Development of Processing Strategies to Enhance the Efficiency of the Growth of the Gammaproteobacterial Methanotroph *Methylomicrobium album* BG8

by

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Abstract

Methanotrophic bacteria can use different one-carbon compounds, including methane and methanol, two common industrial waste byproducts, as their main source of carbon and energy. Interestingly, they can convert these compounds into value-added products such as biofuels and bioplastics. Methanol is a common byproduct of the pulp and paper industry which is usually disposed of as waste. This leads to two issues: potential release in the environment can disturb and disrupt ecosystems, and the costs of disposal and loss of prospective revenues and economic concerns. Using methanotrophs, it is possible to produce high-value compounds from methanol while reducing its negative effects on the environment and the economic prospects of the industry. However, slow growth and low cell densities make the industrial implementation of methanotroph-based bioprocesses difficult. Increasing production and productivity is thus of utmost importance. To do so, bioprocessing strategies must be improved upon. In this study, different bioreactor operation strategies have been developed to improve the productivity for the growth of the gammaproteobacterial methanotroph *Methylomicrobium album* BG8 using methanol as the carbon source.

Copper is known to have a significant impact on the growth of methanotrophs feeding on methane through the regulation of the enzyme methane monooxygenase (MMO); however, its effect on *M. album* BG8 growing on methanol had not yet been determined. In this study, *M. album* BG8 was grown in batch cultures at a range of copper (from no supplementation to 0.3 mM supplemented) and methanol (from 8 to 150 mM). The highest optical densities were obtained when no copper was supplemented to the cultures, and copper addition was generally shown to negatively impact the growth rate. Thus, limiting copper supplementation below 0.01

mM at a methanol concentration of 25 mM was shown to provide optimal conditions for the growth (yield and titer) of *M. album* BG8.

In the second part of the study, various methanol-feeding strategies (pulsing fed-batch, step-wise fed-batch, varying target growth rate and initiation of feeding) were investigated to improve the growth and biomass productivity of *M. album* BG8 growing in fed-batch operation. These results were compared to growth in batch operation as a reference. The step-wise fed-batch operation with a target growth rate of 0.027 h^{-1} proved to have the highest biomass productivity (~0.027 ΔOD/h) while the step-wise fed-batch operation with a target growth rate of 0.0135 h^{-1} resulted in the highest final cell density achieved (6.6 compared to 0.35 in batch). Also, step-wise fed-batch and pulsing fed-batch operation significantly increased productivity compared to conventional batch (with increases of ~2.6 and 3-folds, respectively).

In the third part of this study, comparative metabolomics were used to investigate prospective commercially relevant metabolites produced by *M. album* BