to one of three taxonomic groups Gammaproteobacteria, Alphaproteobacteria and Verrucomicrobia (Kalyuzhnaya et al., 2015; Murrell, 2010; Sharp et al., 2014). These are mainly differentiated

based on their metabolism of methane to generate biomass. Alphaproteobacterial (Alpha-MOB) and gammaproteobacterial (Gamma-MOB) methanotrophs are more closely related to each other as they both belong to the phylum proteobacteria. Methanotrophs from these groups have been isolated since the 1970s (Hazeu & Steennis, 1970; Patt et al., 1974; Whittenbury et al., 1970), while strains from the Verrucomicrobia phylum have only been described since the 2000s (Dunfield et al., 2007). To date, industrial application has only been reported for Alpha-MOB and Gamma-MOB (Table 2.1). The following sections will describe physiology related to Alpha-MOB and Gamma-MOB. A schematic of methane metabolism in Alpha-MOB and Gamma-MOB is shown in Figure 2.1.

Methane metabolism is an essential step for these organisms. The enzyme methane monooxygenase (MMO) catalyzes the first step, the oxidation of methane to methanol, using molecular oxygen (Hanson & Hanson, 1996). Two versions of the MMO enzyme exist. The membrane-bound particulate methane monooxygenase (pMMO) is found in almost all methanotrophs and the soluble methane monooxygenase (sMMO) is found mainly in Alpha-MOB and only certain Gamma-MOB (Semrau et al., 2010). pMMO is arranged in tight arrays within the intracytoplasmic membranes which are characteristic structures of methanotrophs (Koo et al., 2022). pMMO and sMMO differ in terms of their mechanism even though they catalyze the same reaction. The difference lies in the electron transfer mechanism and may be responsible for the lower biomass yield on methane for Alpha-MOB as compared to Gamma-MOB (Bordel et al., 2019). Of the two MMOs, sMMO has been studied more thoroughly, owing to the difficulty of isolation of functional pMMO (Ross & Rosenzweig, 2017). In fact, the structure of pMMO was only recently been described by cryo-EM in a lipid environment (Koo et al., 2022). Functional heterologous expression of pMMO in microbial hosts has not yet been

achieved; thus, methanotrophs the only group of bacteria that can use methane as feedstock in bioconversion operations.

After the oxidation of methane to methanol by MMO, methanol is further oxidized to formaldehyde as catalyzed by methanol dehydrogenase (MDH). Two versions of this enzyme exist. The mxaF1 type is found universally in Alpha- and Gamma-MOB (Khmelenina et al., 2018), while the XoxF type is found in a subset of methanotrophs (Chu & Lidstrom, 2016; Farhan Ul Haque et al., 2015). Understanding the properties of the MDH enzyme is beneficial to industrial production of methanol from methane using methanotrophs. In fact, scaled-up methanol production was achieved in *M. trichosporium* OB3B (Lee et al., 2004) and *M. capsulatus* Bath (Chen et al., 2022) by using sodium chloride and cyclopropanol as MDH inhibitors.

Following methanol oxidation to formaldehyde, unassimilated formaldehyde is further oxidized to formate using the enzyme formaldehyde dehydrogenase. Two forms of that enzymes exist. One is the dye (cytochrome) linked formaldehyde dehydrogenase and other is $NAD(P)^+$ linked dehydrogenase (Zahn et al., 2001). The NAD (P)+ linked enzyme can have further sub categories depending on its need of secondary cofactors such as Thiol compounds, tetrahydrofolate, methylene tetrahydromethanopterin (Vorholt, 2002; Zahn et al., 2001). In addition to direct oxidation by enzymes in methanotroph *M.trichosporium* OB3b H_4MPT pathway was found to be playing the main role in formaldehyde oxidation as determined from its transcriptomic study (Matsen et al., 2013).

Last, formate is oxidized to CO_2 via formate dehydrogenase to produce reducing equivalents in the form of NADH to meet energy requirements of the cell (DiSpirito et al., 2004).

In Gamma-MOB formaldehyde is assimilated by the ribulose monophosphate or RuMP pathway. Through its intermediates (fructose-6 phosphate, glyceraldehyde-3-phosphate, etc.) this pathway is connected to sugar-linked metabolic pathways such as glycolysis, pentose phosphate pathway, and Entner–Doudoroff pathway (Hanson & Hanson, 1996; Trotsenko & Murrell, 2008). Hence Gamma-MOB will be useful when metabolites from these pathways are required for metabolic engineering.

For Alpha-MOB, formaldehyde is assimilated through the serine pathway. Useful features of this pathway include the simultaneous generation of acetyl-CoA as an intermediate along with CO_2 assimilation into biomass (Hanson & Hanson, 1996; Trotsenko & Murrell, 2008). In *M. trichosporium* OB3b up to 60 % biomass carbon is derived from CO_2 (Yang et al., 2013). This makes Alpha-MOB highly suitable for biogas utilization, as it can contain significant amounts of CO_2 (Kalyuzhnaya et al., 2015). This group of MOB has relatively higher acetyl-CoA flux than Gamma-MOB making them useful for generating bioproducts based on carboxylic acids such as PHA. In addition to high acetyl CoA flux multiple strains of Alpha-MOB contain native PHA synthesis pathway leading to these being explored for PHA bioproduction such as *M. trichosporium* OB3b (Rostkowski et al., 2013) , *Methylocystis* sp. Rockwell (Sharma et al., 2022), *Methylocystis parvus* OBBP (Bordel et al., 2019; Rodríguez et al., 2022) In Gamma-MOB, low acetyl-CoA is a bottleneck for PHA production (Hoang Trung Chau et al., 2022). In addition PHA synthesis pathway has been found only in a few members of this group (Luangthongkam, 2019).

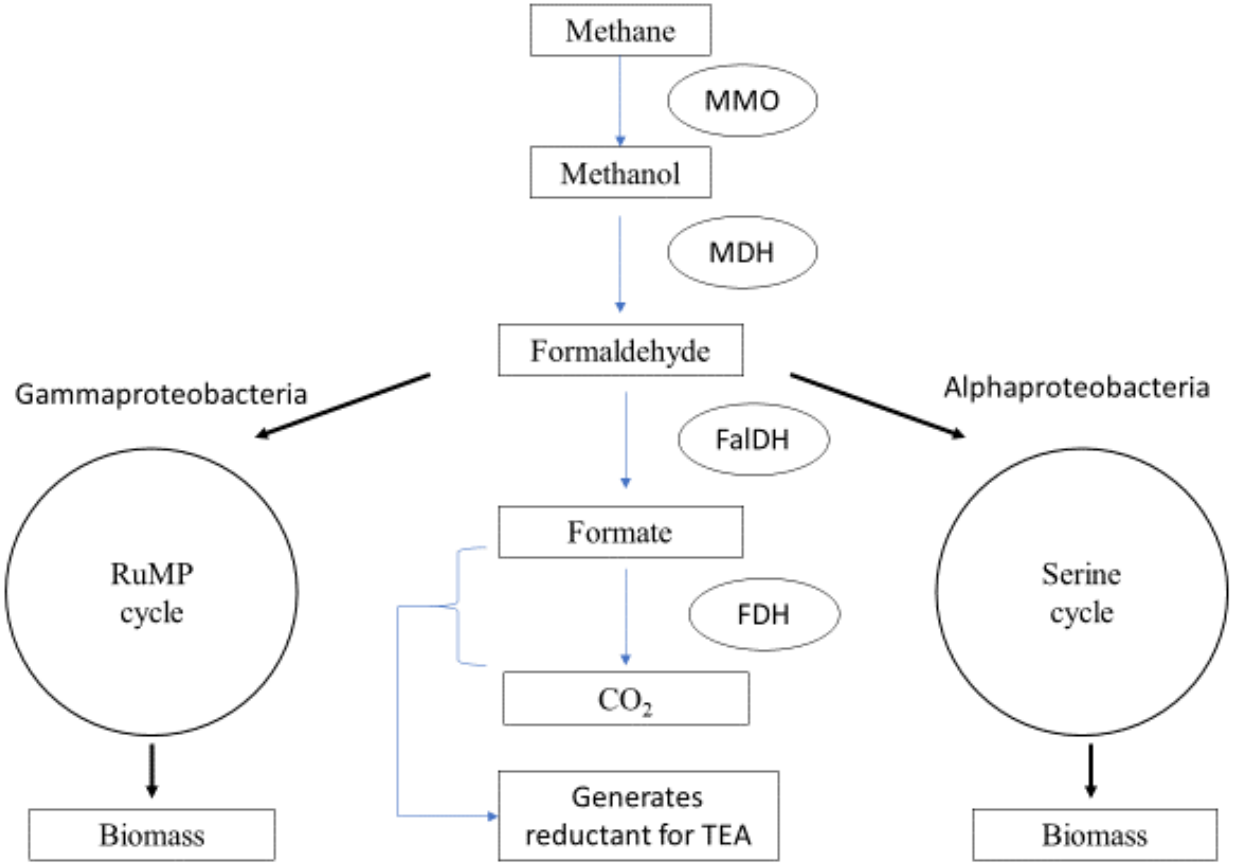

Figure 2.1. A simple schematic of core carbon assimilation in methanotrophs. MMO, MDH, FalDH, FDH stands for methane monoxygenase, methanol dehydrogenase, formaldehyde dehydrogenase, formate dehydrogenase. (DiSpirito et al., 2004; Hanson & Hanson, 1996)

Based on enzymatic efficiency and ATP yield calculations, the yield of the RuMP pathway is significantly greater than the serine pathway (Claassens et al., 2019). This could explain the faster and more efficient growth rates observed in Gamma-MOB as compared to Alpha-MOB (McGenity, 2019). Looking specifically at yield and growth rate, it could be argued that Gamma-MOB should be preferable in industrial applications, but as described earlier that decision should also take into account the feedstock properties, product metabolite requirements, and specific targets for bioproduction.

2.3. Value-added products using methanotrophs

Production of value-added products in methanotrophs has been explored since the 1980s with single cell protein (SCP) production (Kalyuzhnaya et al., 2015) being the most significant. It does not require a genetically engineered strain and a pure methanotroph culture may not be necessary (Øverland et al., 2010). Currently, SCP is closest to commercial scale production using methanotrophic bacteria (https://calysta.com/). SCP generated by methanotrophs has potential to provide 7-11 % of the global protein requirement at an affordable cost of US $ 3-5/kg dry weight (García Martínez et al., 2022). SCP generated from *Methylococcus capsulatus* (Bath) shows promise as an animal feed for pigs, broiler chickens, mink, Atlantic salmon, rainbow trout, and Atlantic halibut (Øverland et al., 2010). SCP does have certain drawbacks. For instance, the high nucleic acid content can affect consumer health. Hence, SCP is currently only certified for use in animal feed (Strong et al., 2015). Nucleic acids can be removed using heat treatment processes (Ritala et al., 2017) making bacterial SCP suitable for human consumption. Another drawback could be a high level of contamination during growth.

However, reports suggest contamination from other bacteria can be helpful in maintaining a continuous culture of *M. capsulatus* Bath for SCP production (Bothe et al., 2002). There is always a distinct possibility of negative effects of contamination such as cell lysis induction leading bioreactor malfunction and crash. The end product maybe less beneficial than the one approved by regulatory agencie (Kalyuzhnaya et al., 2015).

Another highly explored product is polyhrodoxyalkanoate (PHA) that includes polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxy) valerate (PHBV). These compounds ae biodegradable polyesters that can be key to sustainable plastic production (Li et al., 2016). The Alpha-MOB can accumulate PHB in their cell as storage molecules and by changing the nutrient conditions (e.g. nitrogen starvation) PHB can be produced in several Alpha-MOB strains. The nitrogen to carbon ratio is the best way to optimize PHB production in *Methylocystis* sp. Rockwell (Sharma et al., 2022) and by optimizing the nitrogen feeding strategy with *Methylocystis parvus* OBBP growing in bioreactors (Rodríguez et al., 2022). Copolymer of PHB such as PHBV has also been produced in some strains such as *Methylocystis* sp WRRC1 (Cal et al., 2016), *M. trichosporium* OB3b (Myung et al., 2016), and *M. parvus* OBBP (Myung et al., 2017). In most cases, the bacteria were fed with organic acids such as valerate and propionate along with methane and methanol to generate the co-polymer. The single carbon substrates were supplied as the energy and carbon source while the organic acids were incorporated to modify the polymer chain (Myung et al., 2016). Only one case has been reported so far where using only methane as feedstock P(3-hydroxybutyrate-*co*-4-hydroxybutyrate) achieved co-polymer production by an engineered *M. trichosporium* OB3B strain containing the 4-hydroxybutyric acid (4-HB) production pathway (Nguyen & Lee, 2021) These modifications of PHB has led to improved polymer production (Li et al., 2016). Gamma-MOB lack the enzymes necessary for

PHB production. Introducing PHB synthetic genes into the Gamma-MOB *M. alcaliphilum* 20Z allowed for PHB production from methane (Hoang Trung Chau et al., 2022).

Beyond PHB and SCP, production of several other compounds has been validated in methanotrophs such as polymer precursor, isoprenoids, and fatty acids. Some of these products are listed in Table 2.1. Looking at the strategies in the listed studies, it can be inferred that heterologous protein expression and optimization of bioreactor operation are important tools to develop to acquire a methanotrophic strain for industrial applications. Both of these parameters are explored in chapters 3 and 4 of this thess.

Table 2.1 Some value-added chemicals produced in genetically modified methanotrophs

Methanotroph	Product	Strategy applied for production	Reference
Alphaproteobacteria			
Methylosinus trichosporium OB3B	Cadaverine (Polymer precursor)	Heterologous expression lysA, pyc, ldcC, cadB combined with fed-batch operation	Nguyen et al., 2020
	3-hydroxy propionic acid (Polymer precursor)	Heterologous expression of genes of malonyl-CoA pathway combined with fed-batch operation	Nguyen et al., 2020

Methanotroph	Product	Strategy applied for production	Reference
Gammaproteobacteria			
***Methylotuvimicrobium buryatense* 5GB1**	Lactate	Heterologous expression of lactate dehydrogenase combined with continuous stirred tank bioreactor operation	Henard et al., 2016
	Crotonic acid, butyric acid	Heterologous expression of reverse β-oxidation pathway	Garg et al., 2018
***Methylomonas* sp. DH-1**	Succinate	Heterologous expression of glyoxylate shunt in a *sdh* knockout combined with fed batch operation	Nguyen et al., 2019
	Cadaverine (Polymer precursor)	Heterologous expression of cadA and cadB	Lee et al., 2021
***Methylomonas* sp. 16a**	Astaxanthin (carotenoid)	Heterologous expression of crtW and crtZ genes combined with batch operation	Ye et al., 2007

Methanotroph	Product	Strategy applied for production	Reference
***Methylotuvimicrobium alcaliphilum* 20Z**	2,3-Butandiol	Heterologous expression of 2,3-BDO gene cluster combined with fed batch operation	Nguyen et al., 2018
	α-humulene (sesquiterpenoid)	Overexpression of native MEP pathway genes, heterologous expression of α-humulene synthase	Nguyen et al., 2020

2.4. *Methylomicrobium album* BG8

Methylomicrobium album BG8 is a gammaproteobacterial methanotroph (Gamma-MOB) which was first described in 1970 (Whittenbury et al., 1970). It was isolated from freshwater lake samples and was earlier called *Methylomonas albus* BG8. It has been explored over the years for physiological studies. It was shown to co-metabolise chloromethane for better growth (Han & Semrau, 2000). Several studies have explored copper related physiology, such as studying proteins involved in copper sequestration (Berson & Lidstrom, 2006) and the effect of copper on intracytoplasmic membrane formation (Brantner et al., 1997). Recently, the reduction of nitrite to nitrous oxide under hypoxic conditions was shown in this organism as well (Kits et al., 2015).

Only recently have studies have been done to explore the industrial importance of *M. album* BG8. Its genome has been sequenced and annotated (Kits et al., 2013). The growth parameters of *M. album* BG8 were explored and showed its robust growth under a wide range of

conditions (Tays et el. 2018). In this study, *M. album* BG8 had growth using either nitrate or ammonium as a nitrogen source and either methane or methanol as a carbon source (Tays et al. 2018, Sugden et al. 2021). This metabolic flexibility suggests that *M. album* BG8 can utilize diverse feedstocks such as wastewater as the nitrogen source, either methane or methanol as the carbon source, making it highly suitable for industrial application. Furthermore, *M. album* BG8 can be adapted to harsh physiological conditions such as low pH (McDonald, 2019). The transcriptome and metabolome have been described for *M. album* BG8 and a genome scale metabolic model has been constructed (Villada et al. 2022). The transcriptome shows distinct physiological differences between methane and methanol grown cultures (Sugden et al. 2021). This study suggests that genetic tools should be tested under both carbon sources to observe physiological differences in heterologous gene expression.

A knockout system has been developed for *M. album* BG8 (Kang-Yun et al., 2022). Using that tool, *M. album* BG8 mutant cells were generated to investigate their ability to take up methanobactin, a copper-chelating chalkophore, from the environment. This knockout system could be highly useful in industrial applications. Knocking out the native plasmid *M. buryatense* 5GB1 led to its higher transformation efficiency (Puri et al., 2015). In *M. alcaliphilum* 20Z, enhanced ectoine production was achieved by knocking out genes related to ectoine byproduct formation (Cho et al., 2022). Recently, *M. album* BG8 was found to generate outer membrane vesicles (OMV) (Hermary, Mariah K., 2022). This ability could be explored for developing efficient product secretion and recovery, but further study of OMV biogenesis is necessary to develop such a process. Based on the above research, *M. album* BG8 has strong potential as an industrial methanotrophic strain. In this theses, heterologous gene expression tools and bioreactor production of biomass are explored to develop *M. album* BG8 towards that goal.

2.5. Tools for Heterologous protein expression in methanotrophs

Heterologous protein expression tools can be important for engineering bacteria for producing value-added products (Table 2.1). Usually, codon optimized genes of the production pathway is cloned into expression vectors downstream of a promoter and optimzed RBS to express protein. The engineered plasmid is then introduced into the bacterium to generate a product of interest via expression of the introduced genes.

2.5.1 DNA delivery

The most used method to introduce plasmid DNA into methanotrophs is through biparental mating using an *E. coli* S17λ donor strain containing chromosomally integrated helper genes for mobilization. The helper strain can utilize any Gram-negative bacteria as the recipient for conjugative DNA transfer (Simon et al., 1983). Conjugation has been useful in multiple methanotrophs including *M. buryatense* 5GB1 (Puri et al., 2015), *M. alcaliphilum* 20Z (Ojala et al., 2011), *M. trichosporium* OB3B (Nguyen et al., 2020; Lloyd et al., 1999), and *M. capsulatus* Bath (Csáki et al., 2003; Tapscott et al., 2019) to introduce both suicide and expression vectors. Triparental mating can also be useful where another strain containing a helper plasmid, such as pRK2013, can assist the donor strain (Figurski & Helinski, 1979). So far, the tri-parental approach has only been reported in *Methylomonas* sp. strain 16a (Ye et al., 2007). Usually for biparental mating, the process is performed for methanotrophs using nitrate mineral salts (NMS) agar plates containing nutrient broth for supporting donor growth. Both donor and methanotroph recipient strains are grown together on the plate for several days under an atmosphere of air and methane to perform conjugation before selection of transconjugants on marker plates. The recipient methanotroph strain is purified from the donor strain by using naladixic acid (Kang-

Yun et al., 2022) or rifamycin (Puri et al., 2015; Tapscott et al., 2019). Usually, methanotrophs are resistant to these antibiotics whereas the donor strain is sensitive.

Electroporation has been successful in some methanotrophs for introducing plasmid vectors including the knockout vector for *Methylocystis* sp. st.SC2 (Baani and Liesack,2008), linear DNA in *M. buryatense* 5GB1C (Yan et al., 2016), and plasmid DNA for heterologous expression of proteins in *M. buryatense* 5GB1C (Yan et al., 2016), *M. alcaliphilum* 20Z (Hoang Trung Chau et al., 2022), and *Methylomonas* sp. DH-1 (Nguyen et al., 2019). Compared to conjugation, electroporation offers a quicker process for delivering genetic material; however, this process does not work for all methanotrophs.

2.5.2 Broad host range plasmids

Plasmids are loosely categorized as broad or narrow host range based on their ability to transfer into distantly related hosts (Klümper et al., 2015). This classification was introduced as early as 1972 (Datta & Hedges, 1972). Broad host range plasmids such as RK2/RP4 can be transferred between hosts of not only different phyla (e.g. between *E.coli* and *M. trichosporium* OB3B) but also across domains of life, such as between bacteria and yeast (Heinemann & Sprague, 1989). Three broad host range replicons -- RK2/RP4, RSF1010 and pBBR1 -- were developed in this thesis for heterologous gene expression in *M. album* BG8. RK2/RP4 and RSF1010 were isolated from *E.coli* (Jain & Srivastava, 2013) while pBBR1 was isolated from *Bordetella bronchiseptica* (Antoine & Locht, 1992).

Table 2.2. Broad host range replicons and their utility in different bacteria

Replicons	Organism	Purpose
pBBR1	*Rhodobacter sphaeroides*	MEP pathway expression (Wu et al., 2021)
	M. capsulatus Bath	Developing CRISPR/Cas9 gene editing tools (Tapscott et al., 2019), complementation after gene deletion (Welander & Summons, 2012)
	Methylomonas sp. strain 16a	Express Astaxanthin pathway (Ye et al., 2007)
RK2/RP4	*M. buryatense* 5GB1C	Metabolic engineering for lactate and lipid production (Henard et al., 2016, 2017), C4 carboxylic acid production (Garg et al., 2018)
	M. alcaliphilum 20Z	Metabolic engineering for PHB (Hoang Trung Chau et al., 2022), α-humulene (Nguyen et al., 2020), 2,3-butanediol production (Nguyen et al., 2018)
	M. capsulatus Bath	Validate promoter probe vector (Ali & Murrell, 2009),

Replicons	Organism	Purpose
	M. trichosporium OB3B	Metabolic engineering for Cadaverine , 3-hydroxy propionic acid production (D. T. N. Nguyen et al., 2020; T. T. Nguyen et al., 2020)
	Methylomonas sp. DH-1	Metabollic engineering for succinate production (Nguyen et al., 2019)
RSF1010	*M. trichosporium* OB3B	Homologous expression of sMMO (Lloyd et al., 1999)
	M. capsulatus Bath	Test promoter strength (Tapscott et al., 2019)
	Prochlorococcus strain MIT9313	Test promoter strength (Tolonen et al., 2006)

These plasmids have been used over the years in a range of different bacterial hosts that include methanotrophs as well as cyanobacteria and purple non-sulfur bacteria (Table 2.2). In methanotrophs, other than being useful as heterologous expression tools, the vectors have been used to develop a CRISPR based gene deletion system in *M. capsulatus* Bath (Table 2.2). Usually, kanamycin resistance is the most used selectable marker in these vectors (Kalyuzhnaya et al., 2015) but gentamicin resistance has also been useful in some cases (Tapscott et al., 2019).

2.5.3 Fine-tuned heterologous gene expression

To achieve fine-tuned expression of genes, promoters and RBS have been studied in methanotrophs. Both inducible and constitutive promoters have been useful in generating value-added products. Promoter P_{Tet}, inducible with anhydrotetracycline, was useful in generating lactate in *M. buryatense* 5GB1C (Henard et al., 2016) and for cas9 expression in *M. capsulatus* Bath (Tapscott et al., 2019). P_{Tet} is one of the promoters studied in this thesis. However, at a concentration > 1 µg/ml, growth was inhibited in *M. buryatense* 5GB1C (Henard et al., 2016). Thus, to avoid toxicity, other inducible promoters were also included. Recently, a phenol based inducible promoter has been characterized for plasmids transferred to *M. capsulatus* Bath to develop a CRISPR/Cas9 system for a gene knockout system and to express the MVA pathway to generate mevalonate (Jeong et al., 2023).

The synthetic promoter, P_{tac}, has been useful in methanotrophs to drive constitutive heterologous gene expression for generating products such as cadaverine and 3-hydroxypropionic acid production in *M. trichosporium* OB3b (D. T. N. Nguyen et al., 2020; T. T. Nguyen et al., 2020). Endogenous promoters from the genomes of host methanotrophs have also been useful to drive constitutive heterologous gene expression. Using transcriptome data to select and validate endogenous promoters has been a successful approach for creating constitutive promoters for use in heterologous expression systems. In *M. capsulatus* Bath such an approach was used to develop P_{mxa} (the promoter of the Mxa class of methanol dehydrogenase) as a strong promoter for driving gRNA expression (Tapscott et al., 2019). A rationally designed approach using computational tools has been applied in other methanotrophs. In *M. buryatense* 5GB1C, a computational framework was developed based on RNAseq data to identify -35 and -10 motifs of promoters with high levels of reporter gene expression (Wilson et al, 2021).

A combination of promoter prediction tool and 2D gel electrophoresis were applied to generate a promoter library with a broad range of gfp expression levels in *Methylomonas* sp. DH-1. Promoters from this library were used to drive gene expression for cadaverine production (Lee et al., 2021). Surprisingly, when genes were expressed under promoter P_{mxa} to drive high expression, cadaverine was not detected. Interestingly, expression under the much weaker $P_{integrase}$ cadaverine was successful (Lee et al., 2021). One possible This study showed that for industrial application it is appropriate to generate promoters with a range of activity rather than relying on a single strongly active promoter. Keeping this point in mind, multiple constitutive endogenous promoters were tested in *M.album* BG8 in this thesis.

Optimization of the ribosomal binding site (RBS) is a less common strategy for fine tuning heterologous gene expression in bacteria. This approach has been used and validated successfully for production of C4 carboxylic acid in *M. buryatense* 5GB1C. Under the P_{mxa} promoter, expression of genes were optimized using different RBS to achieve a strain with high titer of products (Garg et al., 2018).

2.6. Bioreactor operation

Establishing bioreactor operation is an important aspect of industrial development of microbial strains. The bioreactor can provide the best possible environment for biological production (Mandenius, 2016) as biological reactions of interest occur in a controlled manner. In this thesis, modes of operation for *M. album* BG8 using stirred tank reactor (Liu, 2017) were explored. In such reactors, gas (usually air or oxygen) is provided using sub surface spargers and impellers for efficient gas dispersion (Figure 2.2). The gas supply plays an important role in the

operation. Along with gas supply, pH and temperature are two parameters that can be changed during operation. Different modes of operation exist for stirred tank reactors. In this thesis, batch and fed-batch modes were explored for *M. album* BG8.

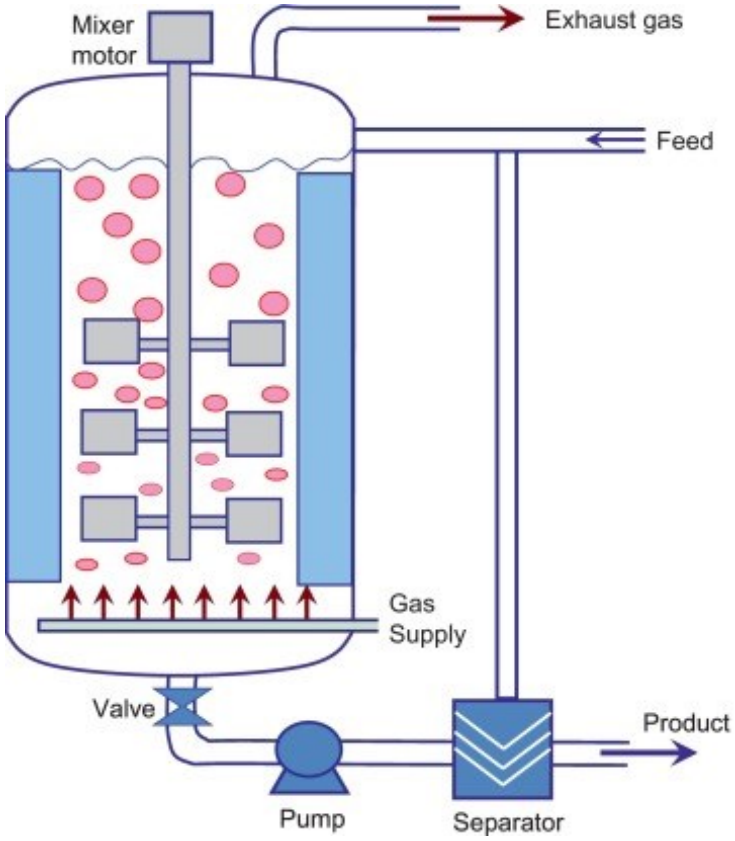

Figure 2.2 A stirred tank reactor showing the gas supply and the mixer for gas dispersion. Reproduced from Liu, 2017

2.6.1 Modes of operation

Broadly, there are three modes of operation with a stirred tank reactor: Batch, Continuous and Fed-Batch (Liu, 2017). Batch operation is the simplest of the three. In a batch operation all the necessary components including media and carbon source for the bacteria are provided at the start. Only air is supplied during the operation. Product is collected at the end of the cycle as

bacterial growth reaches stationary phase. The simplicity of this operation is its most important feature. It allows production of potentially toxic products and running the operation does not require expensive controlling measures. The drawback is its low productivity, separate inoculum preparation for each run and long lag phase (Liu, 2017)

Sequential batch (SBR) or repeated batch is a modified version of batch that addresses some of these drawbacks. SBR has been developed for wastewater treatment since the 1940s (Irvine et al., 1989; EPA 932F99073, 1999). In the wastewater operation, biomass is acclimated to wastewater, which is the feedstock for subsequent cycles. In subsequent cycles, wastewater is supplied to acclimatized biomass and released after treatment, then a new batch of wastewater is supplied. Thus, multiple batches of wastewater are treated using the same biomass, thereby removing the need for lengthy acclimation before each treatment. This mode of operation has been explored for culturing a diverse range of microbes, such as extremely thermophilic (70° C) hydrogen producers (Liu et al., 2008), the cyanobacteria *Spirulina platensis* (Radmann et al., 2007), and PHB producing *Ralstonia eutropha* (Khanna & Srivastava, 2005). Just like wastewater treatment, after the first cycle of growth, part of the culture is kept that acts as inoculum or seed culture for the subsequent cycles. Lag phase can be avoided in subsequent cycles and high growth rate can be maintained throughout the operation (Fabregas et al., 1996).

Continuous mode of operation becomes useful when high productivity is required. Unlike the batch cycle, substrate and media is constantly fed to the culture to maintain a constant growth rate (Liu, 2017) while product is also collected. While it can be very useful for achieving a highly productive high-density culture, achieving continuous production as compared to a batch culture is quite difficult in terms of maintaining a culture at a high specific growth rate (Srivastava & Gupta, 2011). Ccontamination can become a major issue unlike in batch operation

(Maxon, 1955), thus making it difficult to maintain a sterile culture for a long period of time. In addition, maintaining continuous operation of downstream processes, such as extraction, along can become very expensive (Ochoa, 2019). Nonetheless, continuous operation has been successful in producing high volume, low value, product like bioethanol (Liu, 2017).

A fed batch operation could be considered a middle ground between batch and continuous. Just like batch, media is supplied only at the start and product is collected at the end. Like continuous operation, the nutrient required for cell growth and product formation is either fed intermittently or continuously via feed stream during the operation (Lim & Shin, 2013). This nutrient feeding must be highly precise, which could be labor intensive (Srivastava & Gupta, 2011). High density culture can be obtained in a fed-batch culture as in continuous operation. In addition, the closed nature of the reactor, like batch culture, makes maintaining sterility easier in fed-batch cultures.

2.6.2 Bioreactor operation for methanotrophs

Since the start of industrial development of methanotrophs in the 1980s, growth in bioreactors was also explored. The drawbacks of industrializing methanotrophs are their slow rate of growth (Kalyuzhnaya et al., 2015), low aqueous solubility of methane (Lee et al., 2015). Hence it is imperative that efficient bioreactor operation be developed for their application. Both fed batch (Hou et al., 1979) and continuous operation (Papoutsakis et al., 1981) have been explored to generate high biomass in methanotrophs for SCP production using methanol since the 1980s. In recent studies with metabolic engineering, methanotrophs grown in bioreactors has played an important role in increasing product titer as shown in Table 2.1. In *M. trichosporium* OB3b, cadaverine titer was improved 6-fold in a fed-batch compared to flask culture (Nguyen et

al., 2020). Similarly, with *Methylomonas* sp. DH-1, a 1.5 fold increased titer of succinate was obtained using a fed-batch operation compared to flask culture (Nguyen et al., 2019). Exploring multiple operations can be useful in other ways as well. Fed-batch operation for *M. buryatense* 5GB1C showed that inhibition from high methanol concentration can be circumvented to reach a high biomass (Tan et al., 2023). In this context, developing reactor operation strategies for *M. album* BG8 is a worthwhile exercise.

2.7. Isoprenoids

Isoprenoids, sometimes also called terpenoids, are ubiquitous abundant and structurally diverse compounds found in nature. They account for nearly one third of natural products, mainly from plants (Christianson, 2017; Kuzuyama et al., 2013). Isoprenoids have a range of industrial application such as food additives for flavor and fragrance, medicines, biofuel and fuel precursor molecules (Vickers et al., 2014). Even though they are naturally found in plants, low yield has prompted significant research in scaled-up production using microbial hosts (Navale et al., 2021).

2.7.1. Isoprenoids as jetfuel precursor molecules

The aviation industry contributes significantly to greenhouse gas emissions (ICAO,2016). Fuel derived from biomass offers a solution as this approach can substitute for conventional fossil-based jetfuel (CJF) without changing current technologies as long as the fuels have a low freezing point and high energy density (Table 2.3).

Table 2.3. Important parameters of some commonly used aviation fuel and physical properties of isoprenoids of interest in comparison. (Chuck and Donnelly 2014, Liu et al. 2022, Walls and Rios-Solis 2020)

Physical properties of conventional jet fuels and isoprenoids of interest					
	Density (kg/L)	Class of compound	Freezing point (° C)	Flash point (°C)	Energy density (MJ/kg)
Jet A	0.775-0.840	-	<-47	>38	>42.8
Jet A-1	0.775-0.840	-	<-40	>38	>42.8
JP 10	0.94	-	<-79	53	>42.1
β-Pinene	0.866	Monoterpenoid	-61	32	43.22
α-Pinene	0.858	Monoterpenoid	-64	34	43.16
Sabinene	0.842	Monoterpenoid	-40	37	43.05
Limonene	0.842	Monoterpenoid	-74	50	42.88
Farnesene	0.844-0.879	Sesqueterpenoid	-90	43	44

Carbon derived from biomass offers a lower overall carbon footprint during production, although the emission profile from combustion of both biomass-derived and CJF fuels is similar.

The freezing point and energy density of isoprenoid-based fuels makes them favorable as aviation fuels (Table 2.3).

Farnesene, has already been approved for a 10% blend with CJF (Holladay et al. 2020) and limonene has been shown as a possible drop-in replacement (Chuck and Donnelly 2014). Pinene and limonene can be chemically upgraded through dimerization, hydrogenation, and cyclopropanation to improve their density and volumetric NHOC (Net heat of combustion), thereby making them useful as high-performance missile or rocket fuels (Meylemans et al. 2012, Woodroffe et al. 2021, Walls and Rios-Solis 2020).

2.7.2. Production of isoprenoids in microbial hosts

Isoprenoids with potential for aviation fuel have been produced by several microbial hosts like the purple non sulfur bacteria, *Yarrowia lipolytica*, *E. coli* and *Saccharomyces cerevisiae* (Table 2.4). Chemically, isoprenoids are polymers of C_5 isoprene units. Two compounds -- isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) -- serve as building blocks of all isoprenoid synthesis in all living organisms (Withers & Keasling, 2007). These two compounds are derived either using the MEP pathway found in bacteria and plant plastids or the Mevalonate (MVA) pathway found in archaea and eukaryotes from cellular metabolites generated in core carbon metabolism. These IPP and DMAPP precursors are condensed to generate different lengths of isoprenoids starting from C_5 to C_{50} in length. A simple schematic is provided in Figure 2.3 that shows the connection between core carbon metabolism

Table 2.4 Metabollic Engineering for production of isoprenoids useful as jetfuel precursor in microbial hosts

Strain	Isoprenoid	Titer	Metabolic engineering	Reference
Rhodobacter sphaeroides	Pinene	539.84 μg/L (mix of α and β isomer)	Overexpression of idi, dxs, dxr; Heterologous expression of GPPS, PS	Wu et al., 2021
Saccharomyces cerevisiae	α-Farnesene	10.4 g/L	Heterologous expression of Fsso, MVA pathway, inactivation of DPP1, turning the strain prototrophic	Wang et al., 2021
Saccharomyces cerevisiae	Limonene	917.7 mg/L	Heterologous expression of NPPS, LS; Overexpression of native MVA pathway	Cheng et al., 2019
Escherichia coli **BL21(DE3)**	Limonene	3.6 g/L (S isomer)	Heterologous expression of MVA pathway, GPPS, LS gene	Rolf et al., 2020
E.coli **BL21(DE3)**	α-pinene	0.97 g/L	Heterologous expression of MVA pathway, GPPS, α-pinene synthase	Yang et al., 2013
E.coli **BL21(DE3)**	Sabinene	2.65 g/L	Heterologous expression of MVA pathway, GPPS, SabS	Zhang et al., 2014

***E.coli* BL21(DE3)**	α-Farnesene	1.1 g/L	Heterologous expression of MVA pathway, ispA, α-farnesene synthase	Zhu et al., 2014
***E.coli* BL21(DE3)**	β- Farnesene	8.74 g/L	Heterologous expression of MVA pathway, ispA, β-farnesene synthase	You et al., 2017
Synechococcus sp. PCC 7002	Pinene	1.525 mg/L (mix of α and β isomer)	Heterologous expression of GPPS,PS	Yang et al., 2021
Synechococcus sp. PCC 7002	S-Limonene	4 mg/L	Heterologous expression of 4-S-Limonene synthase	Davies et al., 2014
Yarrowia lipolytica	α-Farnesene	259.98 g/L	Heterologous expression of MVA pathway, ispA, α-farnesene synthase	Yang et al., 2016

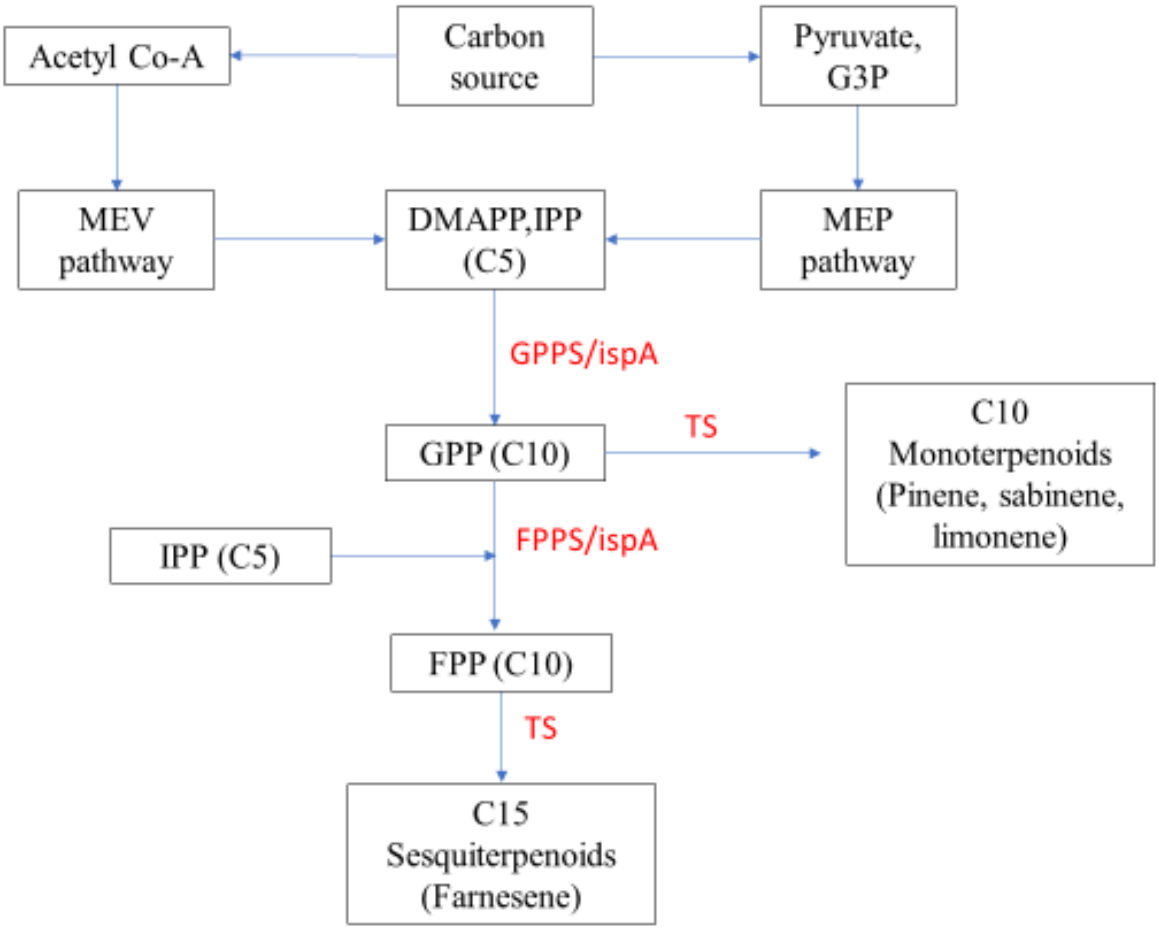

Figure 2.3 Connection between metabolites of core carbon metabolism, DMAPP, IPP, monoterpenoids and sesquiterpenoids. GPP, FPP stands for geranyl pyrophsophate and fernesyl pyrophosphate respectigvely. While GPPS and FPPS are their respective synthases.

to synthesis of monoterpenoids (C_{10}) and sesquiterpenoids (C_{15}) as important precursors of ultraperformance jet fuel.

Engineering microbial hosts for isoprenoid production involves two parts. One part is engineering either the MVA/MEP pathway to increase the DMAPP and IPP isomers, and the second part is engineering the enzymes that can condense DMAPP and IPP to generate either

GPP (C_{10}) or FPP (C_{15}) and a subsequent synthase that generates the respective monoterpenoid or sesquiterpenoid. (Figure 2.3). In between the two, the MEP pathway shows a higher theoretical mass yield compared to the MVA pathway (Li et al., 2020). Yet, the gram scale productions reported were achieved by expression of the MVA pathway (Table 2.4). It is argued that interplay between the MEP pathway and other physiological pathways inside *E.coli* could lead to lower DMAPP/IPP titer even after overexpression of the pathway (Li et al., 2020). It can be infered that the genetic engineering approaches described in Table 2.4 are highly complex. Hence, a robust set of heterologous expression tools need to be developed for *M. album* BG8 in order to produce isoprenoids using this organism.

2.7.3. Toxicity of isoprenoids and alleviating strategies

Isoprenoids, and specially monoterpenoids, are often toxic to microorganisms (Moser and Pichler 2019, Liu et al. 2022). It has been a bottleneck in generating a high titer of these compounds. Several strategies have been devised to circumvent this toxicity. That includes adaptive laboratory evolution (ALE) under isoprenoid stress, like generating a sabinene tolerant strain of *E.coli* BL21(DE3) by serial passaging (Wu et al., 2020) in medium containing increasing amounts of sabinene. These strains showed sabinene tolerance up to 0.6 g/L compared to the sensitive strain, and produced a titer of 191.76 mg/L sabinene, 8-fold higher production compared to the sensitive strain. Expression of an efflux pump in *E. coli* DH1 ΔacrAB led to increased tolerance to limonene. Using the strain expressing the pump, 79% improved production of limonene was achieved (Dunlop et al., 2011). Another highly efficient strategy is addition of an inert organic phase to circumvent toxicity by separating the isoprenoid from the biomass. Limonene was produced at a high concentration of 3.6 g/L in *E. coli* BL21 (DE3)

during fed batch operation using diisononyl phthalate as an inert organic phase (Rolf et al., 2020).

While isoprenoid toxicity in methanotrophs relating to their industrial production has not been studied, their effect on methanotrophs in pure cultures (Amaral et al. 1998) and in soils (Maurer et al. 2008) has been studied. In these studies, α-pinene and limonene negatively affected methane oxidation by *Methylosinus trichosporium* OB3B, and α-pinene, β-pinene and limonene negatively affected methane oxidation in soil. Hence, it is important to explore the toxic effects of isoprenoids on methanotrophs if they are to be developed for bioconversion of isoprenoids. This concept is explored in Chapter 5 of this thesis.

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Chapter 3: Development of genetic tools for heterologous protein expression in *Methylomicrobium album* BG8

3.1 Abstract

Methanotrophic bacteria consume methane and methanol as sole sources of carbon and energy and can convert them into value-added products such as bioplastics, biofuels, and platform chemicals. The methanotroph *Methylomicrobium album* BG8 is notable for its fast and robust growth and rich genetic potential, making it an excellent candidate as a bioconversion platform. Towards this goal, we developed three broad host range plasmids based on the IncP, IncQ and pBBR1 incompatibility families. These plasmids were successfully conjugated and propagated in *M. album* BG8. We also showed that IncQ and pBBR1 could be propagated simultaneously in *M. album* BG8. We successfully tested expression of GFP under the control of three constitutive promoters – P_{MMO} (from a particulate methane monooxygenase), P_{MMA} (from a Mxa type methanol dehydrogenase), and P_{GAP} (from a glyceraldehyde 3-phosphate dehydrogenase) – engineered into the IncQ plasmid, and of the inducible P_{Tet} (tetracycline) promoter engineered into the IncP plasmid. The P_{MMO} and P_{MMA} constitutive promoters led to higher Green fluorescent protein (GFP) expression than the P_{GAP} promoter. In the absence of its inducer, P_{Tet} did not yield GFP expression proving non leaky expression from this promoter. We also confirmed expression and activity of NanoLuc luciferase using the P_{MMA} promoter in both the IncQ and IncP plasmids. Together, this work lays the foundation for using *M. album* BG8 as a microbial platform for bioconversion of methane and methanol into products that require heterologous expression of foreign genes.

3.2 Introduction

Methane and methanol are promising microbial feedstocks for bioindustry (Kalyuzhnaya et al. 2015, Cotton et al. 2020). Methane is a major greenhouse gas that must be reduced to mitigate climate change and its conversion can also add value to the bioeconomy (Shindell et al. 2012). Methanol is derived from natural gas, biomass, and carbon dioxide conversion (Tan et al. 2023) and as a biproduct of turquoise hydrogen production (Banu et al. 2023). Both methane (Gotz et al. 2016) and methanol (Szima and Cormos 2018) can be produced by converting electrochemically derived hydrogen and CO_2. Methane and methanol are emerging as important feedstocks and reusable waste products for establishing a circular carbon economy (Cotton et al. 2020).

Aerobic methanotrophs are a subset of methylotrophic bacteria (Lidstrom 2006). They utilize methane and methanol as sole sources of carbon and energy. Recently multiple strains of aerobic methanotrophs have been developed for industrial production such as *Methylotuvimicrobium alcaliphilum* 20Z (Ojala et al. 2012, Nguyen et al. 2020), *Methylotuvimicrobium buryatense* 5GB1 (Puri et al. 2015), and *Methylosinus trichosporium* OB3b (Ro & Rosenzweig, 2018, Nguyen et al. 2020). In this thesis chapter, the aim was to develop a plasmid-based system for heterologous protein expression in *Methylomicrobium album* BG8 (Kits et el. 2013), a gammaproteobacterial methanotroph that shows robust growth under a wide range of conditions (Tays et el. 2018). *M. album* BG8 can grow reliably on either ammonium or nitrate as the nitrogen source and either methane or methanol as the carbon source (Tays et al. 2018, Sugden et al. 2021). It can be adapted for growth under harsher conditions such as low pH (McDonald, 2019). The transcriptome and metabolome of *M. album* BG8 have been described (Sugden et al. 2021) and a genome scale metabolic model has been constructed

(Villada et al. 2022). Developing genetic tools for heterologous protein expression is the next logical step for industrializing this strain for bioproduction.

Broad host range plasmids containing replicons such as RP4/RK2 (incompatibility group P/IncP), RSF1010 (incompatibility group Q/IncQ), and pBBR1 have been shown to successfully replicate and express heterologous proteins in other methanotrophic bacteria (Puri et al.2015, Ojala et al. 2011, Henard et al. 2016, Tapscott et al.2019). Most recently, an effective CRISPR/Cas9 system was developed for *in vivo* gene editing of for *M. capsulatus Bath* (Tapscott et al. 2019). In the present study, three broad host range plasmids, constitutive and inducible promoters were tested in *M. album* BG8. Green fluorescent protein (GFP) and the luciferase Nanoluc were used to demonstrate effective expression from these plasmid vectors. These results serve as a basis for robust plasmid-based heterologous protein expression in *M. album* BG8.

3.3 Materials and methods

3.3.1 Strain maintenance

M. album BG8 was maintained in 100 ml nitrate mineral salts medium (NMS) (Whittenbury et al; 1970) containing 10 mM potassium nitrate as nitrogen source and 5 µM copper buffered with 1.5 mL phosphate buffer pH 6.8 (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) in Wheaton media bottles (250 mL) closed with butyl-rubber septa caps. For growth with methane, 50 ml of gas headspace was removed from the culture bottle and 2.5 mmol of methane was injected through a 0.22-mm filter-fitted syringe. For growth with methanol, 2.5 mmol high-performance liquid chromatography (HPLC)-grade methanol was directly added. To measure growth, 500 µl of culture was removed with a sterile 1 ml syringe and optical density at 540 nm (OD_{540}) was measured in a 48-well plate using a spectrophotometer (Multiskan Spectrum). For

growth on solid media, bactoagar was added at 1.6% w/v to NMS and allowed to solidify. Plates were inoculated and incubated in a gastight jar containing 30/70 % v/v methane/air. Kanamycin (50 µg/ml) and gentamicin (15 µg/ml) were added as selection markers to maintain genetically modified strains. *Escherichia coli* was cultured in LB medium (200 rpm at 37° C) or on LB agar plates (1.5 % w/v) and incubated at 37 °C. Chemically competent *E. coli* DH10β was used for Gibson assembly and plasmid maintenance, and *E. coli* S17λpir was used for conjugation. Nutrient broth agar (1.5 % w/v bactoagar) was used to detect *E. coli* carryover following conjugation and to test for contamination of *M. album* BG8 cultures.

3.3.2 Plasmid assembly

Plasmids are listed in Table 3.1 and primers are listed in Table 3.2. The primers and codon optimized NanoLuc DNA were synthesized (Integrated DNA Technologies). Plasmid backbones and DNA inserts were amplified using Q5 High-Fidelity Polymerase (NEB) and wereeither PCR or Gel purified using PCR or Gel purification kits (Qiagen). The selected promoter regions from the *M. album* BG8 genome (Genbank accession number CM001475.1; Table 3.3) were amplified using genomic DNA as a template. Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen). The plasmids were assembled using NEBuilder® HiFi DNA Assembly Master Mix following the reaction protocols. The resulting plasmids were transformed into chemically competent *E. coli* DH10β. Transformed colonies were confirmed via colony PCR and sequencing of the plasmid vector. Purified plasmids were then transformed into chemically competent donor *E. coli* S17λpir for conjugation into *M. album* BG8.

Table 3.1 List of strains and plasmids used in this study.

Name	Description	Reference
Strain		
E. coli DH10B	Fφ80lacZΔM15 Δ(lacZYA-argF)U169 deoR nupG recA1 endA1 hsdR17(rK- mK+) phoA glnV44 (supE44) thi-1 gyrA96 relA1, λ	Henard et al. 2016
E. coli S17-1 λ pir	Tpr Smr recA thi pro hsd(r - m+)RP4-2-Tc::Mu::Km Tn7 λpir	Henard et al. 2016
M. album BG8	Wildtype strain	Whittenbury et al. 1970
Plasmid		
pQCH	RSF1010 replicon (IncQ) based broad host range vector and sfGFP coding sequence	Tapscott et al. 2019
pCAH01	RP4/RK2 replicon (IncP) based broad host range vector with P_{Tet} promoter TetR sequence	Henard et al. 2016
pGLO	Plasmid containing cyclic GFP coding sequence	Biorad
pBBR1MCS5	pBBR1 replicon based broad host range vector	Tapscott et al. 2019,
Name	**Description**	**Reference**
$pQSDP_{MMO}$sfgfp	pQCH with *M. album* BG8 P_{MMO} cloned upstream of sfGFP	This study
$pQSDP_{MMA}$sfgfp	pQCH with *M. album* BG8 P_{MMA} cloned upstream of sfGFP	This study
$pQSDP_{GAP}$sfgfp	pQCH with *M. album* BG8 P_{GAP} cloned upstream of sfGFP	This study
$pQSDP_{PYC}$sfgfp	pQCH with *M. album* BG8 P_{PYC} cloned upstream of sfGFP	This study
$pPSDP_{tet}$gfp	pCAH01 with GFP from pGLO cloned downstream of P_{Tet}	This study
$pQSDP_{MMA}$nluc	Nanoluc under P_{MMA} promoter in IncQ backbone	This study
$pPSDP_{MMA}$nluc	Nanoluc under P_{MMA} promoter in IncP backbone	This study

Table 3.2. Primers used in this study. The flanking region for Gibson assembly primers are shown in small letters.

Primer	Sequence	Description
P_{MMA}_fwd	aggcatgttccgcgtccttgcaataCGGATTTTTTCCTAGCCGAATT	To copy *M. album* BG8 P_{MMA} in pQCH to generate pQSDP$_{MMA}$sfgfp
P_{MMA}_rev	gttcttctcctttgctcatAATTCCCCCTCCAAGGGT	
P_{MMO}_fwd	aggcatgttccgcgtccttgcaataCCGGCCTGTTTGAGTGCT	To copy *M. album* BG8 P_{MMO} in pQCH to generate pQSDP$_{MMO}$sfgfp
P_{MMO}_rev	gttcttctcctttgctcatTTCTACCTCCTAAAAATTTAACAATCCC	
P_{GAP}_fwd	aggcatgttccgcgtccttgcaataGACACCGCAAAAAACCGC	To copy *M. album* BG8 P_{GAP} in pQCH to generate pQSDP$_{GAP}$sfgfp
P_{GAP}_rev	gttcttctcctttgctcatTGAGTCTCTCCAGAGTGATGAG	
P_{PYC}_fwd	aggcatgttccgcgtccttgcaataCGGGATCATATCCCGTCC	To copy *M. album* BG8 P_{PYC} in pQCH to generate pQSDP$_{PYC}$sfgfp
P_{PYC}_rev	gttcttctcctttgctcatAACAGGGTGTGGGAGAAC	
pQCH_fwd	ATGAGCAAAGGAGAAGAA	To generate IncQ backbone with sfGFP
pQCH_rev	TATTGCAAGGACGCGGAAC	
pQSDP$_{MMA}$_fwd	AGGCATCAAATAAAACGAAAG	To generate IncQ backbone with P_{MMA} promoter and T1 terminator
pQSDP$_{MMA}$_rev	AATTCCCCCTCCAAGGG	
pCAH01_fwd	AAGCTTGACCTGTGAAGTG	To generate IncP vector with P_{Tet} promoter
pCAH01_rev	TTCACTTTTCTCTATCACTGATAG	
GFP_fwd_pCAH01	cagtgatagagaaaagtgaaATGGCTAGCAAAGGAGAAG	To generate GFP from pGLO to clone under P_{Tet} promoter
GFP_rev_pCAH01	tcacttcacaggtcaagcttTTATTTGTAGAGCTCATCCATG	

IncP_fwd	TTGTCGGGAAGATGCGTG	To generate IncP backbone only
IncP_rev	CAGCTCACTCAAAGGCGG	
nanoluc_fwd	agggggaattATGGTATTTACGCTGGAAG	To generate NanoLuc fragment to clone downstream of P_{MMA}
nanoluc_rev	tttgatgcctTTAATGGTGGTGATGATGGTGTGCCAGGATGCGTTC	
P_{MMA}sfgfpT1ter_fwd	taccgcctttgagtgagctgCGGATTTTTTCCTAGCCGAATTAC	To generate fragment with P_{MMA}, T1 terminator and sfGFP to generate pPSDP$_{MMA}$sfGFP
P_{MMA}sfgfpT1ter_rev	atcacgcatcttcccgacaaCATCCGTCAGGATGGCCTTC	
IncPPmma_fwd	AGGCATCAAATAAAACGAAAGGC	To generate IncP with P_{MMA}, T1 terminator
IncPPmma_rev	AATTCCCCCTCCAAGGGTTG	
IncPOriT FWD Set 2	TGCGAATAAGGGACAGTGAAG	For determining plasmid copy number of IncP vectors
IncPOriT REV Set 2	TGCCAAAGGGTTCGTGTAG	
IncQMobA FWD Set 1	AAGCTATGGCTAACCGTGATG	For determining plasmid copy number of IncQ vectors
IncQMobA REV Set 1	CTGCAAAGTCCTGTCGTTCT	
pBBR1Rep FWD Set 1	GTCCAGAGAAATCGGCATTCA	For determining plasmid copy number of pBBR1 vectors
pBBR1Rep REV Set 1	CGTGGGTTTCCTCGCAATAA	
KanR Fwd	GGTATAAATGGGCTCGCGATAA	To generate amplicon of Kanamycin resistance gene
KanR Rev	CCGACTCGTCCAACATCAATAC	
GenR Fwd	ATGTTACGCAGCAGCAAC	To generate amplicon of Gentamicin resistance gene
GenR Rev	TTAGGTGGCGGTACTTGG	

Table 3.3. List of promoter gene sequences from *Methylomicrobium album* BG8 (Genbank Accession Number: CM001475.1) and synthesized NanoLuc sequence.

Promoter or gene	Sequence (5' to 3')
Mxa type Methanpol dehydrogenase promoter (P_{MMA}) (Upstream of Metal_2469)	CGGATTTTTTCCTAGCCGAATTACAGGTTTATTCCGTAACGCCCGCCTCTCCC GCTCGGCTACCATGTCGCTTGCTCGGATCCGCGCTTCGGTACCCCTGTGAACG TTTTGATCAAGAAGGCCGAATCCAATTTTACCGAGGCGCCGGATCCAGGCTT TTCAAATTCAGCTCAATAAAGACAACCCTTGGAGGGGGAATT
Particulate methane monoxygenase promoter (P_{MMO}) (Upstream of Metal_3593)	CCGGCCTGTTTGAGTGCTGAGCGATTTTTTTGACGAAAATATTTTTTGTCTTG ACAAGGTGCTTCGAGCTGGTAAACTGACCGCTCACCAAACTGGACTCGGTGC GGTTCCTGGCCAAAAGCCAGTGAATGTGGAAATGGAGTTGAACAAGAAACT CCCAGCCAAATAGCTGGGGATTGTTAAATTTTTAGGAGGTAGAA
Glyceraldehyde-3-phosphate dehydrogenase (P_{GAP})(Upstream of Metal_0282)	GACACCGCAAAAAACCGCGATCATTTTGCCTCCGGCACCTGAAAATTCACCC GCAAGCTTTAGTGCAAGGGAAAAAGATTTTTCCGCCCATGTCAATAGACGAA TACCCCCCGTTTTTGGTATAGTGAGCGGATTTTAGGGTTGCTCTATAACCCCT CATCTTAGTTGGTTTTTCATACTCATCACTCTGGAGAGACTCA
Pyruvate carboxylase (P_{PYC}) (Upstream of Metal_0090)	CGGGATCATATCCCGTCCGGCATTTGGACCGTTGGCGCGCTGCGTGGATCTTC GGAAAAAAGGACATAGCGCGATTGCCCTGAAGAAACGCTCCCTATGACGCC AAACCGGTCAAGAATCTGATATATTGGCAGCCTGTCGCCAATGGCGTCGTTT GTCGGCTCAATAACAACGGTTTCTCCGTTCTCCCACACCCTGTT
NanoLuc codon optimized	ATGGTATTTACGCTGGAAGATTTCGTTGGCGACTGGCGACAAACTGCTGGAT ATAATTTGGATCAAGTCTTGGAACAAGGCGGTGTATCCTCCTTATTCCAAAAC TTAGGCGTCTCCGTTACCCCGATTCAACGGATCGTTTTGAGTGGCGAAAACG GCTTAAAAATCGATATCCACGTCATCATCCCATACGAAGGCTTGTCCGGTGA CCAAATGGGTCAGATCGAAAAGATTTTCAAAGTCGTGTATCCGGTTGACGAT CATCATTTCAAAGTGATTTTGCATTATGGTACCTTGGTGATCGATGGTGTTAC ACCAAATATGATCGATTACTTCGGTAGACCGTACGAAGGCATTGCAGTATTC GACGGCAAAAAAATTACAGTCACCGGTACTTTATGGAACGGTAACAAAATTA TTGATGAGCGTTTAATCAATCCTGATGGTAGTCTGTTATTCCGGGTAACGATC AACGGCGTTACGGGTTGGCGGTTGTGCGAACGCATCCTGGCATAA

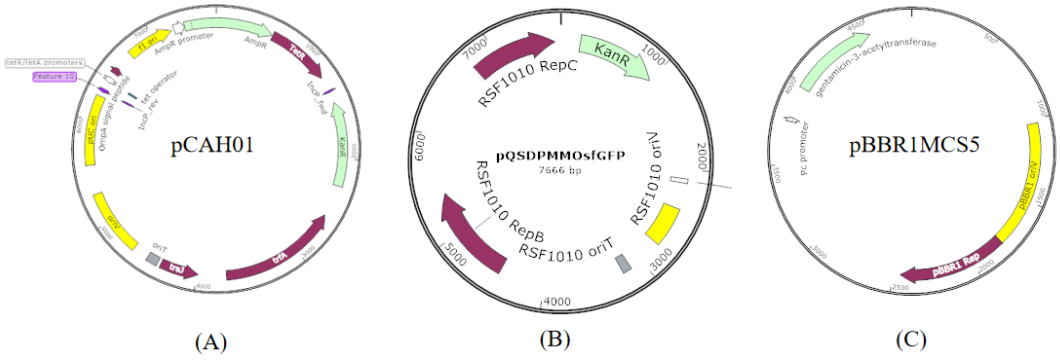
3.3.3 Conjugation of plasmids into M. album BG8

The conjugation method was described previously for *M. buryatense* 5GB1 (Puri et al. 2015) and was used with a modification of the *E. coli* donor clean-up procedure. *M. album* BG8

cells were grown for 72-96 h on NMS agar plates in a gastight jar with methane (30% v/v). The resulting biomass was transferred to a mating plate (NMS agar containing 15% v/v nutrient broth) and incubated overnight at 30°C without methane. At the same time, *E. coli* S17λpir harboring the desired constructed plasmid vector was grown overnight on LB agar plates containing antibiotic. *M. album* BG8 and *E. coli* S17λpir were mixed on the mating plate and incubated for 48 h at 30°C without methane. After 48 h, the biomass was transferred to NMS agar plates containing antibiotic and grown at 30°C in a gastight jar with methane (30% v/v) for 7 days. Colonies of *M. album* BG8 arising on the plates were individually selected and grown in 35 ml serum vials containing 7 ml NMS media supplemented with 15 µl high grade methanol as the sole carbon source. The appropriate antibiotic corresponding to the marker on the plasmid was also added, and vials were incubated at 30°C for 72-96 h. After passaging the cultures 2-4 times with methanol as the carbon source, the absence of *E. coli* S17λpir donor cells was confirmed via spot test on nutrient agar plates. The pure *M. album* BG8 culture containing the desired plasmid was then grown in 100 ml NMS in 250 ml Wheaton bottles as described above in section 3.3.1.

3.3.4 Dry weight and cell count

Dry weight was calculated after 48 h of growth of *M. album* BG8 in NMS and methane by vacuum filtration of 50 ml of culture onto pre-tared filter paper discs. The filter paper discs were dried at 30 °C for 48 h. The pre-tared mass was subtracted from the dry mass to determine the biomass of bacteria per volume of culture, or mg/ml. Direct cell counts were performed on cultures grown for 48 h using a Helber Bacteria Counting Chamber and a phase contrast light microscope. Single factor ANOVA was used to compare the similarity of values across samples.



(A) (B) (C)

Figure 3.1. The three broad host range plasmids successfully transformed into *M. album* BG8 and propagated. Genetic elements associated with plasmid replication and conjugation are highlighted. A) pCAH01 KanR (kanamycin resistance), TetR (transcriptionally fused tetracycline repressor), oriV/oriT (IncP-based origin of replication/transfer), trfA (oriV replication initiation protein), B) pQSDMMOsfGFP OriT/OriV (IncQ-based origin of replication/transfer) RepARepBRepC (Required for plasmid replication) , C) pBBR1MCS5 Gentamicin resistance , Rep (Required for plasmid replication)), OriV (pBBR1 origin of replication)

3.3.5 Determination of promoter activity

Promoter activity was quantified using fluorescence spectroscopy to detect GFP, as described previously for *M. buryatense* 5GB1 (Henard et al. 2016). *M. album* BG8 transformed with promoter-plasmid combinations were grown to stationary phase in 250-ml Wheaton bottles containing 100 ml NMS with either 2.5 mmol methane or 2.5 mmol methanol as the carbon source. Anhydrotetracycline (Cayman chemicals, Michigan) was added at increasing concentrations from 0-5 µg/ml to induce the P_{tet} promoter. A growth curve was created by measuring OD_{540} every 24 h up to 96 h. Fluorescence intensity (λ_{abs} = 485 nm and λ_{ext} = 520 nm) was recorded after 48 h of growth by adding 200 µl culture into 96-well black colored plates (Cytation5 plate reader). The fluorescence intensity was normalized to the OD readings (NFI),

after which the relative intensity (I_R^P) was calculated by dividing the NFI of the plasmid-containing *M. album* BG8 strain to that of the wildtype, or non-conjugated strain.

$$I_R^P = \frac{F_{norm}^P}{F_{norm}^{WT}} \quad (1)$$

$$F_{norm}^{P/WT} = \frac{F^{P/WT}}{OD_{540}^{P/WT}} \quad (2)$$

Where *P* is the index for data related to a promoter P, *WT* indicates wildtype or non-conjugated strain, *F* is fluorescence, OD_{540} is the optical density of the *M. album* BG8 culture measured at 540 nm, and I_R^P is the relative fluorescence intensity of promoter P.

Fluorescent images of *M. album* BG8 actively expressing GFP were taken using a ZEISS Axio Imager M2 fluorescence microscope **.** For these images, 1 ml culture was collected using a sterile 1-ml syringe, cells were centrifuged at 13,000 rpm for 3 min (Eppendorf 5425), and the supernatant was removed. The cell pellet was then resuspended in 100 µl phosphate buffer, and 10 µl of the resulting cell suspension was used for image capture.

3.3.6 Expression of active luciferase protein

M. album BG8 containing pPSDP$_{MMA}$nluc and pQSDP$_{MMA}$nluc were grown in 100 ml NMS in 250-ml Wheaton bottles containing 2.5 mmol methane. After 48 h of growth, 1 ml of culture was collected using a sterile 1-ml syringe. OD_{540} was determined and the culture was diluted to OD_{540}=0.1 and then further diluted in 5-fold increments using NMS media. Luciferase activity was determined by the conversion of furimazine to furimamide (England et al. 2016; Promega). 25 µl of the culture dilutions was mixed with 25 µl of reagent and added to 96-well opaque white plates. The plates were incubated for 10 min at room temperature, after which luminescence was measured (Cytation 5 plate reader).

3.3.7 Determination of plasmid copy numbers

Plasmid copy numbers (PCN) per transformed *M. album* BG8 cell were determined by qRT-PCR for three promoter-plasmid combinations: $pPSDP_{tet}gfp$ for IncP, $pQSDP_{MMO}sfgfp$ for IncQ, and $pPSDP_{tet}gfp$ for pBBR1. This method was adapted from protocols for transformed *E. coli* and mammalian CHO cells (Carapuça et al., 2007, Skulj et al., 2008). Briefly, *M. album* BG8 was collected via vacuum filtration using supor 0.22 µM membrane filter (PALL) after 48 h of growth. The filter paper was transferred in 15 ml falcon tubes containing nuclease-free water. Cells were resuspended from the filter paper in the water by gentle vortexing to achieve a 5X concentrated solution of ~10^9 cells per ml as determined by direct cell count (see above). The solutions were stored at -20 °C prior to PCN determination. Frozen cells were incubated in a 95° C water bath for 15 min to break the cells, and 1 µl of the resulting lysate was used for a 10 µl qPCR reaction (QuantStudio 3, Applied Biosystems). Amplicons of 100-150 bp from the OriT, MobA gene and Rep gene were used to determine the PCN of IncP, IncQ and pBBR1, respectively. Primer sequences are listed in Table 3.2 The qPCR condition was as follows: 2 min initial denaturation at 95 °C, 40 cycles of 15 sec denaturation at 95 °C, and 50 sec combined annealing and elongation at 60 °C. The qPCR reaction was followed by a melt curve analysis to confirm the presence of single amplicons. Standard curves were generated for each gene product using five points starting with 1000 pg and subsequent 5X dilutions down to 1.6 pg per reaction. The PCN values were determined by comparing signal to a standard curve of dilution, divided by the cell number.

3.4 Results:

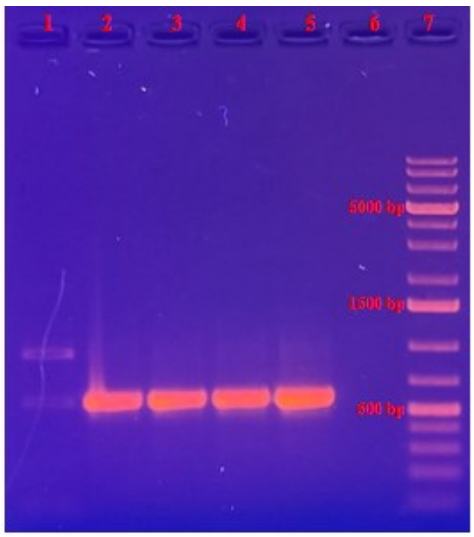
3.4.1 Transformation and propagation of broad host range plasmids in M. album BG8

The successful transformation and propagation of broad host range plasmids into *M. album* BG8 were confirmed by PCR. Broad host range plasmids pCAH01, pQSDP$_{MMO}$sfGFP and pBBR1MCS5 containing the replicons RP4/RK2, RSF1010 and pBBR1 (Figure 3.1 and Table 3.1) were successfully introduced into *M. album* BG8 (Figure 3.2). The presence of plasmid was confirmed by PCR using cell biomass of recombinant *M. album* BG8 containing the plasmid as template. pCAH01, pQSDP$_{MMO}$sfGFP resence was confirmed by generating amplicon of Kanamycin resistance gene (Figure 3.1 and 3.2 A). The presence of pBBR1MCS5 was confirmed by generating amplicon of Kanamycin resistance gene (Figure 3.1 and 3.2 B). The strong bright band in the same position as that of the positive control confirms the presence of plasmid. Plasmid propagation is further confirmed using plasmid copy numbers as described in later section. Efficiency of transformation was not calculated since the precise amount of host and donor biomass was not measurable using the plate-to-plate conjugation method. As pQSDP$_{MMO}$sfGFP and pBBR1MCS5 are from different incompatibility groups, they were both successfully transformed and propagated simultaneously into *M. album* BG8 (Figure 3.2).

The dry weight and cell number of plasmid-containing *M. album* BG8 cultures after 48 h of growth were recorded in Table 3.4. Both dry weight and cell number were similar between recombinant *M. album* BG8 and the wildtype, suggesting that plasmid propagation did not affect growth . While all three replicons could be propagated successfully, conjugation of *M. album* BG8 with pBBR1MCS5 took multiple efforts with inconsistent results. Hence, the subsequent gene expression experiments were performed using the RP4/RK2 and RSF1010 system.

Table 3.4. Cell counts and dry weights of transconjugant and non-conjugated (wildtype) *M. album* BG8. Transconjugants maintained the plasmids IncP, IncQ, pBBR1MCS5 or pBBR1MCS5/IncQ. Data were obtained after 48 h growth. Single factor ANNOVA values were calculated for Dry weight ($P< 0.15$) and cell number ($P<0.5$), suggesting that the values were not statistically different. Data showed is average from n=9 replicates

Sample	**Dry Weight (mg/ml) Cell**	**Cell Count (10^8 per ml)**
Wildtype	0.130±0.004	3.17±0.51
$pPSDP_{Tet}GFP$	0.133±0.013	2.64±0.54
pBBR1MCS5	0.122±0.009	2.40±0.71
$pQSDP_{MMO}sfGFP$	0.121±0.01	3.03±0.92
pBBR1MCS5/ $pQSDP_{MMO}sfGFP$	0.123±0.021	2.89±0.49



Lane 1: Wildtype BG8 negative control

Lane 2: BG8 containing pCAH01

Lane 3: BG8 containing pQSDP$_{MMO}$sfGFP

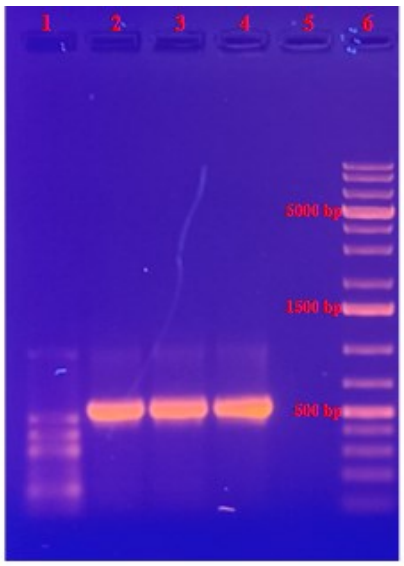
Lane 4: BG8 containing pQSDP$_{MMO}$sfGFP and pBBR1MCS5

Lane 5: pQSDP$_{MMO}$sfGFP plasmid positive control

Lane 6: Vacant

Lane 7: Generuler 1 Kb plus ladder

A



Lane 1: Wildtype BG8 negative control

Lane 2: BG8 containing pBBR1MCS5

Lane 3: BG8 containing pQSDP$_{MMO}$sfGFP and pBBR1MCS5

Lane 4: pBBR1 plasmid positive control

Lane 5: Vacant

Lane 6: Generuler 1 Kb plus ladder

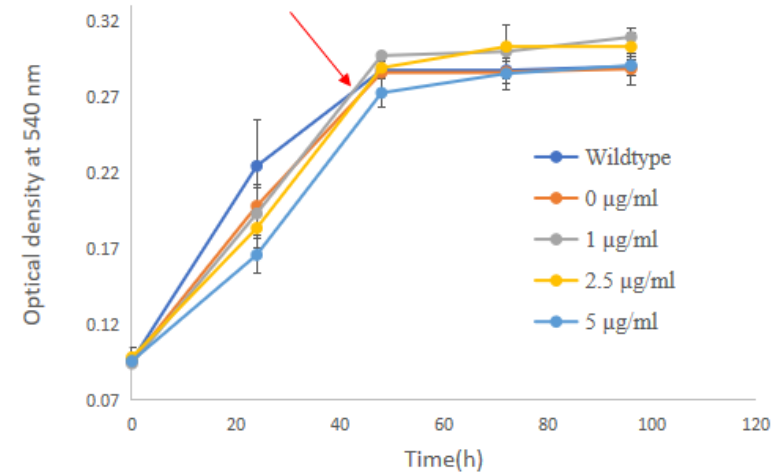
B

Figure 3.2. Confirmation of broad host range plasmid in M. album BG8 recombinant strains by generation of plasmid specific amplicon using PCR followed by agarose gel electrophoresis (A) PCR Result of amplicon from Kanamycin resistance gene to confirm presence of pCAH01 and pQSDP$_{MMO}$sfGFP (B) PCR Result of amplicon from Gentamicin resistance gene to confirm presence of pBBR1MCS5. Biomass was from *M. album* BG8 grown with 2.5 mmol methane as carbon source. 3 µl of GeneRuler 1 kb Plus DNA Ladder (Thermo scientific) was loaded for each gel.

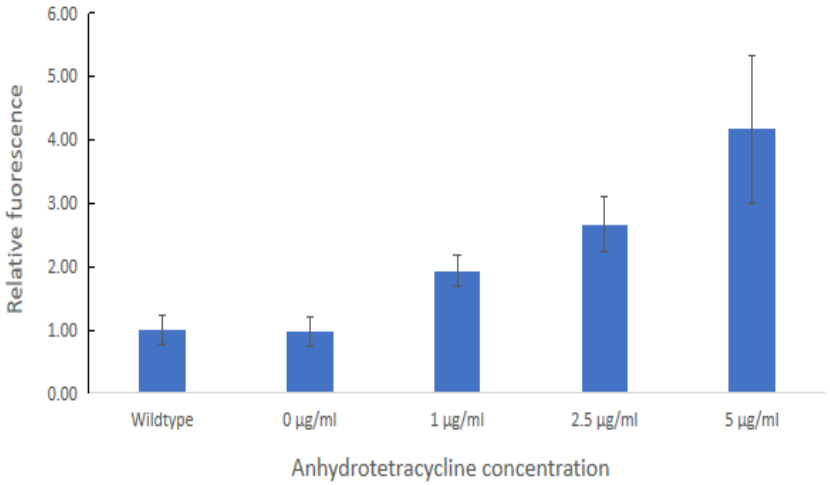
3.4.2 Broad host range vectors with constitutive and inducible promoters

Once successful propagation of the plasmids was confirmed, we fine-tuned the heterologous gene expression in *M. album* BG8 using well-characterized inducible and constitutive promoters controlling expression of GFP. The tetracycline promoter (P_{Tet}) was selected for testing inducible GFP expression. The cyclic GFP gene was amplified from pGLO and cloned downstream of the P_{Tet} promoter in the RP4/RK2 replicon (pCAH01) to generate pPSDP$_{Tet}$GFP. GFP fluorescence showed a steady increase with an increasing amount of aTc (Figure 3.3) added to the media. Furthermore, leaky expression of GFP was not detected in the absence of inducer. There was no growth inhibition of *M. album* BG8 in the presence of aTc inducer up to the 5 µg/ml as observed in Figure 3.3.

Regions which putatively included constitutive promoters from *M. album* BG8 that showed different levels of gene expression activity in a transcriptomic study (Sugden et al., 2021) were selected. These included the regions upstream of the particulate methane monooxygenase (P_{MMO}), MXA type methanol dehydrogenase (P_{MMA}), glyceraldehyde-3-phosphate dehydrogenase (P_{GAP}), and pyruvate carboxylase (P_{PYC}). In each case, 200-bp upstream regions of the start codon (Table 3.3) were cloned into the RSF1010-based vector (pQCH) upstream of the sfGFP (superfolded GFP) gene (Figure 3.1). Distinct levels of sfGFP expression were observed for each promoter in transformed *M. album* BG8 with either methane and methanol as carbon source (Figure 3.4). The P_{MMO} and P_{MMA} showed the highest levels of fluorescence



A



B

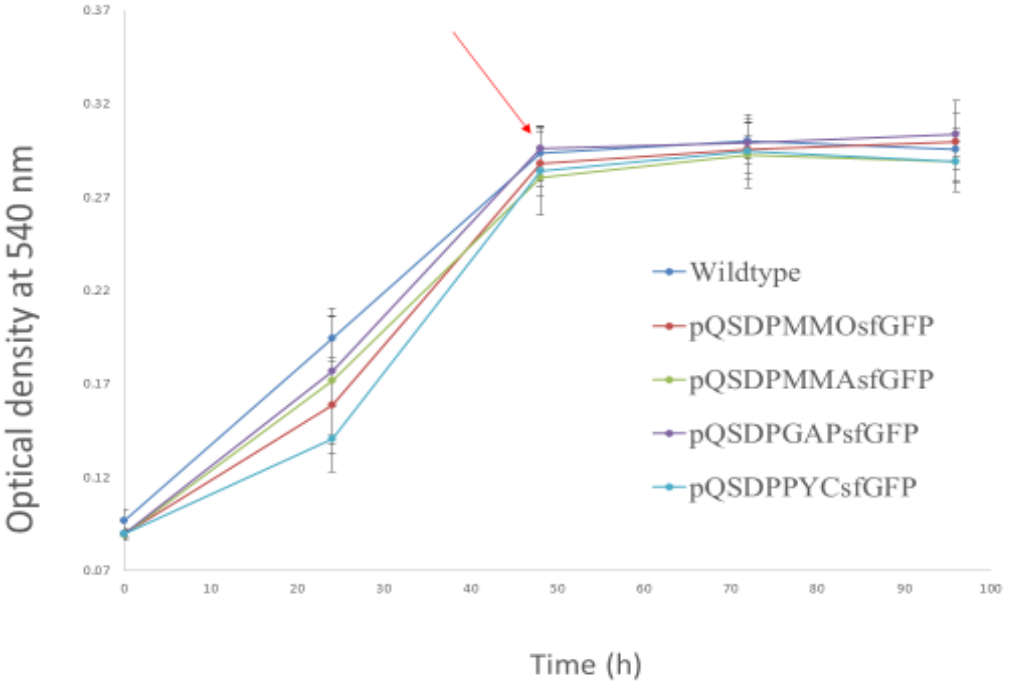
Figure 3.3 Characterization of inducible P_{Tet} in IncP based broad host range vector pPSDP$_{Tet}$GFP in *M. album* BG8 grown with 2.5 mmol methane as carbon source (A) Growth curve of *M. album* BG8 at different concentrations of anhydrotetracycline added at the red arrow showing data collection point for reading cyclic GFP fluorescence, (B) Expression of cyclic GFP driven by P_{Tet} from pPSDP$_{Tet}$GFP induced with increasing anhydrotetracycline concentration in the media measured as relative fluorescence.Data showed is average from n=6 replicates.

suggesting high expression, while P_{GAP} was weaker (Figure 3.4). The fluorescence signal for P_{PYC} was almost indistinguishable from the wild type, suggesting that this promoter cannot drive expression of sfGFP (Figure 3.4). sfGFP expression for the three active promoters was higher with methane than with methanol as carbon source. Neither plasmid replication nor sfGFP expression negatively affected cell growth, regardless of the carbon source (Figure 3.3). GFP expression was further validated by observing fluorescence of *M. album* BG8 cells under fluorescence microscope transformed with pPSDP$_{Tet}$GFP, pQSDP$_{MMO}$sfGFP, pQSDP$_{MMA}$sfGFP, pQSDP$_{GAP}$sfGFP and pQSDP$_{PYC}$sfGFP (Figure 3.5).

During routine sequencing of *E. coli* DH10β glycerol stocks containing the pQSDP$_{MMO}$sfGFP promoter-plasmid combination random point mutations were found in the RBS region of the P_{MMO} promoter (Figure 3.6). Mutation in RBS region could negatively effect heterologous protein expression This led to the conclusion that P_{MMO} should not be used for future studies involving gene expression work in *M. album* BG8 due to its structural instability.

3.4.3 Determining plasmid copy number of pCAH01, pQSDP$_{MMO}$sfGFP and pBBR1MCS5

Plasmid copy numbers for pCAH01, pQSDP$_{MMO}$sfGFP and pBBR1MCS5 were determined via qRT-PCR of amplicons from the OriT region, the MobA region, and the Rep region, respectively (Table 3.5). These regions are specific to the backbone of each of the three plasmids. The measured copy number of pBBR1MCS was the highest found in *M. album* BG8 among the three plasmids. Interestingly, we routinely observed an IncQ copy number of <1 using this method. From this data, we surmised that IncQ may not be suitable as a stable replicating vector for *M. album* BG8 due to its inability to be propagated at a minimum of 1 copy per cell.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0.27
0.22
0.17
0.12
0.07
0
10
20
30
40
50
60
70
80
90
100
Wildtype
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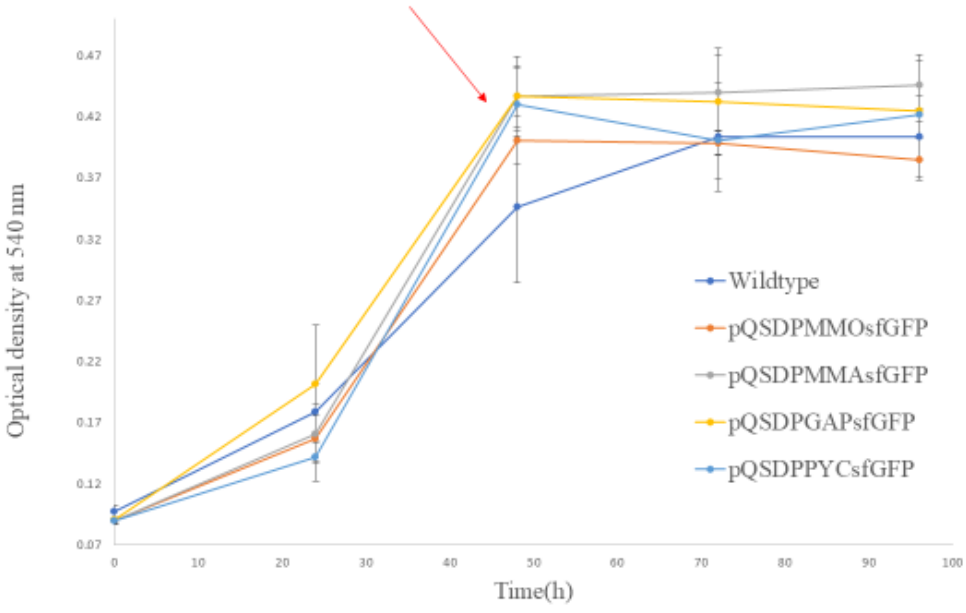
pQSDPMMAsfGFP
pQSDPGAPsfGFP
pQSDPPYCsfGFP
Time (h)

A


Optical density at 540 nm
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0.17
0.12
0.07
0
10
20
30
40
50
60
70
80
90
100
Wildtype
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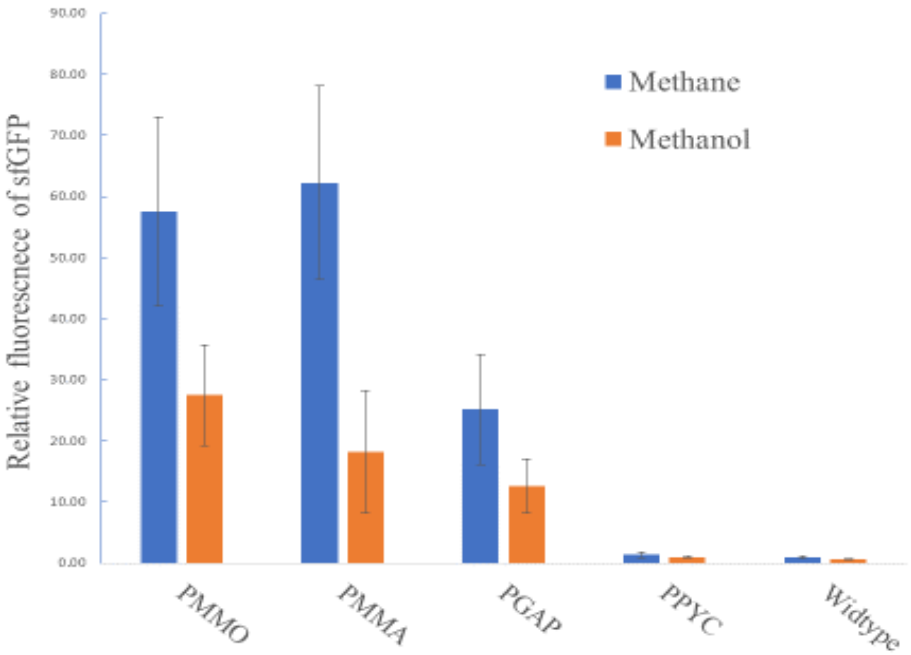
pQSDPMMAsfGFP
pQSDPGAPsfGFP
pQSDPPYCsfGFP
Time(h)

B



C

Figure 3.4 Characterization of constitutive promoters in IncQ based broad host range vector pQCH containing sfGFP (pQSDP$_{MMO}$sfGFP, pQSDP$_{MMA}$sfGFP, pQSDP$_{GAP}$sfGFP and pQSDP$_{PYC}$sfGFP described in Table 3.1 in M. album BG8. Growth curve of M. album BG8 containing expression vectors with promoters driving sfGFP expression using (A) 2.5 mmol methane and (B) 2.5 mmol methanol as carbon source, red arrow indicating data collection point for reading sfGFP fluorescence (C) Relative strength of the P$_{MMO}$, P$_{GAP}$, P$_{MMA}$ and P$_{PYC}$ promoters driving sfGFP expression measured as relative fluorescence in M. album BG8 grown in either 2.5 mmol methane or 2.5 mmol methanol. Data showed is average from n=9 replicates

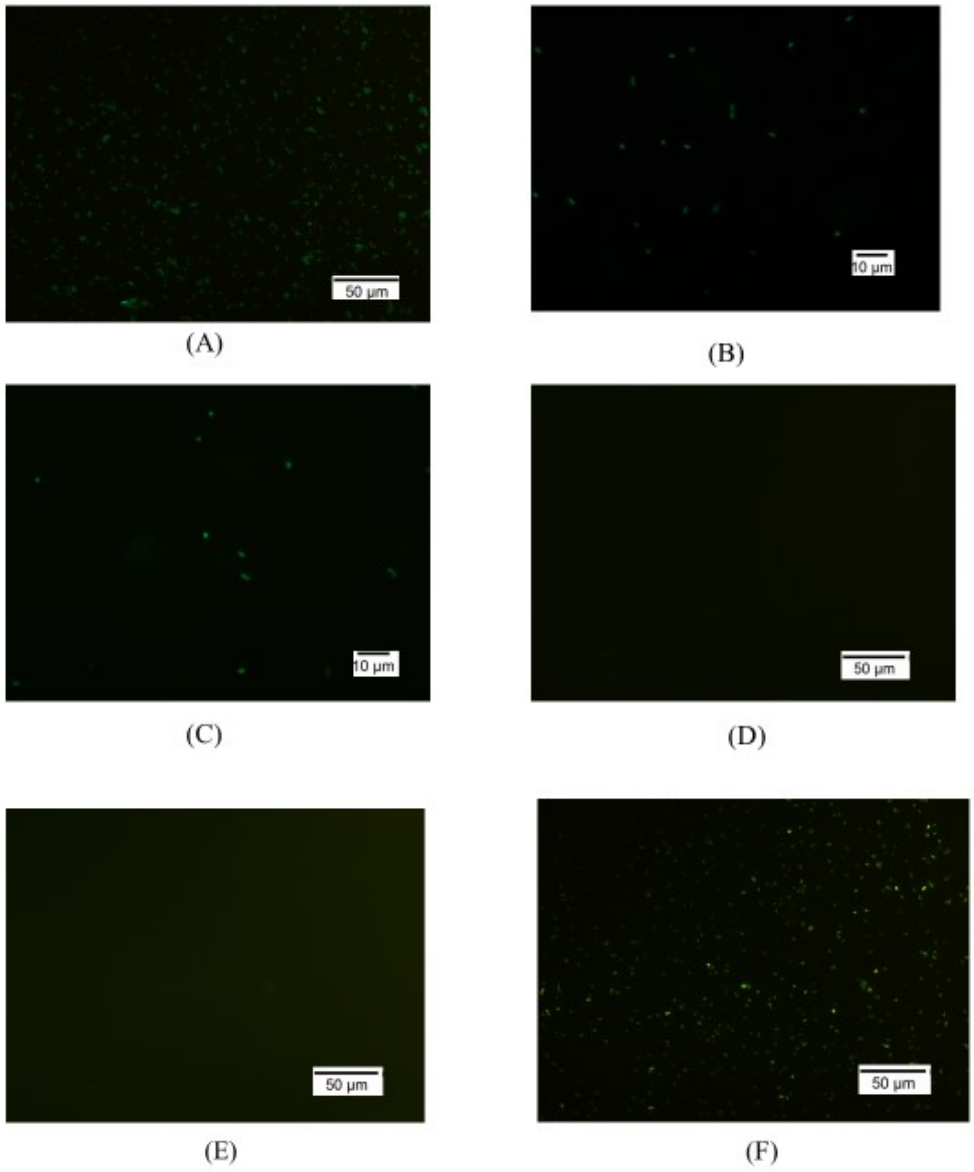


Figure 3.5. Fluorescence image of different recombinant *M. album* BG8 grown with 2.5 mmol methane for 48 h containing A) pQSDP$_{GAP}$sfGFP at 400X magnification, B) pQSDP$_{MMA}$sfGFP at 1000X magnification, C) pQSDP$_{MMO}$sfGFP at 1000X magnification D) pQSDP$_{PYC}$sfGFP at 400X magnification E) Wildtype *M. album* BG8 400X magnification F) pPSDP$_{Tet}$GFP with 5 µg/ml anhyfrotetracyckine in the media at 400X magnification

Figure 3.6. Comparison of multiple extraction of plasmid with the Pmmo promoter (pQSDP$_{MMO}$sfGFP) from multiple glycerol stocks. The names on the left denotes the glcyerol stock name. The Shine-Dalgarno sequence/RBS region is not conserved among cultures, indicating a fast mutation rate for this promoter when maintained in a plasmid. All stocks were sequenced once.

3.4.4 Expressing an active luciferase protein under P_{MMA} in IncP and IncQ vectors

The luciferase gene (NanoLuc) was codon-optimized to remove hindrance in expression arising from codon bias (sequence provided in Table 3.3). NanoLuc was cloned downstream of the P$_{MMA}$ promoter within the IncP backbone to generate the pPSDP$_{MMA}$nluc, and under the P$_{MMA}$ promoter within the IncQ backbone to generate the pQSDP$_{MMA}$nluc. *M. album* BG8 cells transformed with these two separate vectors showed a similar level of luciferase activity,

Table 3.5 Determination of plasmid copy numbers of the three broad host range plasmids in *M. album* BG8 grown with 2.5 mmol methane for 48 h. PCN determined via qRTPCR of amplicons from OriT, MobC and Rep region of pCAH01, pQSDP$_{MMO}$sfGFP and pBBR1MCS5 respectively. Data showed is average from n=9 replicates

Sample	Plasmid family	Average (per cell)
pCAH01	IncP	6±4
pBBR1MCS5	pBBR1	11±8
pQSDP$_{MMO}$sfGFP	IncQ	0.47±0.25
pQSDP$_{MMO}$sfGFP in pBBR1MCS5 and pQSDP$_{MMO}$sfGFP combination	IncQ	0.33±0.17
pBBR1MCS5 in pBBR1MCS5 and pQSDP$_{MMO}$sfGFP combination	pBBR1	19±6

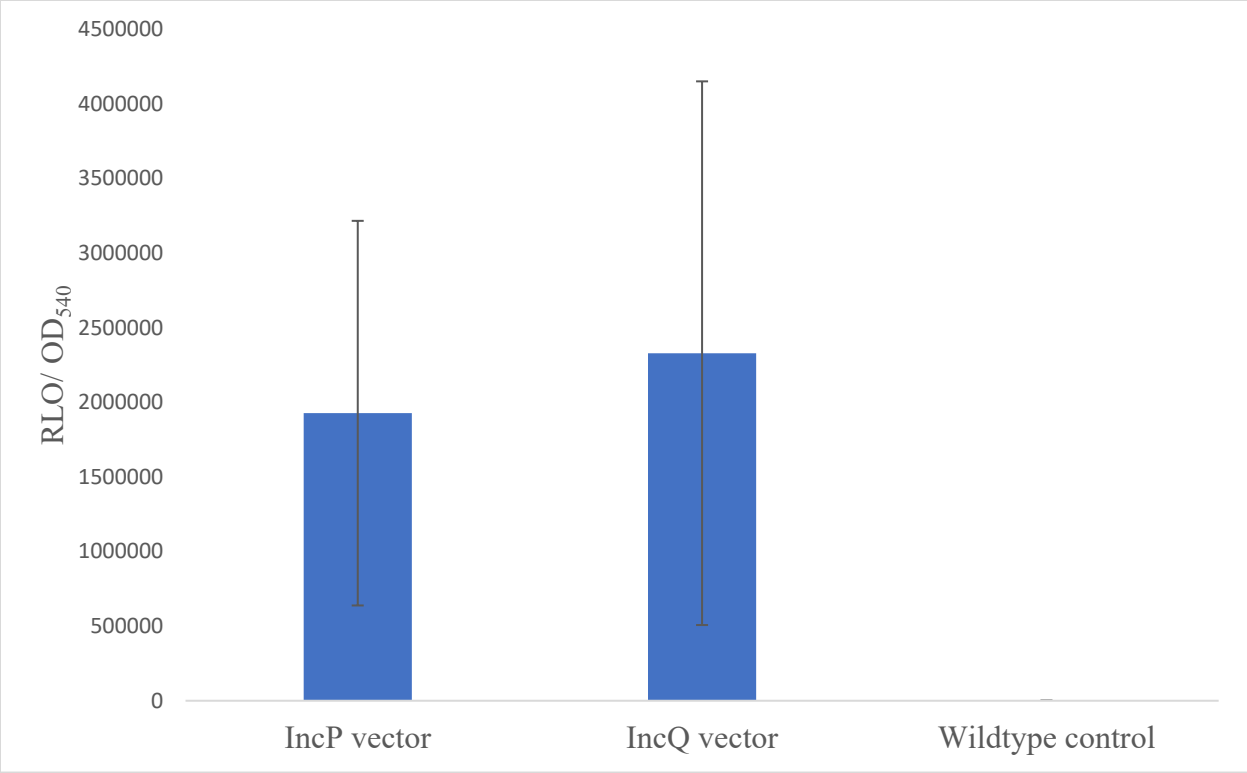


Figure 3.7. Expression profile of NanoLuc luciferase protein under control of P_{MMA} in either IncP or IncQ plasmid vectors (pPSDP$_{MMA}$nluc, pQSDP$_{MMA}$nluc as described in Table 3.1). *M. album* BG8 cultures were grown for 48 h with 2.5 mmol of methane as carbon source. Activity is reported relative luminescence per OD540 (RLO/ OD540) unit of M. album BG8 culture. Data showed is average from n=5-7 replicates

suggesting an equivalent level of gene expression from P_{MMA} for the two plasmid backbones tested (Figure 3.7.).

3.5 Discussion

Developing genetic tools for heterologous protein expression is an important step towards developing a strain of *M. album* BG8 for industrial purposes. An initial step was the

development of broad host range plasmid vectors that can be stably maintained in methanotrophic bacteria (Henard et al., 2017; Lloyd et al., 1999; Mustakhimov et al., 2016; Tapscott et al., 2019) and to determine if these plasmids can be propagated. Both conjugation and propagation of the three plasmids were successful, further confirming their utility in genetic engineering of methanotrophs. Conjugation of a suicide vector for mutational analysis was already successfully implemented in *M. album* BG8 by conjugation (Kang-Yun et al., 2022), and the present work successfully introduced replicating broad host range plasmids using the same method.

In addition to single plasmid systems, the ability of *M. album* BG8 to propagate both RSF1010 and pBBR1 plasmids simultaneously could be useful in creating expression systems incorporating genes from two different pathways, as shown in the production of taxol precursor (Ajikumar et al., 2010) and farnesene (You et al., 2017) in *E.coli*. In those studies, genes from two different pathways were expressed separately from two plasmids for optimized production. Simultaneous propagation of broad host range vectors RP4/RK2 and pBBR1 was achieved in the methanotroph *M. capsulatus* Bath (Tapscott et al. 2019).

Both inducible and constitutive promoters were characterized using GFP as a proof-of-concept for heterologous protein expression. An IncP-based expression vector with the inducible P_{Tet} promoter has previously been used in the methanotroph *M. buryatense* 5GB1C for lactate production (Henard et al. 2016), and in *M. trichosporium* OB3B for cadaverine production from methane (Nguyen et al., 2020). Here, we showed concentration-dependent GFP expression in *M. album* BG8 transformed with pPSDP$_{Tet}$GFP in the presence of the inducer aTc (Figure 3.3). In addition, growth inhibition was not observed in the range of Atc concentration 0-5 µg/ml, unlike what has been observed with *M. buryatense* 5GB1C, where growth was inhibited at

concentrations ≥ 2.5 µg/ml (Henard et al., 2016). Hence, the IncP-based expression vector containing the P_{Tet} promoter can be a successful approach for the inducible, modulated expression of proteins in *M. album* BG8.

Using transcriptome data to select and validate endogenous promoters for use in expression vectors has been a successful approach for creating expression systems in other methanotrophic bacteria. For instance, in *M. capsulatus* Bath, promoters from highly expressed genes were used to drive expression of reporter genes from introduced plasmids (Tapscott et al, 2019). In *M. buryatense* 5GB1C, a computational framework was developed based on RNAseq data to identify -35 and -10 motifs of promoters with high levels of reporter gene expression (Wilson et al, 2021). In the present study, we also used RNAseq data to select P_{MMO} and P_{MMA} promoters that can drive high expression levels of sfGFP, while P_{GAP} led to low expression levels. The P_{PYC} promoter showed no activity. This promoter in this study was selected as a negative control to test any leaky expression. In the transcriptome data the pyruvate carboxylase gene showed extremely low level of expression suggesting reporter gene expression from the promoter of this gene should be very low or close to the negative control. Since this was found to be true from our result this promoter could be used in future to test any leaky expression of genes. Due to the high mutation rate of the RBS region of P_{MMO} while manipulating the cloning and donor *E. coli* strains (Figure 3.6), P_{MMO} was excluded from this study, and P_{MMA} was selected as the driver of high gene expression using the IncQ-based expression vector. The P_{GAP} promoter could also be useful when weaker levels of gene expression are necessary; for instance, in the production of pinene, an isoprenoid-based biofuel precursor (Table 2.3). The P_{GAP} promoter could limit production of the toxic pinene precursor geranyl pyrophosphate (GPP),

whereas the P_{MMA} promoter could drive high expression of pinene synthase (PS) to circumvent possible GPP toxicity by increasing the rate towards pinene.

While both P_{MMA} and P_{GAP} maintained distinct sfGFP expression levels when *M. album* BG8 was growing on either methane or methanol, the relative fluorescence value decreased considerably when methanol was used as a carbon source (Figure 3.3). Transcriptome and metabolome studies have suggested that methanol can lead to stress responses affecting cell growth (Sugden et al., 2021), which could explain the lower sfGFP expression under these promoters when methanol is used as carbon source. In future bioconversion work with *M. album* BG8, methanol effects on heterologous gene expression should be taken into consideration, especially since *M. album* BG8 growth has shown to be robust when using methanol as a carbon source (Figure 3.4).

Plasmid copy number (PCN) is an important property of expression vectors affecting plasmid retention and protein expression. The low copy number of pCAH01(RP4/RK2) (6±4) and pQCHP$_{MMO}$sfGFP (RSF1010) (0.47± 0.25) and comparatively higher number for pBBR1MCS5 (pBBR1) (11±8) follows from the fact that plasmid size generally negatively affects the PCN (Smith & Bidochka, 1998). pBBR1MCS5 is smaller at 4.7 Kb compared to the other two plasmids at 7.6 Kb. This is similar to the trend observed in *E. coli* where the former two replicons had lower copy number compared to pBBR1 (Jahn et al., 2016). A low PCN was observed for an IncP-based vector in *M. alcaliphilum* 20Z (Mustakhimov et al., 2016). However, PCN was determined per chromosome copy instead of per cell making a direct comparison between these two studies difficult, and there is no standardized definition of an appropriate PCN (Jahn et al., 2016). The higher PCN of pBBR1MCS5 suggests a high potential as a stable expression vector in *M. album* BG8. This vector has been used for complementation studies in

mutant strains of *M. capsulatus* Bath (Welander & Summons, 2012), and for driving gRNA expression for CRISPR tool development (Tapscott et al., 2019). This vector could be modified to improve conjugation levels as has been done for RSF1010-based vectors in cyanobacteria (Bishé et al., 2019). The PCN values <1 seen for pQCHP$_{MMA}$sfGFP could be caused by plasmid-free sub-populations of cells, as was suggested for plasmid RSF1010 in *E.coli* DH5α (Jahn et al., 2016), or by incomplete lysis of cells leading to lower than expected amplification of signal during in the qPCR reaction.

While the PCN data suggests that IncP is a more stable vector than IncQ in *M. album* BG8, the NanoLuc expression data suggests that both vectors have equivalent heterologous protein expression in this strain. In fact, NanoLuc was expressed at a similar level in *M. album* BG8 under P$_{MMA}$ in both IncP and IncQ plasmids (Figure 3.6). Studies in *E.coli* earlier suggest that high copy number plasmid can be a metabolic burden to the cells and may not necessarily lead to higher protein expression(Rosano & Ceccarelli, 2014). The growth parameters of *M. album* BG8 containing the two different plasmids could be further explored in future study to check if there is metabolic burden from plasmid with higher PCN. In addition, as luminescence is the result of an enzymatic reaction, this data showed that these vectors could produce active heterologous enzyme conformations in *M. album* BG8. In previous studies, active cathecol dioxygenase XylE was expressed using the RP4/RK2 based expression plasmid in the methanotrophs *M. buryatense* 5GB1, *M. capsulatus* bath (Puri et al., 2015; Stolyar et al., 2001). This work expands the range of plasmids and strains effectively used for heterologous expression of active enzymes.

The IncP -based expression vector has been more commonly reported in methanotrophs, including with *M. buryatense* 5GB1C, *M. trichosporium* OB3B, and *M. alaciphilum* 20Z (Garg

et al., 2018; Henard et al., 2016, 2017; Mustakhimov et al., 2016; A. D. Nguyen et al., 2020; T. T. Nguyen et al., 2020) for both homologous and heterologous gene expression. IncQ has only been reported so far for homologous gene expression in *M. trichosporium* OB3B (Lloyd et al., 1999). The present study showed that both broad host range plasmids can be used as expression vectors in *M. album* BG8 with both inducible (P_{Tet}) and constitutive (P_{MMA}, P_{GAP}) promoters.

3.6 Conclusion

In this study, a successful conjugation protocol has been developed to transform plasmids from three broad host range family RP4/RK2, RSF1010 and pBBR1 in *M. album* BG8. The replicon systems can be introduced into *M. album* BG8 by conjugation. Moreover, three promoters (the inducible promoter P_{Tet} and the constitutive promoters P_{MMA} and $P_{GAP.}$) were characterized using GFP and NanoLuc as demonstration proteins for heterologous gene expression. Two expression vectors based on the IncP replicon and IncQ replicon were validated with identical expression of NanoLuc under the P_{MMA} promoter. pBBR1 and IncQ plasmids were simultaneously propagated by *M.album* BG8, suggesting this strain can maintain plasmids from different incompatibility families. This study also suggests that using methanol as a carbon source, while producing high biomass might lead to lower heterologous gene expression as compared to methane as a carbon source. The three expression vectors and three promoters investigated can be utilized in various combinations for expression heterologous genes towards the production and recovery of commercially useful products in *M. album* BG8 using methane or methanol as feedstock.

3.7 Reference

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Chapter 4: Developing batch, sequential batch and fed-batch strategies for methanol bioconversion by *Methylomicrobium album* BG8

4.1 Abstract

Methanol is an abundant low-cost carbon feedstock that has the potential to be a strong driver of the future bioindustry. It can be sourced from biological processes, carbon dioxide capture and conversion, or blue hydrogen production. Methylotrophs are a group of microbes which can utilize methanol as sole sources of carbon and energy and can convert them to value-added products such as bioplastics, biofuels, and platform chemicals. The methylotrophic bacterium *Methylomicrobium album* BG8 is notable for its fast and robust growth and rich genetic potential, making it an excellent candidate for bioproduction from methanol. Towards this goal, batch, sequential batch and fed-batch operation of *M. album* BG8 were developed to improve biomass production. Results from batch operation showed that high methanol concentration, up to 50 mM, can be used without inhibiting growth. Additionally, the use of biomass from batch operation as inoculum for subsequent runs – as sequential batches – substantially decreased the lag phase. In fed-batch operation, the maximum optical density achieved (16.6) – 10-fold increase compared to results from batch operation (1.52) – was comparable to that of more widely studied strains for methanol bioconversion. This study, when supplemented with genetic engineering of *M. album* BG8, lays the basic framework for developing advanced bioreactor operation of this bacterium as an industrial platform for methanol bioconversion.

4.2 Introduction

Methanol is a cheap and abundant carbon feedstock that can be derived from natural gas, biomass (Poluzzi et al., 2022) and carbon dioxide conversion (Tan et al. 2023,Zhang et al., 2021), and as a byproduct of turquoise hydrogen production (Banu et al. 2023). Recently, the viability of bulk methanol production from a CO_2-to-methanol plant has been demonstrated, with a capacity of 110,000 tonnes of methanol per year (*World's Largest CO2-to-Methanol Plant Starts Production*, 2022). The liquid nature methanol allows easy storage and transportation than [what?]. Its high solubility in water makes it easily accessible to biological systems. These favorable properties translate into the fast emergence of methanol as an important feedstock and reusable waste product in the establishment of a circular carbon economy (Cotton et al. 2020).

The bioconversion of methanol has been explored using a group of organisms called methylotrophs. These microbes can utilize compounds with no carbon–carbon bonds, also referred to as C1 compounds, such as methane, methanol and methyl amine, as sole sources of carbon and energy (Chistoserdova & Kalyuzhnaya, 2018). Methylotrophs that can utilize both methane and methanol are specifically called methanotrophs and they are subset of this group (Lidstrom 2006). This ability to utilize methanol has enabled the bioproduction of a diverse range of compounds using methylotophs, such as amino acids (Brautaset et al., 2007), vitamins like riboflavin (Klein et al., 2023), bioplastics like poly(hydroxybutyric acid) (PHB) (Bourque et al., 1992), and platform chemicals like 3-hydroxypropionic acid (Yang et al., 2017).

In most bioprocesses, the conversion of a feedstock to a desired product using the organism of interest is carried out in bioreactors (Mandenius, 2016), vessels in which environmental parameters necessary for growth of the organism can be controlled to achieve

optimal growth thereby optimal production (Jaibiba et al., 2020). As such, understanding the growth dynamics of methylotrophs in bioreactors is important to achieve efficient methanol conversion. Three modes of operation are of interest in this study. Batch operation represents the simplest process for bioconversion and can be useful in scenario where the product can be toxic (Liu, 2017). It can also be used to identify possible substrate toxicity issues, such as growth inhibition, as was reported for another methanotroph, *Methylotuvimicrbium buryatense* 5GB1C (Tan et al. 2022). In sequential batches, an actively growing culture can be passaged from a batch to the next possibly skipping lag phase (by, for example, keeping a small volume in the reactor at the end of a batch to serve as inoculum for a subsequent batch) thereby a short operation downtime leading to higher productivity (Huang et al., 2008). This can be particularly useful since slow growth is one of the drawbacks of using methanotrophs for bioconversion. Fed-batch operation is typically used to achieve high biomass density (Çalık et al., 2010; Lim & Shin, 2013). It was shown to be effective for the industrially important methanotrophs *M. buryatense* 5GB1C (Tan et al. 2022) and *Methylosinus trichosporium* OB3b (Adegbola, 2008; Dubencovs et al., 2022) using methanol as carbon source.

Methylotroph growth dynamics in bioreactors has been investigated since the 1980s. In studies with *Methylomonas* L3 (Chu & Papoutsakis, 1987; Papoutsakis et al., 1981), high cell density was achieved for single-cell protein production. From there, reaching high cell density has remained one of the main objectives of methylotroph bioreactor studies, including work performed with *Methylobacterium extorquens* AM1 *(*Bourque et al., 1995) and *M. trichosporium* OB3b (Adegbola, 2008; Dubencovs et al., 2022). High cell density can be beneficial in obtaining higher product titers, as was seen for mevalonate produced by *M. extorquens* AM1 in which fed-batch operation led to a 10-fold increase in titer compared to growth in flasks (Zhu et al., 2016).

Varying growth parameters can also improve product quality, as shown in the production of high-molecular weight PHB production by *M. extorquens* AM1 through adjusting methanol to oxygen feed in fed-batch operation (Bourque et al., 1995).

In this context, investigating growth in bioreactors is an important step towards achieving the overarching goal of this thesis, i.e. the development of *Methylomicrobium album* BG8 as biological platform for isoprenoid production from C1 sources. This organism is an aerobic methanotroph (Whittenbury et al., 1970), a subset of methylotrophic bacteria (Lidstrom 2006). Hence it can be developed for methanol and/or methane – another potential feedstock for bioindustry which cannot be utilized by some common methylotrophs such as *M.extorquens* AM1 and *Bacillus methanolicus* (Singh et al., 2022) – bioconversion. In addition, *M. album* BG8 can grow reliably utilizing either ammonium or nitrate as nitrogen source (Tays et al. 2018, Sugden et al. 2021). It can be adapted for growth under various conditions, such as low pH (McDonald, 2019). The transcriptome and metabolome of *M. album* BG8 have been described under different carbon- and nitrogen-source conditions (Sugden et al. 2021), and a genome-scale metabolic model has been constructed (Villada et al. 2022).

While several challenges exist towards achieving methanol bioconversion at industrial scale, production and productivity in bioreactors remain an important bottleneck. With this in mind, this chapter will focus on understanding batch, sequential batch and fed-batch growth of *Methylomicrobium album* BG8 in bioreactors. These bioreactor operation strategies will help us lay the framework to further develop this strain for efficient methanol bioconversion to value-added products.

4.3 Material and methods

4.3.1 Bacterial strain and media

M. album BG8 was routinely cultured in 100 ml nitrate mineral salt (NMS) medium (Whittenbury et al; 1970) containing 10 mM potassium nitrate as nitrogen source and 5 µM copper buffered with 1.5 mL phosphate buffer pH 6.8 (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) in Wheaton media bottles (250 mL) closed with butyl-rubber septa caps. High-performance liquid chromatography (HPLC)-grade methanol was added to a concentration of 25 mM. The cultures were incubated at 30°C and 150 rpm in a walk-in incubator on a shaker [New Brunswick G10]. Stationary phase cultures were stored at room temperature and used as inocula for precultures grown in Wheaton bottles as described above. These precultures were then used as inocula for bioreactor operation. Precultures were routinely checked for contamination by doing spot tests on Nutrient broth (NB) agar plates (Fisher chemicals]). 100 µl of culture was taken using a sterile syringe and deposited as drops on NB agar plates, which were then incubated at 30° C for 48 h in a walk-in incubator. Any appearance of colony was considered as evidence the culture was contaminated.

4.3.2 Bioreactor operation

Batch, sequential batch and fed-batch operations were carried out in an Infors Multifors 2 system. The bioreactor system was equipped with a temperature sensor, heating blanket, air inlet with controller, a sampling port, and peristaltic pump to supply media during operation. The working volume was kept at 400 ml, temperature was maintained at 30° C and agitation was maintained at 600 rpm with a double Rushton impeller. Antifoam SE-15 [Sigma] was added as required. Aeration was varied depending on the operation as explained in section 4.3.3 below.

Methanol was manually added using sterile serological pipettes for batch, sequential batch and pulse feeding experiments.

Initial inoculation was performed by adding 40 ml of preculture to the bioreactor for all operation modes. In sequential batch operation, subsequent cycles were inoculated using a modified protocol in which 40 ml of cell culture from the previous cycle was collected using a sterile 60-ml syringe and transferred to another bioreactor containing 360 ml of medium.

Two feeding modes were used for fed-batch experiments: pulsing feeding and continuous feeding. In the latter, NMS containing 8 % v/v (methanol (3.16 M) was prepared and supplied to the bioreactor using a feeding pump at a predetermined controlled flowrate.

In all experiments, the culture was tested for contamination at the end of operation, as described earlier.

4.3.3 Optical density, pH, and dry weight measurement

Bioreactor cultures were assessed throughout operation by measuring optical density at 540 nm (OD_{540}) using a spectrophotometer (Ultrospec 50, Biochrom). The specific growth rate for fed-batch run was determined from the slope of growth during exponential growth (linear section of log OD_{540} vs time). pH was measured using a pH meter and electrode (Denver Instruments Ultrabasic, Sartorius). Yield was measured based on OD_{540} divided by the total amount of methanol added to that point. Dry weight measurements were performed using 3.8 ml of culture. A sample was centrifuged at 16,000 g for 5 minutes in a bench top centrifuge (Eppendorf, Model 5425). The supernatant was discarded, and the pellet was resuspended in 500

µl of deionized ultrafiltered (miliQ) water. The resuspension was put on a pre-tared aluminum weighing dish (Fisher), and dried in a desiccator at room temperature for 96 h. The dried sample was weighed to determine the sample biomass content in g/L.

4.4 Result

4.4.1 Batch operation

Experiments were first performed in batch reactors to assess the impact of initial methanol concentrations on growth, factors leading to potential growth inhibition, and different aeration strategies. Previous studies of batch cultures of *M. album* BG8 grown in 250-ml Wheaton bottles reported no growth inhibition up to 25 mM methanol (Tays et al. 2018, Sugden et al. 2021). Hence, batch runs were performed at methanol concentrations of 25 mM and 50 mM using 10 % inocula of late log phase *M. album* BG8 previously grown in Wheaton bottles with methanol at 25 mM. Five batch runs (B1to B5) were performed , and the results are reported in Table 4.1 and Figure 4.1.

For the first three batches performed at either 25 or 50 mM methanol (B1 to B3), aeration was continuous at a flow rate of 70 ml/min. As can be seen in Figure 1 and Table 4.1, the growth pattern, lag phase and maximum OD_{540} were similar for all three runs, regardless of the initial methanol concentration used. This suggested that no growth inhibition took place in the bioreactor runs at methanol concentrations up to 50 mM. Hence, all further batch runs were initiated at 50 mM methanol.

Experiments were then performed to establish whether methanol, a volatile substance, was lost through evaporation in the course of a batch run. B4 and B5 were performed with air being supplied at 140 ml/min for 20 min prior to inoculation, at which point aeration the

bioreactor was sealed and aeration was stopped. Maximum OD_{540} of these closed bioreactor runs (B4 and B5) were found to be substantially higher than those of continuous airflow runs (B1 to B3) (Figure 4.1, Table 4.1). Lastly, the lag phase was found to be between 24 and 27.5 h in all five runs, regardless of initial methanol concentration or aeration (Table 4.1).

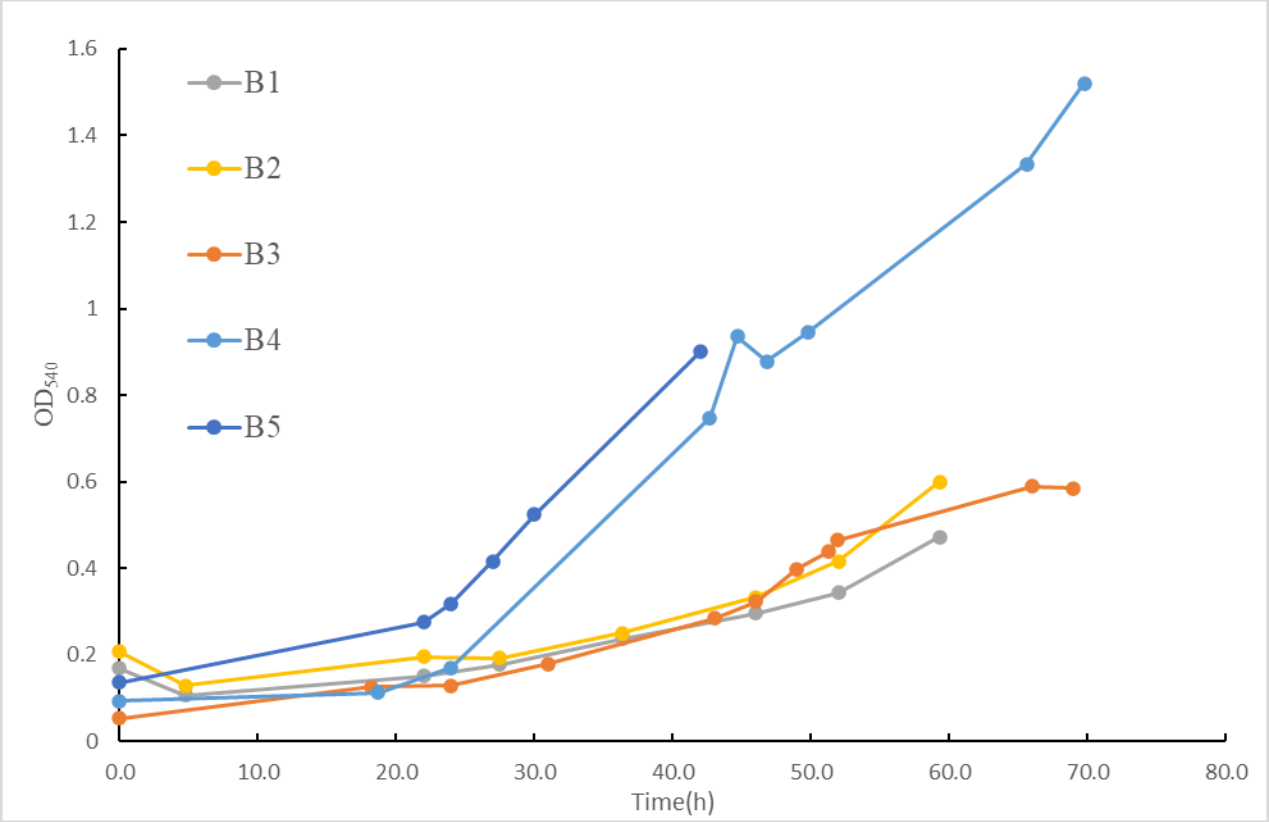

Figure 4.1 Batch cultures of *M. album* BG8 at different methanol concentrations and aeration. B1 with 25 mM and B2, B3, B4, B5 with 50 mM methanol. B1, B2, B3 with continuous airflow and B4, B5 with 20 min aeration prior to inoculation.

Table 4.1 Batch operation parameters of *M. album* BG8

Run	Initial Methanol concentration (mM)	Air supply	Lag phase (h)	Maximum OD_{540}
Batch run 1 (B1)	25	Continuous at 70 ml/min	27.5	0.535
Batch run 2 (B2)	50	Continuous at 70 ml/min	27.5	0.59
Batch run 3 (B3)	50	Continuous at 70 ml/min	24	0.758
Batch run 4 (B4)	50	20 minute aeration at 140 ml/min	24	1.52
Batch run 5 (B5)	50	20 minute aeration at 140 ml/min	24	0.9

4.4.2 Sequential batch operation

Sequential batch operation was performed to investigate its impact on growth. *M. album* BG8 biomass grown in bioreactors to an $OD_{540} > 0.9$ was used to inoculate (10% inoculum) subsequent batches. These experiments were denoted as SB1, SB2 and SB3 . Again, the initial methanol concentration was set to 50 mM and the air was supplied for 20 min prior to

inoculation of every cycle (based on results from runs B4 and B5). In all three sequential batch runs the lag phase decreased substantially after the first cycle (Figure 4.2, Table 4.2). For all three SB runs, an OD_{540} of 1 was reached within 25 h in cycle 2, which was faster than in batch runs (Figure 4.1, Table 4.1). In the case of SB3 a culture was successfully maintained until cycle 3.

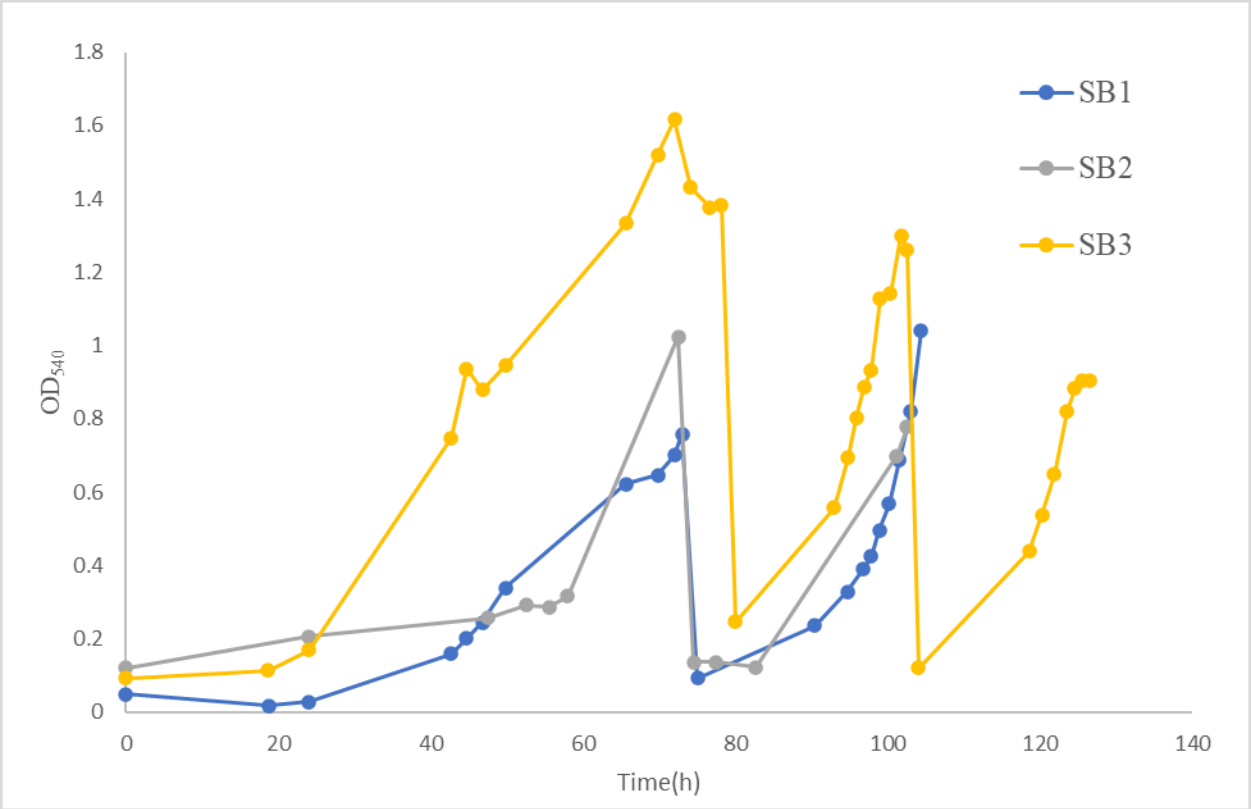

Figure 4.2 Growth curve of sequential batch operations for *M. album* BG8. All three runs with initial methanol concentration 50 mM and aeration for 20 min prior to inoculation for each cycle.

Table 4.2 Sequential batch operation parameters of *M. album* BG8

Sequential Batch Run	Initial Methanol concentration (mM)	Air supply	Lag phase first cycle (h)	Lag phase later cycles (h)
SB1	50	20 min aeration at start of every cycle at 140 ml/min	42.7	No lag phase
SB2	50	20 min aeration at start of every cycle at 140 ml/min	47.5	10
SB3	50	20 min aeration at start of every cycle at 140 ml/min	24	No lag phase

4.4.3 Fed-batch operation

Fed-batch operation is commonly used to generate high biomass density to improve product titer and productivity. This was implemented for cultures of *M. album* BG8. Fed-batch was initiated based on the conditions previously determined for batch operation: initial methanol at 50 mM, air supplied at 140 ml/min for 20 min prior to inoculation or continuously at 70 ml/min, and inoculum from a previous bioreactor batch run. Once an OD_{540} of ~0.8-1 was reached, (at about 26-28 h), methanol addition (either pulsed or continuous) was initiated. Six different modes of feeding and aeration were investigated, with three conditions undergoing

pulsed methanol addition (FB1-FB3) and the three others undergoing continuous methanol addition (FB4-FB6). The details of operation and results are presented in Table 4.3.

Two parameters were investigated to improve fed-batch operation: methanol feeding strategy and aeration strategy. Methanol was either added every 24 h as a 40 mmol pulse (FB1-FB3) or continuously fed using a feed pump (FB4-FB6). For the latter runs, two different strategies were tested: 1) constant addition of 40 mmol over 24 h (FB4-FB5), and 2) addition of 20 mmol over the first 24 h and increasing by an additional 10 mmol every subsequent 24 h (FB6).

Two approaches were taken for air supply: 1) pulsing air at 140 ml/min for 20 min every 24 h, and 2) supplying air continuously at a flow rate of 70 ml/min. The flow rate of 70 ml/min was selected to avoid rapid accumulation of foam, which was observed at higher air flow rates in preliminary experiments, even with the addition of antifoam.

The different feeding strategies used in this study and growth parameters observed are detailed in Table 4.3, and the growth patterns are shown in Figure 4.3. The maximum OD_{540}, time to reach maximum OD_{540}, methanol fed to reach this point, yield – as maximum OD_{540} per mmol methanol fed to the system –, and pH increase were all recorded (Table 4.2). Cell dry weight at stationary phase were recorded for pulse feed run FB3 and continuous feed run FB6.

Table 4.3 Parameters for fed-batch operation of *M. album* BG8

Parameter	Fed-batch operation					
	FB1	FB2	FB3	FB4	FB5	FB6
Methanol feeding strategy	Pulse feeding 40 mmol every 24 h	Pulse feeding 40 mmol every 24 h	Pulse feeding 40 mmol every 24 h	Continuous feeding 40 mmol/24 h	Continuous feeding 40 mmol/24 h	Continuous feeding starting at 20 mmol/24 h and increasing +10 mmol/24 h
Air supply strategy	20 min of aeration every 24 h at 140 ml/min	Continuous at 70 ml/min	Continuous at 70 ml/min	20 min of aeration every 24 h at 140 ml/min	Continuous at 70 ml/min	Continuous at 70 ml/min
Initial pH	6.89	7	6.9	6.82	7	6.92
Final pH	7.06	8.38	8.61	6.63	8.38	8
Time to reach max OD_{540} (h)	142.5	74	72	191	74	96
Max OD_{540}	10.51	10.96	9.52	5.49	11.38	16.56
Methanol fed to reach Max OD_{540} (mmol)	220	140	100	260	98.83	111.25
Dry weight (g/l)	ND	ND	2.32	ND	ND	3.76
Yield (OD/mol CH_3OH)	48	78	95	21	115	149
Specific growth rate (h^{-1})	0.015	0.049	0.038	0.009	0.05	0.04

* ND stands for not determined

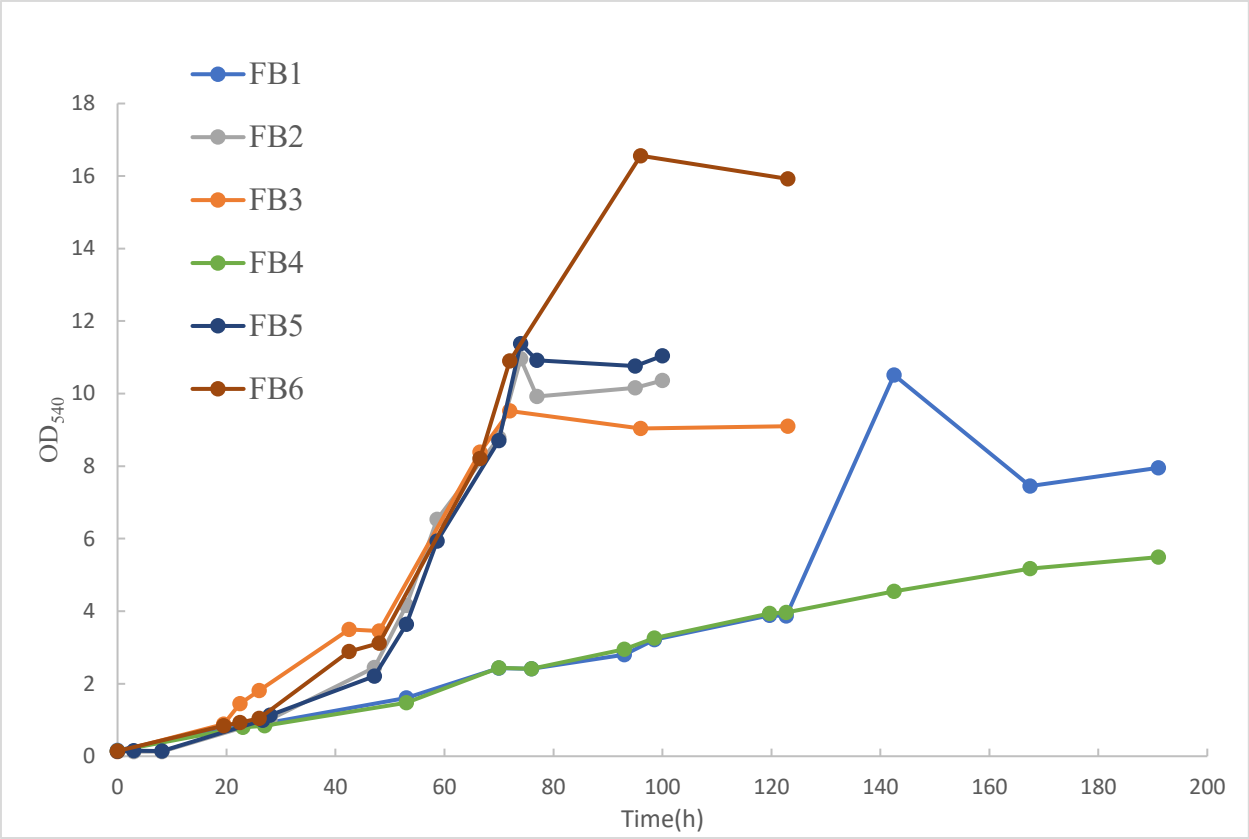

Figure 4.3 Growth curve of fed-batch operation of *M. album* BG8. Fed-batch runs were started after 24-30 h of batch operation with pulse feed runs receiving methanol every 24 h and continuous feed runs receiving methanol via feed pump at a constant rate. Feeding strategies consisted of pulse feeding (FB1-FB3) and continuous feeding (FB4-FB6) during the fed-batch periods. Pulse feed run FB1 and continuous feed run FB4 received air every 24 h while for all other runs received continuous airflow following the initial batch period. Specific conditions for each run are described in Table 4.3.

Looking at the effect of different methanol feeding approaches, it was observed that pulse-feeding methanol led to an almost two-fold increase in maximum OD_{540} and a 2.5-fold

increase in yield compared to continuous feeding for runs in which air was pulsed every 24 h (Pulse feed FB1 vs Continuous feed FB4; Table 4.2). On the other hand, when air was continuously fed to the reactor, continuous methanol feeding (both at a constant rate – FB5 – and at increasing rates – FB6) led to higher maximum OD_{540} and yield than pulsed methanol feeding (Pulse feed run FB2 and FB3; Table 4.3). In fact, continuous methanol feeding at increasing rate (Continuous feed run FB6) led to the highest maximum OD_{540} and yield of all conditions tested.

It should be noted that, for cultures in which methanol was continuously fed, continuous airflow led to higher OD_{540} compared to pulse airflow (Continuous feed run FB5 and FB6 vs. run FB4; Figure 4.3 and Table 4. 3. The best strategy tested for aeration in fed-batch was then identified to be continuous airflow after the initial batch step.

Another observation was that pH increased in all fed-batch operations (Table 4.3), which suggests *M. album* BG8 may excrete a basic metabolite to its surrounding medium.

4.5 Discussion

In this study batch, sequential batch and fed-batch operation for *M. album* BG8 cultures were investigated with the aim to improve the bioconversion of methanol to biomass and, eventually, value-added products at larger scale.

The best strategy for batch operation involved the initiation of the run with 50 mM methanol, aeration for 20 min prior to inoculation (Table 4.1, Figure 4.1). This methanol concentration is close to the initial methanol concentration of 53 mM used in a study of fed-batch operation with the methylotroph *M. extorquens* AM1 (Bourque et al., 1992). In that study this

value was determined by plotting growth rate against initial methanol concentration and using Luong's model (Luong, 1987). It was however higher than the maximum methanol concentration observed for the methanotroph *M. buryatense* 5GB1C grown in bioreactors, where strong growth inhibition was observed at initial methanol concentration of 15 mM or above (Tan et al. 2023). Although in other studies, no growth inhibition was observed in other strains of the same species at much higher methanol concentration; *M. buryatense* 5GB1 at 125 mM in a batch operation and *M. buryatense* 5G up to 1 M in shake flask culture(Gilman et al., 2015; Eshinimaev et al., 2002).

While batch operation has some limitations for methanol bioconversion due to lower final biomass and long lag phase, it can provide valuable information on the effective range of initial methanol concentration and efficient aeration strategies. It also serves as the first step of both sequential batch or fed-batch operation, and it is thus important to determine the conditions for effective batch operation.

The demonstration of sequential batch operation of *M. album* BG8, with a clear reduction in lag phase, bodes well for future bioconversion. Based on this result it can be concluded that a growth cycle can be completed within a 24-h window, which is much shorter than a batch operation. In this study, up to three sequential batch cycles were tested, but this can be extended to a larger number of cycles. For example, sequential batch operation with high numbers of cycle from 4 to up to 17 cycles has been tasted in other bacteria, such as cyanobacteria (Radmann et al., 2007), *Streptococcus zooepidemicus* (Huang et al., 2008), thermophilic hydrogen producing bacteria (Liu et al., 2008), *Rhodobacter sphaeroides* S10 (Pattanamanee et al., 2012). Thus in this mode of operation a productive culture can be maintained for a longer period of time compared to a batch operation. After the first batch the broth from the previous batch acts as

inoculum\, only fresh medium is required for the next set of cycles. Hence time and labor are saved associated with inoculum preparation. These are the main advantages of sequential batch over the batch reaction. These advantages of this operation has been argued in multiple bacterial culture hyluronate production in *Streptococcus zooepidemicus, Acetobactor xylinum* subsp. sucrofermentans BPR3001A culture to make bacterial cellulose (Naritomi et al., 2002)

While enabling higher overall productivity, sequential batch operation alone is plagued by the same limitations in biomass yield as traditional batch operation (e.g. the max OD_{540} ~ 1.5 obtained in this study (Table 4.2) was similar to batch results). In this context, fed-batch operation can come in play. Results obtained with different methanol addition (pulsed, continuous at steady rate, and continuous at increasing rate) and aeration (pulsed and continuous) strategies helped establish processing conditions for enhanced biomass production. Experiments showed that continuous aeration was preferable (Table 4.3 and Figure 4.3) to the closed system operation, and both pulse and continuous feeding of methanol led to high biomass yield. The need for continuous aeration could be explained by the increased oxygen requirement linked to the increased addition of the carbon source, methanol, which must be oxidized to generate biomass. In addition there may be increased oxygen demand due to more biomass being present. Of the three different methanol feeding strategies tested, continuous feeding at increasing addition rate (FB6) – from 20 mmol/24 h to 60 mmol/24 h – led to the highest biomass yield. The maximum OD_{540} reached in this condition (16.56) was comparable to that reported in studies investigating fed-batch for *M. extorquens* AM1 (Bourque et al., 1992; Zhu et al., 2016). Similarly, the highest biomass yield of 149 $OD_{540} \cdot mol^{-1}$ was much higher than yield of 16 $OD_{540} \cdot mol^{-1}$ obtained in fed-batch operation of another widely used methanotroph *M.*

trichosporium OB3B (Dubencovs et al., 2022). This firmly establishes the fact that *M. album* BG8 has a high potential for methanol bioconversion, especially under fed-batch operation.

An interesting observation from fed-batch operation growth curves (Figure 4.3) was that the OD_{540} of the culture undergoing continuous airflow remained the same after reaching its maximum, regardless of the methanol feeding strategy used (Figure 4.3). One possible reason for this could be the increasing oxygen demand as biomass increases, leading to reductions in dissolved oxygen in the medium. For example, in batch cultures of *M. buryatense* 5GB1, dissolved oxygen decreased with increasing OD (Gilman et al., 2015). A similar situation can arise with *M. album* BG8, especially under fed-batch operation. Oxygen is crucial for the growth of methanotrophs as it is directly involved in methane and methanol oxidation and the central carbon metabolism (Hanson & Hanson, 1996). Another possibility that could explain the stagnation of OD_{540} could be a buildup of unused methanol in the medium. Methanol can be toxic to methanotrophs at high concentration especially Alpha-MOB (Tan et al., 2023; Tays et al., 2018; Van Dijken & Harder, 1975). In future fed-batch studies both methanol concentration and DO of the media should be monitored to get a more complete understanding of the impact of the mode of operation on growth.

The increase in pH observed during fed-batch operation was unexpected since methanotrophs typically release formate, an organic acid that decreases pH, when grown on methanol. Formate release from *M.buryatense* 5GB1 and 5G undergoing Batch (Gilman et al., 2015) operation and in shale flask cultures(Eshinimaev et al., 2002) respectively have been reported earlier. While formate production has not been previously reported in *M. album* BG8 cultures, the transcriptomic study of this organism with different carbon and nitrogen sources (Sugden et al., 2021) has shown that glutathione-dependent formaldehyde detoxification to CO_2

is upregulated when methanol is the carbon source, and one of the intermediate of that pathway is formate. Hence, when exposed to high methanol concentration, this strain may release formate. Yet the opposite was observed in the study at hand, suggesting the strain released some basic metabolite over the bioreactor operation. This needs to be explored further to establish the actual source and mechanism for this observation. In *M. buryatense* 5GB1 cultures, glycogen is reported as a carbon sink; a potential analogous response could take place in *M. album* BG8. The carbon sink is not available as a product and hence undesirable for developing strains for commercial purpose.

Fed-batch operation has shown to be useful to improve titer of products in methanotrophs. In *M. trichosporium* OB3B cadaverine titer was improved 6-fold in a fed-batch compared to flask culture (Nguyen et al., 2020). In *Methylomonas* sp. DH-1, a 1.5-fold increase in succinate titer was obtained using fed-batch operation compared to flask culture (Nguyen et al., 2019). Similarly, the fed-batch strategy developed in this study could be applied to a recombinant *M. album* BG8 containing heterologous genes for generating a product of interest with the aim of improving product titer.

With *M. album* BG8 showing high biomass production, the biomass itself could be explored for the production of single cell protein (SCP) from methanol using the fed-batch strategy developed here. While SCP from this organism has not been explored SCP generated *Methylococcus capsulatus* (Bath) shows promise as animal feedstock for pigs, broiler chickens, mink, Atlantic salmon, rainbow trout, and Atlantic halibut (Øverland et al., 2010). One of the requirements of a good SCP is lower nucleic acid content and in biomass obtained from methanotrophic consortium its content was found to be much lower than maximum permissible dose (Kuźniar et al., 2019). In addition, this biomass was found to be rich in essential minerals

such as iron, magnesium and potassium. Analysis of the amino acid profile of combined biomass of *Methyloparacoccus murrelli* LMG 27482 with hydrogen oxidizing bacteria *Cupriavidus necator* LMG 1201 was found to be better than soybean to cover need of an adult human (Kerckhof et al., 2021). *M. album* BG8 biomass can be further analyzed for its protein and nucleic acid content to determine its suitability as animal feed in future.

4.6 Conclusion

In this study, conditions for batch, sequential batch and fed-batch operation of *M. album* BG8, a potential methanotrophic strain for industrial methanol bioconversion, have been investigated. [Batch was used to establish conditions such as aeration protocol, initial methanol concentration] Sequential batch operation was demonstrated up to three cycles. This operation could be useful to maintain a productive culture for a long period of time thereby reducing downtime. High amounts of biomass could be reached using the fed-batch operation within 96 h. In the fed-batch process using the best condition of continuous airflow and increasing methanol addition, biomass was increased almost 15 times compared to batch operation. The optical density obtained was similar to that of *M.extorquens* AM1, which has been explored widely for methanol bioconversion. All these outcomes of different bioreactor operation explored in this study shows a strong potential of *M. album* BG8 for methanol bioconversion]

4.7 Reference

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Chapter 5: Toxicity of isoprenoids on methanotrophic bacteria

5.1 Abstract

Isoprenoids can be used for a wide variety of applications, including high performance jet fuels. Methane and methanol can be used as inexpensive and readily available feedstocks for the production of isoprenoids by methanotrophic bacteria. Prior research for the microbial production of isoprenoids showed that these molecules have toxic effects on microbes, including *E.coli*, yeast, and methanotrophs. Previous studies on methanotrophs showed rate of methane oxidation decreased in presence of isoprenoids. Neither their effect on biomass production nor on cell viability were determined. In this thesis chapter, six isoprenoid molecules that are of interest as biofuel precursors – α-pinene (+), α-pinene (-), β-pinene, D-limonene, sabinene, and farnesene – were selected to examine their toxicity on the growth and activity of methanotrophic bacteria. Two alphaproteobacterial methanotrophs, *Methylocystis* sp. WRRC1 and *Methylosinus trichosporium* OB3b, and two gammaproteobacterial methanotrophs, *Methylomicrobium album* BG8 and *Methylotuvimicrobium alcaliphilum* 20Z were compared for their physiological responses to isoprenoid exposure. The three pinene isomers were the least toxic to the growth and survival of methanotrophs, whereas sabinene and D-limonene were the most toxic. Farnesene addition caused floccule formation, preventing analysis of microbial growth rates through optical density measurements. *M.album* BG8 and *M.trichosporium* OB3b tolerated the highest concentrations of pinene among the strains tested. The addition of an n-dodecane overlayer in microbial cultures containing isoprenoid precursor molecules led to a higher tolerance and the reduction of lag phase for all methanotroph strains tested. Together, the data

suggest that application of an n-dodecane overlayer will facilitate industrial production of isoprenoid molecules by methanotrophic bacteria.

5.2 Introduction

The aviation industry is one of the most important facilitators of modern civilization, while also contributing significantly to greenhouse gas emissions (ICAO,2016). Aviation has a high growth potential that will lead to four times more GHG emissions by 2050 than in 2015 if petroleum-based conventional jetfuel (CJF) continues to be used (Doliente et al. 2020). Fuels derived from biomass, often referred to as sustainable aviation fuel (SAF), offer a short-term solution as they can substitute for CJF without changing current technologies as long as the fuels have a low freezing point and high energy density (Table 4.1). SAF derived from biomass offers a lower overall carbon footprint during production, although the emission profile from combustion of both biomass-derived and CJF fuels is similar. The freezing point and energy density of isoprenoid-based fuels makes them favorable as aviation fuels (Table 2.1). Farnesene, has already been approved for a 10% blend with CJF (Holladay et al. 2020) and limonene has been shown as a possible drop-in replacement (Chuck and Donnelly 2014). Pinene and limonene can be chemically upgraded through dimerization, hydrogenation and cyclopropanation to improve their density and volumetric net heat of combustion (NHOC), thereby making them useful as high-performance aviation, missile or rocket fuels (Meylemans et al. 2012, Woodroffe et al. 2021, Walls and Rios-Solis 2020).

Production of isoprenoid-based fuels has been reported in microbial hosts such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Walls and Rios-Solis 2020, Ren et al. 2020, Cao et al. 2014). While there are multiple challenges in developing microbial

hosts for industrial production of isoprenoids, toxicity is a primary hindrance. The isoprenoids shown in Table 4.1, barring farnesene, show high levels of toxicity towards *E. coli* and *S. cerevisiae* (Liu et al. 2022, Brennan et al. 2012, Dunlop et al. 2011). Farnesene has been reported to be toxic to *Synechocystis* sp PCC6803 (Hellier et al., 2013). The mechanism of pinene and limonene toxicity is likely the disruption of the lipid membrane of *E. coli* (Liu et al. 2022), and the disruption of both cell membrane and organelle membranes in *S. cerevisiae* (Brennan et al. 2012). While isoprenoid toxicity in methanotrophs has not been studied in the context of industrial bioproduction, their effects on methanotrophs in pure cultures (Amaral et al. 1998) and in soils (Maurer et al. 2008) has been studied. In those studies, α-pinene and limonene negatively affected methane oxidation by *M. trichosporium* OB3B, and α-pinene, β-pinene and limonene negatively affected methane oxidation in soil.

Methanotrophs can be used to produce isoprenoid precursor molecules from methane and/or methanol. Methane is available from a number of sources, including landfill waste, the oil and gas industry and possibly through atmospheric capture (Alvarez et al. 2018, Holmes and Smith 2016, Zhou et al. 2020). Similarly, methanol is available from plant sources, conversion of methane, or hydrogenation of atmospheric CO_2 (Pfeifenschneider et al., 2017, Zhang et al. 2021)

In this thesis chapter, the toxicity of some isoprenoids suitable as SAF precursors against methanotrophic bacteria will be determined by assessing their impact on the growth of two alphaproteobacterial and two gammaproteobacterial methanotrophs, with and without an overlayer of n-dodecane acting as an isoprenoid sink. Furthermore, the effect of isoprenoid precursor molecules on growth, biomass production, methane oxidation and viability of the two strains that showed the highest resistance to isoprenoid toxicity was examined in detail. This

information is useful for selecting methanotrophs and appropriate isoprenoid target molecules for industrial bioproduction from low-cost single-carbon feedstocks.

5.3 Materials and Methods

5.3.1 Bacterial strains and cultivation

For this study *M. album* BG8, *Methylocystis* sp. WRRC1 and *M. trichosporium* OB3B were grown in nitrate mineral salts medium (NMS) (Whittenbury et al; 1970) containing 10 mM potassium nitrate as nitrogen source and buffered with 15 mL/L phosphate buffer pH 6.8 (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) with methane (25% v/v gas headspace) as the carbon source. For *M. alcaliphilum* 20Z, a modified NMS medium containing 10 mM potassium nitrate as nitrogen source, 15g/L sodium chloride, and buffered with 20 mL/L phosphate buffer pH 6.2 (5.44 g/L KH_2PO_4, 8.96 g/L Na_2HPO_4), 45 mL/L of 1 M $NaHCO_3$, and 5 ml of 1 M of Na_2CO_3 carbonate was used to accommodate its growth at high pH and salinity (Ojala et al., 2011). For regular maintenance strains were grown in 100 ml media in 250 ml Wheaton bottles. To add methane, 50 ml of gas headspace was removed from the culture bottle and 60 ml of methane was injected through a 0.22-mm filter-fitted syringe.

5.3.2 Determination of isoprenoid LD_{80}, LD_{50} and LD_{20}

For isoprenoid toxicity assays, 5% inoculum of strains grown in Wheaton bottles was added to 10 ml NMS medium into 35-ml glass serum vials. Six types of isoprenoids (Figure 5.1) were purchased from Sigma (Sabinene purity >75 %, rest were >98%). Those were added starting at 0.03 % v/v to max 1 % v/v or till lethal concentration is determined whichever is the lowest . For reporting the concentration in tables or for plotting optical density vs concentration

curve the percentage concentration was converted to mg/ml. Looking at the isoprenoid concentration reported in literature (Table 2.4) the highest values reported were between 8-10 mg/ml corresponding to ~ 1 % v/v concentration. This was our rationale for keeping maximum concentration tasted at 1 % v/v. 7 ml methane was injected through a 0.22-mm filter-fitted syringe into the gas headspace to achieve a 25% v/v headspace concentration. The control culture was inoculated without addition of isoprenoid, and the negative controls were uninoculated. Cultures were grown at 30° C with shaking at 200 rpm in an incubator shaker (New Brunswick G10). Growth was assessed at 96 h using plate reader at OD_{540}. This time-point was selected since since at that point untreated cultures of all four strains reach late stationary phase (Figure. 5.2) To determine growth inhibition, the ratio of OD_{540} with isoprenoid added to the OD_{540} without isoprenoid added for each culture or normalized OD_{540} was plotted against each tested isoprenoid concentration as described before. From these plots the LD_{80}, LD_{50} and LD_{20} values were obtained for each culture and each isoprenoid. These values were assessed as following:

- LD_{80}: Concentration of isoprenoid at which normalized OD_{540} is below 0.2. In the graph it is the point of intersection between the normalized OD_{540} vs concentration plot and normalized OD_{540} = 0.2 line.
- LD_{50}: Concentration of isoprenoid at which normalized OD_{540} is below 0.5. In the graph it is the point of intersection between the normalized OD_{540} vs concentration plot and normalized OD_{540} = 0.5 line.
- LD_{20}: Concentration of isoprenoid at which normalized OD_{540} is below 0.8. In the graph it is the point of intersection between the normalized OD_{540} vs concentration plot and normalized OD_{540} = 0.8 line.

The same set of experiments was repeated for cultures containing a 2 ml n-dodecane (Thermo Scientific) overlay on 8 ml of NMS medium to keep 10 ml total volume of culture with the same amount of methane.

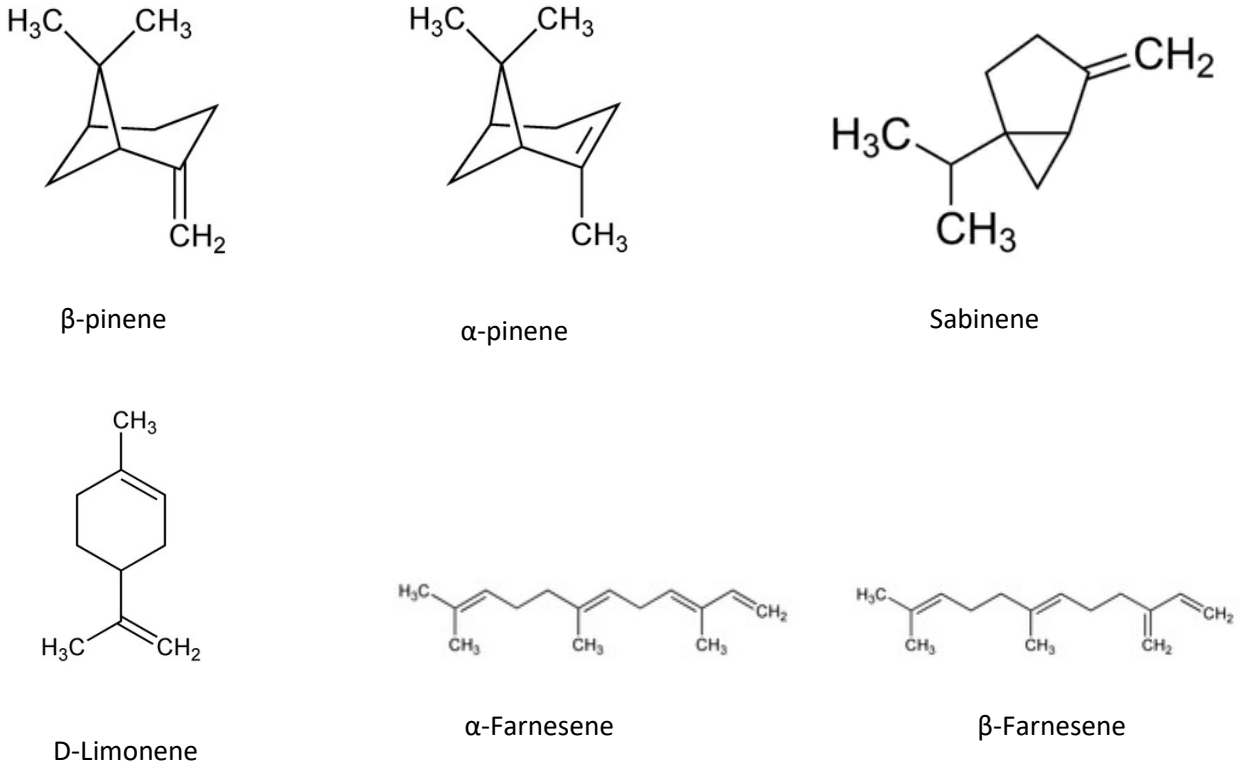

Figure 5.1 Structure of isoprenoids used in this study. The farnesene used was a mixture of both α and β isomers. (Brennan et al, 2012; Amaral et al., 1998; You et al., 2017)

5.3.3 Bacterial viability in the presence of isoprenoid precursor molecules

Methanotroph cultures (10 ml) grown in the presence of isoprenoid precursor molecules as described above were collected by filtration (0.22-µm sterile membrane filter, PALL) and used as inoculum to determine cell viability. The filter containing the collected cells was transferred to a sterile vial (35 ml) containing 10 ml NMS medium. The positive control contained 0.5 ml culture from the prior experiment's positive control and 10 ml additional NMS, which was then filtered and the filter paper was added to a sterile vial containing 10 ml NMS media. The biomass attached to the filter paper was the inoculum for the assay. The negative control contained 10 ml of uninoculated filtered NMS medium, and the filter was transferred to a vial with 10 ml NMS medium. Each vial received 7 ml methane injected through a 0.22-mm filter-fitted syringe into the headspace. Cultures were grown at 30° C with shaking at 200 rpm. Once growth was initiated (as determined by visualization), the OD_{540} was measured every 12 h as described earlier in 5.3.1 until achieving stationary phase or 96 h had elapsed. No growth at 96 h suggested that the inoculum was not viable. The time point of growth initiation was put down as lag phase. Growth rate was determined from the following formula using the OD_{540} of two successive data point in the linear range of exponential growth.

$$Growth\ rate = 2.303 * \frac{(\log_{10} OD_{540}^{t2} - \log_{10} OD_{540}^{t1})}{t_2 - t_1}$$

where OD_{540}^{t2} is the optical density at 540 nm at time 2 (in h) and OD_{540}^{t1} is the OD_{540} at time 1.

5.3.4 Methane oxidation assay

Strains were inoculated into 200 ml of NMS media in 500-ml Wheaton bottles sealed with caps inlaid with rubber septa, as described earlier. 100 ml of air was removed and 120 ml of methane was injected through a 0.22-µm filter-fitted syringe to achieve 40% v/v methane in the

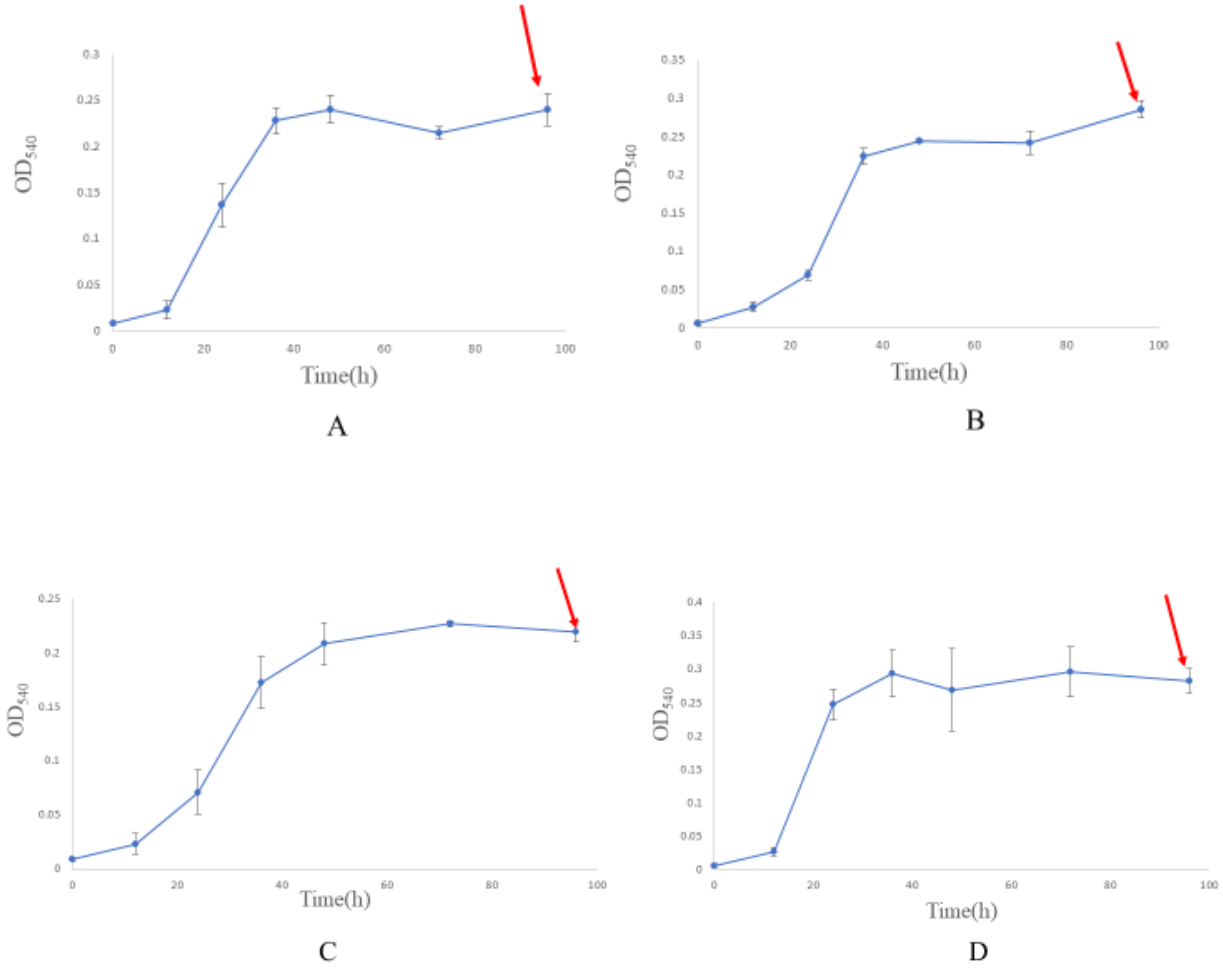

Figure. 5.2. Growth curves of four methanotrophic strains used in this study: (A) *M. album* BG8, (B) *Methylocystis* sp WRRC1, (C) *M. trichosporium* OB3B, and (D) *M. alcaliphilum* 20Z. Cultures were grown in 35-ml butyl rubber capped serum vials with 10 ml NMS media and methane at 30% v/v as the carbon source. Data represented from n=3. Arrows pointing at the biomass collection for toxicity assay.

gas headspace. Using higher methane led to more biomass production for use in this assay. Cultures were grown at 30° C with shaking at 200 rpm to achieve late log phase (36-48 h). Cells were collected by filtration (0.22 µm membrane filter, non-sterile, Sigma). For *M.album* BG8, cells were concentrated 25X while for *M. trichosporium* OB3b, cells were concentrated 100X. Cells were enumerated using a Helber Bacteria Counting Chamber (Hawksley). As negative control, cells were heat killed by transferring 2 ml of concentrated cells in microcentrifuge tubes and then incubated for 1 h in a 90℃ water bath (Fisherbrand). 2 ml of concentrated cells were added to 15-ml butyl rubber capped serum vials. 3 ml methane was added through a 0.22-mm filter-fitted syringe and isoprenoid was added to the appropriate concentration to be tested. The vials were kept at room temperature. Methane and oxygen concentrations were measured at 0, 2, 4, 6, and 8 h by Gas chromatography thermal conductivity detector (Shimadzu, Porapak Q column). The injection temperature was kept at 120 °C while that of the column at 80° C. 100 µ l headspace gas was collected using a Hamilton 500 µl syringe fitted with 22-gauge needle and was injected in the GC. The concentration of each gas was determined by generating standard curves of known concentrations using pure methane or oxygen (Praxair). Amounts of methane and oxygen in the gas headspace of each culture were plotted against time. The slope was divided by the cell count for each culture to determine the rate of methane and oxygen consumption determined as mole per h per cell.

5.3.5 Growth of methanotrophs in the presence of minimal toxic concentration of isoprenoid precursor molecules

Methanotroph cultures were inoculated (5% v/v) into 15 ml NMS media in 70-ml septum-capped vials, as described above. 15 ml methane was added through a 0.22-mm filter-

fitted syringe to achieve 25% v/v methane in the gas headspace. Isoprenoid precursor molecules were added at the minimal toxic concentration as determined in the initial toxicity assay. Cultures were grown at 30° C with shaking at 200 rpm. The OD_{540} was measured every 12 h up to stationary phase. The OD_{540} was plotted against time; lag phase and growth rate were determined as stated in section 5.3.3.

5.4 Results

5.4.1 Relative toxicity of isoprenoid precursor molecules to methanotrophic bacteria

Determination of different lethal doses for each isoprenoid precursor against each of the four methanotroph strains was the first round of screening in this study. The toxicity plot of each isoprenoids barring farnesene is shown in Figure 5.3. Due to flocculation in the presence of farnesene, the LD values could not be determined. The LD_{80}, LD_{50} and LD_{20} are determined from the plot as described in section 5.3.2 and are tabulated in Table 5.1. The lower the value of LDs the higher the toxic effect of isoprenoids. Specifically, a low LD_{80} would suggest severe toxicity. The opposite is true for LD_{20} where a high value would suggest low toxic effect. Sabinene was the most toxic as the LD_{80} of all methanotrophs for sabinene is very low at 0.21 mg/ml (Table 5.1). While both isomers of α-pinene were the least toxic to methanotrophs as evident from their high LD_{80} values (> 1 mg/ml) for all methanotrophs. Among the methanotroph strains, *M. album* BG8 and *M. trichosporium* OB3b were the most tolerant towards isoprenoids and had the highest LD values (Table 5.1 Specifically, with isomers of α-pinene no lethal effect was observed for *M. trichosporium* OB3b and *M. album* BG8 and. Both had higher LD_{50} values for β-pinene (1.68

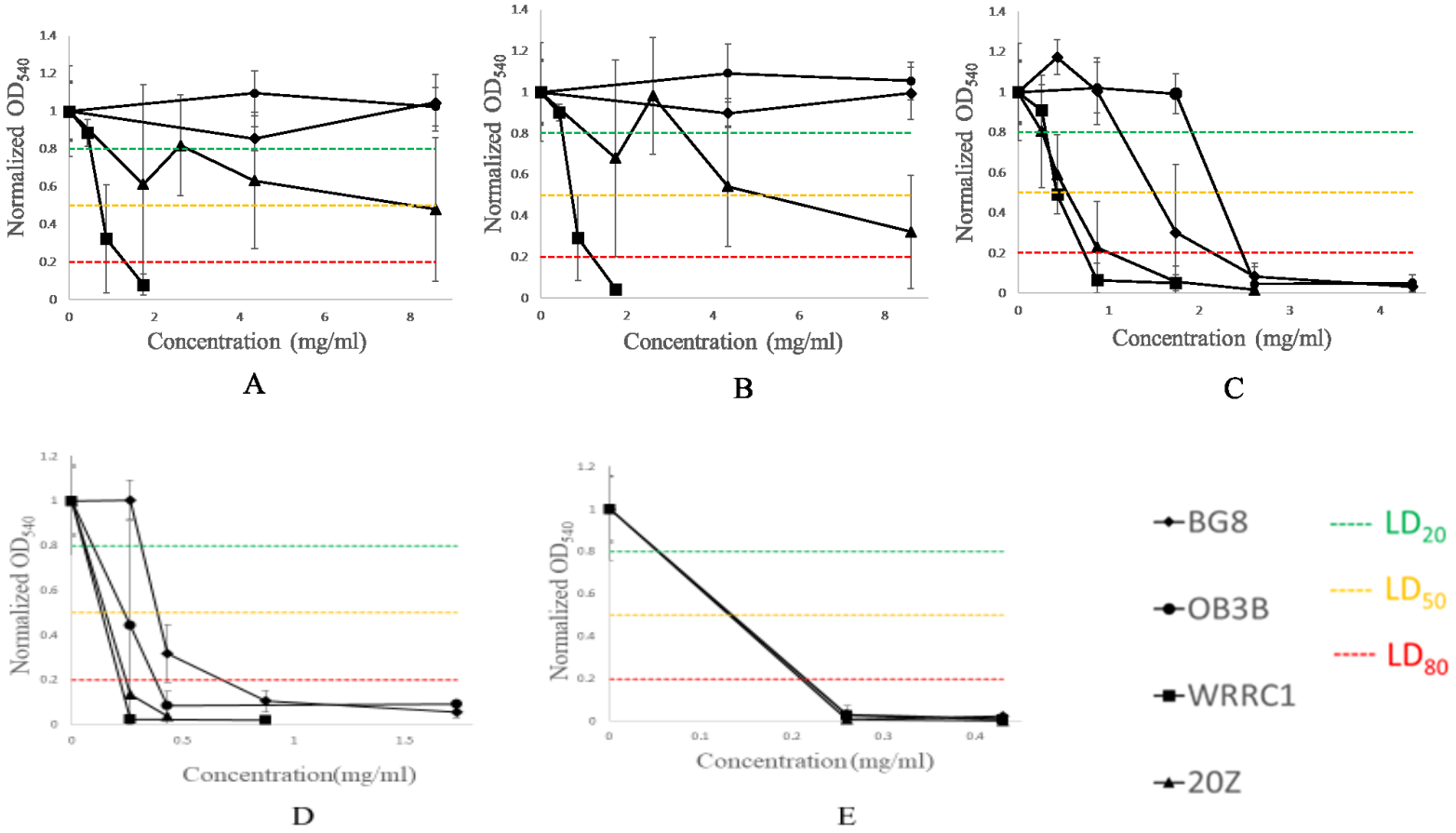

Figure. 5.3. Toxic effect of isoprenoids on methanotrophic bacteria with increasing concentrations shown with plot of normalized OD540 after 96 h of growth vs isoprenoid concentration (mg/ml) (A) α-pinene (+), () α-pinene (-), (C) β-pinene, (D) D-Limonene, (E) Sabinene. All cultures were grown with 25 % v/v methane as carbon source with 10 ml respective media. The LD80, LD50, LD20 lines in each graph corresponds to normalized OD540 of 0.2, 0.5, 0.8 respectively. Data showed is average of n=9-12 replicates.

Table 5.1. Estimated LD_{80}, LD_{50} and LD_{20} values for isoprenoid toxicity on four methanotrophic strains. Values were determined from the graphs shown in Fig. 5.3. NT stands for not toxic as normalized OD_{540} did not go below even 0.8 in these cases. BR stands for beyond range since in these cases only a particular lethal dose could not be determined as it was beyond the range of concentration tasted here.

Isoprenoid		BG8 (mg/ml)	20Z (mg/ml)	WRRC1 (mg/ml)	OB3B (mg/ml)
α-Pinene(+)	LD_{20}		2.78	0.5	
	LD_{50}	NT	8.02	0.73	NT
	LD_{80}		BR	1.31	
α-Pinene(-)	LD_{20}		3.33	0.5	
	LD_{50}	NT	5.16	0.73	NT
	LD_{80}		BR	1.31	
β-Pinene(-)	LD_{20}	1.12	0.17	0.23	1.92
	LD_{50}	1.68	0.73	0.49	2.19
	LD_{80}	2.24	1.28	0.76	2.47
Sabinene	LD_{20}	0.05	0.05	0.05	0.05
	LD_{50}	0.13	0.13	0.13	0.13
	LD_{80}	0.21	0.21	0.21	0.21
D-Limonene(+)	LD_{20}	0.31	0.06	0.05	0.19
	LD_{50}	0.38	0.15	0.13	0.33
	LD_{80}	0.67	0.24	0.21	0.47

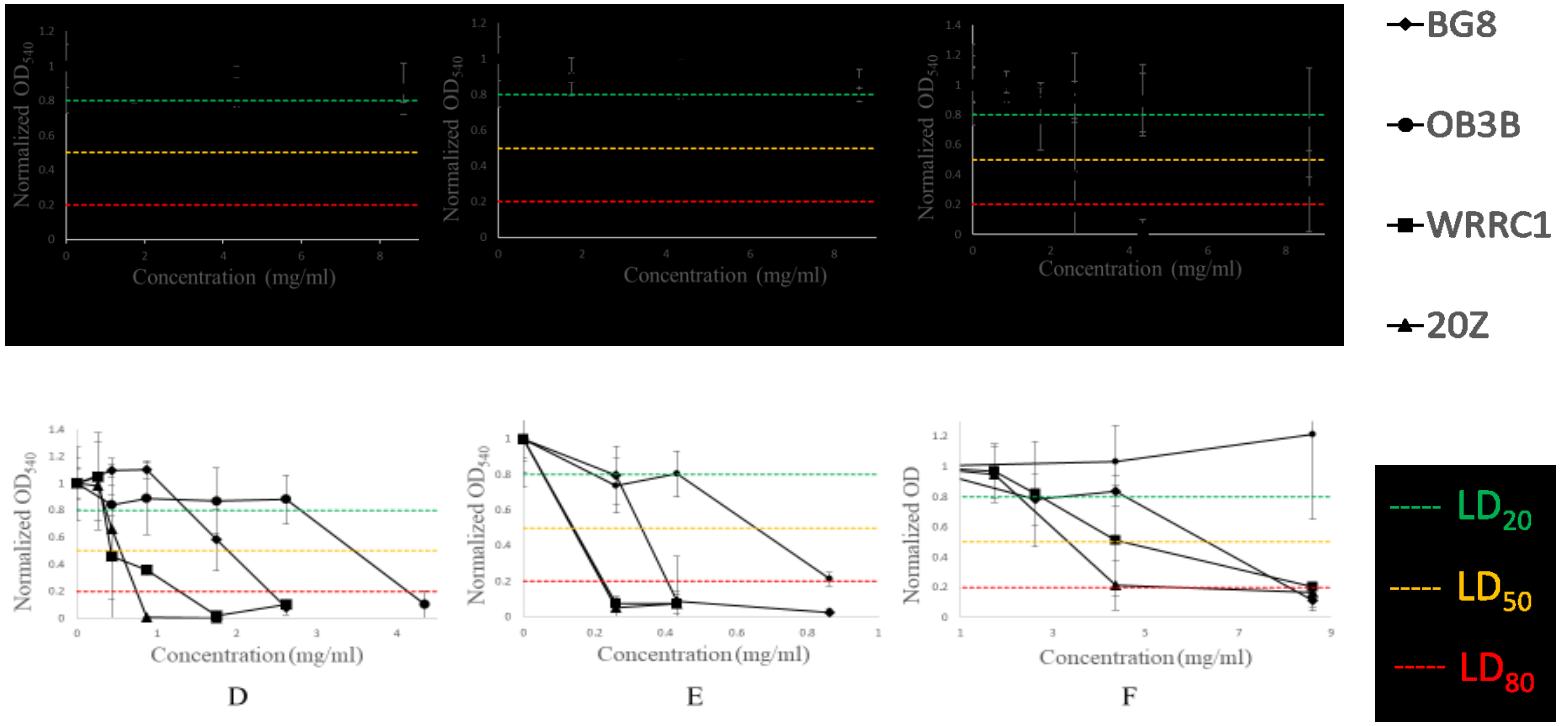

Figure 5.4. Toxic effect of isoprenoids on methanotrophic bacteria in presence of n-dodecane with increasing concentrations shown with plot of normalized OD540 after 96 h of growth vs isoprenoid concentration (mg/ml)) (A) α-pinene (+), (B) α-pinene (-), (C) β-pinene (D) D-Limonene, (E) Sabinene, (F) Farnesene. Culture was grown with 25 % v/v methane, 8 ml respective media and 2 ml n-dodecane top layer. LD80, LD50, LD20 lines in each graph corresponds to OD540 ratio of 0.2, 0.5, 0.8 respectively. Cross section of plot and these lines were determined and tabulated as LD80, LD50, LD20 values in Table 5.1. Data showed is average of n=9-12 replicates.

Table 5.2. Estimated LD_{80}, LD_{50} and LD_{20} values for isoprenoid toxicity on four methanotrophic strains in presence of n-dodecane. Values were determined from the graphs shown in Fig. 5.4. NT stands for not toxic as normalized OD_{540} did not go below even 0.8 in these cases. BR stands for beyond range since in these cases only a particular lethal dose could not be determined as it was beyond the range of concentration tasted here.

Isoprenoid		BG8 (mg/ml)	20Z (mg/ml)	WRRC1 (mg/ml)	OB3B (mg/ml)
α-Pinene(+)	LD_{20}				
	LD_{50}	NA	NT	NT	NA
	LD_{80}				
α-Pinene(-)	LD_{20}				
	LD_{50}	NA	NT	NT	NA
	LD_{80}				
β-Pinene(-)	LD_{20}	5.02	1,1	1.84	6.92
	LD_{50}	7.12	2.41	2.22	BR
	LD_{80}	BR	3.72	2.6	
Sabinene	LD_{20}	0.26	0.05	0.06	0.43
	LD_{50}	0.33	0.14	0.14	0.65
	LD_{80}	0.4	0.22	0.22	0.87
D-Limonene(+)	LD_{20}	1.38	0.34	0.33	2.79
	LD_{50}	1.89	0.53	0.42	3.46
	LD_{80}	2.4	0.72	1.27	4.13
Farnesene	LD_{20}	4.58	2.27	2.72	
	LD_{50}	6.34	3.34	4.42	NT
	LD_{80}	8.1	4.44	8.66	

and 2.19 mg/ml respectively) and D-limonene (0.38 and 0.33 mg/ml respectively) compared to *Methylocystis* sp. WRRC1 and *M. alcaliphilum* 20Z. In the case of *M. alcaliphilum* 20Z growth

in the presence of the two α-pinene isomers, the growth vs concentration line intersect the LD_{20} line twice. The higher value of LD_{20} was reported in Table 5.1

Similar experiments were conducted with a 2-ml n-dodecane overlay in the cultures (Figure 5.4). In a similar fashion LD_{80}, LD_{50} and LD_{20} were determined and tabulated in Table 5.2 In the presence of n-dodecane, higher LD_{80}, LD_{50} and LD_{20} values were achieved for all four strains, with the highest value (lowest toxicity) observed for α-pinene (Fig. 5.4, Table 5.3). Since the prior experiment without n-dodecane showed that α-pinene was not toxic to *M. album* BG8 or *M. trichosporium* OB3Bb, this isoprenoid was omitted. Floccules that had formed with farnesene were not apparent with the n-dodecane overlay, which allowed the measurement of LD values. This result suggest n-dodecane can play a crucial role in isoprenoid engineering in methanotrophs owing to its ameliorating effect.

This LD-based screening method was based on final biomass obtained after 96 h. This screening did show the extremely toxic nature of sabinene and limonene (Figure 5.3 and Table 5.1). Further questions still need to be answered. Even at non-inhibitory concentrations (< LD_{20})*I*soprenoids could negatively *a*ffect the strains through loss of viability, longer lag phase, *and* reduced rate of methane consumption. Th*is* could not be determined in th*ese* experiment*s*. Conversely strains may not grow in presence of isoprenoids at inhibitory concentration*s (*> LD_{80}*)* but could still be viable. The next set of experiments *we*re carried out to determine whether the isoprenoids tested led to such effects. Owing to their relatively lower toxicity, pinene isomers were selected to determine these effects on two strains *M. album* BG8 and *M. trichosporium* OB3*b* which showed the most tolerance towards these compounds. With α-pinene isomers 8.6 mg/ml concentrations was used for both strains. This value corresponds to 1% v/v, maximum

Table 5.3. Methane and oxygen consumption rate in presence of pinene isomers for *M.trichosporium* OB3b and *M.album* BG8. All the numbers are in nmole/h/10^10 cells. Data shown is average of n=6 samples

Sample	Methane consumption	Oxygen consumption
Methylosinus trichosporium OB3B		
Positive control	5.5 ± 0.5	6.9 ± 1.2
β-pinene 1.74 mg/ml (non toxic)	4.2± 0.2	4.8± 0.9
β-pinene 2.61 mg/ml (inhibitory)	4.1± 0.8	5.6± 0.5
α-Pinene(+) 8.6 mg/ml (non toxic)	4.2 ± 0.2	5.2 ± 1.1
α-Pinene(-) 8.6 mg/ml (inhibitory)	3.9 ± 0.3	5.1 ± 0.3
Methylomicrobium album BG8		
Positive control	28.1 ± 2.9	32.2 ± 4.1
β-pinene 0.87 mg/ml (non toxic)	19.2 ± 1.6	22.8 ± 4.1
β-pinene 2.61 mg/ml (inhibitory)	16.4± 3.2	18.8± 2.4
α-Pinene(+) 8.6 mg/ml (non toxic)	15.3 ± 1.7	18.1 ± 1.9
α-Pinene(-) 8.6 mg/ml (non toxic)	16.4± 1.8	20.5± 2.5

concentration tested in this study. For *M. album* BG8 with β-pinene(-), the highest non-lethal and lowest lethal concentrations tested were 0.87 mg/ml and 2.61 mg/ml , respectively (Figure 5.3).

For *M. trichosporium* OB3b with β-pinene(-), the highest non-lethal and lowest lethal concentrations tested were 1.74 mg/ml and 2.61 mg/ml , respectively (Figure 5.3).

5.4.2 Effect of pinene isomers on methane oxidation in M.trichosporium OB3b and *M.album* BG8

Methane and oxygen consumption in *M.trichosporium* OB3b and *M.album* BG8 cells at rest exposed to pinene isomers at concentrations listed in Table 5.3 and described in section 5.4.1. All three pinene isomers led to decreased methane and oxygen consumption in both strains, irrespective of their concentration. That suggest that pinene can affect methane consumption even at non toxic concentrations.

5.4.3 Cell viability of M.trichosporium OB3b and *M.album* BG8 *after exposure to pinene isomers*

M. album BG8 and *M. trichosporium* OB3b viability was assessed after exposing them to pinene isomers for 96 h. For both strains α-Pinene concentration was 8.6 mg/ml which was non-toxic (Figure 5.3). For *M. album* BG8 with β-pinene(-), the non toxic and lowest inhibitory concentrations were 0.87 mg/ml and 2.61 mg/ml respectively while for *M. trichosporium* OB3b with β-pinene(-), the non-lethal and lethal concentrations were 1.74 mg/ml and 2.61 mg/ml respectively. Neither strains could grow following their exposure to non toxic concentration of α-pinene isomers when collected and inoculated into fresh media (Table 5.4). In the case of β-pinene, *M. trichosporium* OB3b cells could grow following their exposure to inhibitory

Table 5.4. Determination of cell viability of *M.trichosporium* OB3b and *M.album* BG8 following exposure to pinene isomers. Strains were grown following exposure in 10 ml NMS media with 25 % v/v methane in headspace. Empty cells suggest no growth.

Isoprenoid treatment	Lag phase (h)	Growth rate (h^{-1})	Maximum optical density (OD_{540})
Methylosinus trichosporium OB3B			
No treatment	24	0.10±0.04	0.19±0.03
β-pinene 1.74 mg/ml	-	-	-
β-pinene 2.61 mg/ml	29±10	0.08±0.03	0.21±0.06
α-pinene (+) 8.6 mg/ml	-	-	-
α-pinene (-) 8.6 mg/ml	-	-	-
Methylomicrobium album BG8			
Control	12	0.07±0.05	0.27±0.04
β-pinene 0.87 mg/ml	28±9	0.08±0.02	0.27±0.01
β-pinene 2.61 mg/ml	30±6	0.07±0.03	0.28±0.03
α-pinene (+) 8.6 mg/ml	-	-	-
α-pinene (-) 8.6 mg/ml	-	-	-

Table 5.5. Growth assay of *M.trichosporium* OB3b and *M.album* BG8 at nonlethal pinene concentration with and with out n-dodecane overlay. Strains were grown with 25 % v/v methane in headspace as carbon source. Data is shown for n=3-6 samples

Isoprenoid treatment	Lag phase (h)	Growth rate (h^{-1})	Maximum optical density (OD_{540})
Methylosinus trichosporium OB3b			
No treatment	12	0.1 ± 0.02	0.22 ± 0.02
With n-dodecane overlay	24	0.11±0.01	0.19±0.03
β-pinene 1.74 mg/ml	60 ± 8	0.07±0.03	0.18 ± 0.02
β-pinene 1.74 mg/ml with n-dodecane	20 ± 5	0.09±0.01	0.13 ±0.02
α-pinene (+) 8.6 mg/ml	44±5	0.06 ± 0.01	0.18 ±0.01
α-pinene (+) 8.6 mg/ml with n-dodecane	20±5	0.08 ± 0.02	0.14 ± 0.01
α-pinene (-) 8.6 mg/ml	48	0.09 ± 0.01	0.21± 0.01
α-pinene (-) 8.6 mg/ml with n-dodecane	16±5	0.05 ± 0.01	0.13± 0.04
Methylomicrobium album BG8			
No treatment	13±4	0.07 ± 0.05	0.28 ± 0.02
With n-dodecane overlay	12	0.1±0.03	0.33±0.02
β-pinene 0.87 mg/ml	54 ± 8	0.06±0.02	0.25 ± 0.02

Isoprenoid treatment	Lag phase (h)	Growth rate (h^{-1})	Maximum optical density (OD_{540})
β-pinene 0.87 mg/ml with n-dodecane	19 ± 8	0.08±0.03	0.36 ±0.03
α-pinene (+) 8.6 mg/ml	36±11	0.1 ± 0.03	0.27 ±0.02
α-pinene (+) 8.6 mg/ml with n-dodecane	28 ± 11	0.1 ± 0.05	0.38 ± 0.06
α-pinene (-) 8.6 mg/ml	28 ± 5	0.08 ± 0.03	0.22 ± 0.03
α-pinene (-) 8.6 mg/ml with n-dodecane	16 ± 5	0.11 ± 0.04	0.39 ± 0.04

concentration while growth was not observed following exposure to non-toxic concentration. It is possible that the mechanism of β-pinene effecting cell growth and cell viability is different for this strain. The nature of the stress causing this behaviour was not determined in this study so this can not be explained further.

M. album BG8 could grow following their exposure to either inhibitory or non-toxic concentration of β- pinene (Table 5.4). When growth was observed following exposure, lag phase was longer but neither growth rate nor max OD_{540} was different compared to positive control (Table 5.4). The longer lag phase and loss of cell viability observed in this assay would suggest pinene isomers can cause cellular stress, irrespective of its concentration.

5.4.4 Growth of M.trichosporium OB3b and *M.album* BG8 *at non-lethal pinene concentration*

When exposed to non-toxic pinene concentrations, both strains grew to similar final biomass levels than non-treated cells (Figure 5.3). On the other hand, results from viability and methane oxidation assays (Table 5.3 and 5.4) suggest pinene can have detrimental effect even at

these non-toxic concentrations. Hence, final biomass cannot be the only parameter used to determine toxicity; other growth parameters might be useful indicators. So, both strains were grown using methane as carbon source in the presence of pinene isomers at non-toxic concentrations and growth was monitored to determine lag phase, growth rate and max OD_{540}. Growth was also monitored with an n-dodecane overlayer to determine its potentially beneficial effect.

Longer lag phase was observed in the presence of all pinene isomers (Table 5.5). The longer lag phase could be an effect of cells adapting themselves to pinene. Interestingly, the presence of n-dodecane shortened the lag phase, suggesting a beneficial effect on cell growth. The growth rate and maximum OD_{540} did not decrease between treated and non-treated samples, barring the *M.trichosporium* OB3b α-pinene samples grown with n-dodecane. Interestingly, with *M.album* BG8 non treated cells in presence of n-dodecane overlay had a slightly higher OD_{540} than cells without the overlay.

5.5. Discussion

Isoprenoids, and especially monoterpenoids, are often toxic to microorganisms (Moser and Pichler 2019, Liu et al. 2022). In studies with *E.coli,* the toxicity of pinene and sabinene were determined by plotting stationary phase biomass levels (as measured by Optical density at 600 nm) against isoprenoid concentration (Dunlop et al., 2011; Wu et al., 2020). A similar approach was applied here to determine toxicity of the selected isoprenoids towards methanotrophs. The lethal dosage was determined to establish the toxic effects of the isoprenoids tested (Figure 5.3, Table 5.1). The same experiment was replicated with n-dodecane as an overlayer to determine whether it could act as an isoprenoid sink, reducing the isoprenoid

concentration in the aqueous phase and thus benefiting biomass growth. Previous studies have shown that overlaying solvent in the growth medium of *S. cerevisiae* and *E. coli* cultures that are producing isoprenoids can overcome issues with product extraction and toxicity (Liu et al. 2022, Brennan et al. 2012). Using dibutyl phthalate as an organic overlay and extraction solvent, the MIC of D-limonene was increased to to 42.1 mg/ml (a 702-fold increase) in *S. cerevisiae* (Brennan et al. 2012). A dibutyl phthalate overlayer was also used to reach limonene titers of 3.2 mg/ml in *E.coli* by alleviating the toxicity (Rolf et al., 2020). The solvent n-dodecane has already been shown to be useful as an extraction solvent for sesquiterpenoid isoprenoids α-humulene and bisabolene in *M. alcaliphilum* 20Z (Nguyen et al. 2020, Nguyen et al. 2021). While A 20 % n-dodecane overlay led to alleviation of growth inhibition of *Synechocystis* sp. PCC6803 in the presence of farnesene (Hellier et al., 2013) Hence n-dodecane was explored to determine if it can alleviate toxicity in methanotrophs.

Sabinene was the most toxic to all methanotrophs with an LD_{80} of 0.21 mg/ml, improving marginally to an LD_{80} of 0.87 mg/ml against *M. trichosporium* OB3b in the presence of the n-dodecane overlay (Table 5.1 and Table 5.2). Reported production of sabinene by microorganisms has been as high as 2.65 mg/ml (Zhang et al. 2014). The toxicity of sabinene to methanotrophs suggests that it is not a proper candidate for bioproduction by this group of bacteria unless strain improvement can increase their tolerance (Wu et al. 2020). D-limonene was similarly toxic to *Methylocystis* sp. WRRC1 and *M. alcaliphilum* 20Z, and the presence of n-dodecane did not improve their growth or survival to an appropriate level for bioproduction (Ren et al., 2020). However, the two enantiomers of limonene and sabinene were not treated independently, and one might be less toxic than the other (Ren et al. 2020, Zhang et al. 2014, Wu et al. 2020, Brennan et al. 2012, Liu et al. 2022). Addition of farnesene, a sesquiterpenoid, to the cultures

caused flocculation, except when n-dodecane was added as an overlayer. With n-dodecane, farnesene was tolerated to high concentrations by both *M. trichosporium* OB3b and *M.,album* BG8. No lethal effect was observed for *M. trichosporium* OB3b with farnesene. While with *M.,album* BG8 LD_{50} was 6.34 mg/ml. This being said, flocculation could be helpful to promote for product recovery of 145arnesene in multiphase fermentation (Da Costa Basto et al. 2020). Since flocculation of methanotrophs is a poorly understood phenomenon, Farnesene was not deemed unsuitable as a product of interest.

Pinene isomers were found to be relatively less toxic based on LD values, and *M. trichosporium* OB3B and *M. album* BG8 showed the highest tolerance to these compounds. The initial LD_{20} values for pinene for *M. trichosporium* OB3B and *M. album* BG8, 1.12 mg/ml and 1.92 ml respectively, were comparable to titers of pinene produced heterologously by *E.coli* (Yang et al. 2013, Zhou et al. 2023). In the presence of n-dodecane, the tolerance for β-pinene vastly improved. Hence, the effects of pinene isomers on cell viability and methane oxidation were further studied.

In addition to decreased toxicity of the isoprenoids in presence of n-dodecane overlay, it is possible it could even improve growth of the bacteria. In case of *M.album* BG8 the final OD_{540} improved in presence of n-dodecane overlay (Table 5.5). There have been studies on *E.coli* which showed that presence of n-dodecane improved growth and bioproduction of value added chemicals(Ciobanu et al., 2020; Zhang et al., 2018). In those studies the better biomass and yield was attributed to higher oxygen availability to the microbe in presence of n-dodecane. While it is beyond the objective of this toxicity study this improved growth in presence can certianly be explored in future studies with *M. album* BG8.

Pinene isomers have been shown to inhibit methane consumption in resting cell assays in *M. trichosporium* OB3b at a concentration of 0.25 mg/ml, much lower than the values observed in this study (Amaral et al., 1998). In the present study, resting cell assays of both *M. trichosporium* OB3b and *M. album* BG8 showed decreases in methane consumption. Although the extent of inhibition was much lower in this study compared to the study by Amaral et al., where as much as 90 % inhibition was observed with α-pinene (-) at 0.24 mg/ml which was found to be non-toxic for all four strains tested (Table 5.1). Decreased methane consumption even at non-lethal concentrations suggests that methane consumption will decrease as the organism starts producing pinene.

Viability of bacterial cells would be defined as their ability to form progeny (Cangelosi & Meschke, 2014). If cells start becoming less viable it will be difficult to maintain long bioreactor operations (more than 100 h) reported in chapter 4. Cell viability loss has been reported with *S. cerevisiae* for the isoprenoid limonene (Brennan et al., 2012). Hence, effect on cell viability was determined in this study. In the present study, a protocol was developed for determining viability, whereby growth following exposure to isoprenoids was used as proof of cell viability. Using this protocol, it was determined that exposure to non-lethal pinene concentrations led to loss of cell viability (Table 5.4), as they were not able to re-grow when inoculated into fresh media. In addition, when grown in the presence of pinene at non-lethal concentration, lengthy lag phase was observed in both *M. trichosporium* OB3B and *M. album* BG8 cultures, compared to the untreated control. Longer lag phase usually suggests stress, such as cold stress leading to 3-4 fold increase in the lag phase of *Campylobacter* spp. (Lanzl et al., 2020). It can be concluded that cells were under stress even at non-lethal concentration of pinene. The presence of n-dodecane

substantially decreased the length of the lag phase observed with exposure to pinene isomers in both *M. trichosporium* OB3b and *M. album* BG8, suggesting an alleviation of toxicity and stress.

Usually for biomanufacturing engineered strains are grown in bioreactors. Pinene isomers may be suitable for production using *M.trichosporium* OB3B or *M. album* BG8, but only in batch production which is not always very efficient for getting high titer. N-dodecane can be added during batch operation to alleviate stress and allow the strains to tolerate higher amounts of pinene. More complex operation such as fed batch has been used to generate improved titer of product in methanotrophs such as cadaverine in *M. trichosporium* OB3B (Nguyen et al., 2020). But the combination of lower methane consumption, cellular stress, cell viability loss after 96 h exposure to pinene would suggest when pinene is being produced in long fed batch operation lasting 100 h or more, cells will start losing viability as more pinene is generated and released in the media. It is possible even with the addition of nutrients the bioreactor operation will crash in absence of sufficient number of viable cells. Hence these compounds cannot be produced in these operations. It can be argued that maybe β-pinene will be a good product to grow using *M. album* BG8 since the bacteria does not loose viability in its presence. Unfortunately, the pinene synthase generates pinene as a mix of α and β isomers. And same enzyme can lead to different isomer ratio in different hosts. This has been observed with expression of pinene synthase derived from *Abies grandis* in *E.coli* and *Deinococcus radiodurans* (Helalat et al., 2021*).* That rules out the approach of selective β-pinene production.

5.6. Conclusion

In conclusion it was determined that isoprenoids are toxic and negatively affect methanotrophic growth depending on the type of isoprenoid, its concentration, and the strain of

methanotroph involved. Pinene isomers allow for initial growth of *M. album* BG8 and *M. trichosporium* OB3b at industrially relevant concentrations, but it led to cell stress and viability loss. In addition, methane consumption was lowered in presence of pinene isomers. Economically feasible production of the isoprenoids tasted in this study using complex bioreactor operation will be difficult to achieve in methanotrophs due to these toxic effects. The possible role of n-dodecane in alleviating stress and facilitating pinene recovery from cultures could play an important role in industrial production of pinene isomers.

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Chapter 6: Summary, Conclusion, and future direction

6.1. Summary and conclusion

Methanotrophic bacteria are being widely explored for bioconversion of methane and methanol to value added chemicals. Towards that end, *M. album* BG8, a gammaproteobacterial methanotroph, was studied to determine its suitability for such microbial platform. Isoprenoids were selected as the chemical of interest due to their potential as ultraperformance fuel precursors.

In chapter 3, tools for heterologous gene expression in *M. album* BG8 were developed. Based on earlier studies done on methanotrophs (Henard et al., 2016; Kang-Yun et al., 2022; Ojala et al., 2011) a conjugation method using *E.coli* S17λpir was developed to transfer BHR plasmids pCAH01, pQSDP$_{MMA}$sfGFP and pBBR1MCS5 of IncP, IncQ and pBBR1 family into *M. album* BG8. The method was successful and *M. album* BG8 could propagate those plasmids. It was also shown *M. album* BG8 can propagate BHR from two families simultaneously when a strain containing both pQSDP$_{MMA}$sfGFP and pBBR1MCS5 was generated. PCN for all plasmids were determined which showed IncQ with the lowest copy number, pBBR1 highest and IncP in between. Three promoters were characterized using GFP for fine-tuned heterologous gene expression. These are the inducible promoter P$_{Tet}$ and the constitutive promoters P$_{MMA}$ and P$_{GAP.}$ Two expression vectors based on the IncP replicon and IncQ replicon were validated with expression of luciferase under the P$_{MMA}$ promoter. The luciferase expression data also suggested that protein expression is probably not related with PCN as the IncP and IncQ expression vectors showed similar expression levels.

Results from batch, sequential batch and fed batch operation in bioreactors were described in chapter 4. The results of batch operation suggested that *M. album* BG8 can tolerate up to 50 mM initial methanol concentration similar to the methylotroph, *M. extorquens* AM1, a strain widely explored for methanol bioconversion. The batch operation was associated with a long lag phase. Sequential batch operation was found to be useful in circumventing lag phase duration. Sequential batch operation can become a useful strategy for exploring production of a valuable, but highly toxic product, in *M. album* BG8. Providing air supply at the start of the operation rather than continuous supply was found to be effective. Fed batch operation was run using the batch condition as the first cycle. A continuous air supply at 70 ml/min and continuous methanol feeding at a rate of 20 mmol/24 h with rate of increase of 10 mmol every 24 h was the most suitable for reaching high density biomass over a short length of time. OD_{540} reached in these conditions were comparable to *M. extorquens* AM1 and *M. buryatense* 5GB1, two strains studied for their potential application in methanol bioconversion (Tan et al., 2023; Zhu et al., 2016).

The toxic effects of isoprenoids on methanotrophs were reported in chapter 5. The study included a first round of screening wherein lethal doses for isoprenoids were quantified based on their LD_{80} values. The pinene isomers -- β-pinene, α-pinene(+) and α-pinene (-) -- were found to be least toxic. The addition of n-dodecane to the toxicity screens increased the concentration of lethal doses for all isoprenoids, suggesting an ameliorating effect of n-dodecane. Furthermore, comparing across the different strains it was determined that *M. album* BG8 and *M. trichosporium* OB3b have the best tolerance to pinene isomers. In a second round of toxicity screening, the cell viability was determined for *M. album* BG8 and *M. trichosporium* OB3b after treatment with pinene isomers. Treatment with α-pinene isomers at non lethal concentration led

to cell viability loss in both strains, while the opposite was observed with β-pinene with no loss of cell viability even after treatment at the LD_{80} concentration. Currently, there is no way to predict which isomers will be produced by the pinene synthesizing enzyme, pinene synthase (Helalat et al., 2021). It was concluded that while pinene can be produced using these two strains, producing at high titer in a bioreactor might be difficult as accumulation of these compounds can reduce their viability, thereby causing a stable bioreactor to collapse.

With the genetic tools for heterologous protein expression, genes of isoprenoid biosynthesis pathways, such as isoprenoid synthases and GPPS synthase (Helalat et al., 2021), can be potentially engineered into *M. album* BG8. Pinene could be a potential compound for bioconversion from methane and methanol since *M. album* BG8 can tolerate it up to a certain concentration. Such recombinant strains can be grown in bioreactors using the fed-batch operation for maximum biomass production. However, the negative effect of pinene isomers on cell viability could limit a high titer during growth. Hence, with the information gathered in this thesis, further optimization and methods to ameliorate isoprenoid toxicity, such as inclusion of an n-dodecane overlay in the bioreactor, will be required for *M. album* BG8 to produce isoprenoids at high titer.

The results from this thesis are important in future biotechnological application of *M. album* BG8 and other methanotrophs. The tools for heterologous protein expression and bioreactor operation protocol can be utilized for isoprenoids and other chemicals of interest. Gammaproteobacterial methanotrophs have potential to produce other valuable compounds like lactic acid (Henard et al., 2016) and putrascine (Nguyen & Lee, 2019) among others. If found to be nontoxic, unlike the isoprenoids, these could be excellent targets for production.

6.2 Future directions

Results of this thesis can be explored further in following ways:

1) **Determining protein expression during bioreactor operation.** High biomass production in bioreactors may not necessarily match levels of heterologous gene expression from introduced plasmids. Gene expression studies in bioreactors could use *M. album* BG8 strains with the the luciferase reporter contruct to determine the relationship between growth and heterologous gene expression.
2) **Effect of dissolved oxygen in bioreactor operation**. Oxygen is necessary for growth of aerobic methanotrophs. In earlier studies with *M. buryatense* 5GB1 (Gilman et al., 2015), dissolved oxygen (DO) decreased as biomass increased. The change of DO during the *M. album* BG8 fed-batch run can be measured and strategies can be explored to increase DO to optimize biomass production.
3) **Bioreactor operation of *M. album* BG8 using different carbon and nitrogen sources**. *M. album* BG8 has the ability to assimilate both nitrate and ammonium as a nitrogen source and methane and methanol as a carbon source (Tays et al., 2018). Fed-batch operations should be tested to determine if more robust growth of *M. album* BG8 can be achieved with different combinations of carbon and nitrogen sources.
4) **Developing a strategy for circumventing toxicity**. While pinene isomers negatively affected cell viability, it could be explored if an n-dodecane overlay could prevent toxicity in bioreactors. N-dodecane has been shown to increase the lethal dose values of all the isoprenoids for all tested methanotroph strains. Alternatively, efflux pumps can be over-expressed in *M. album* BG8 as a potential mechanism to lower isoprenoid toxicity. Similar strategies has been useful in lowering pinene tolerance in *E.coli* (Dunlop et al., 2011).

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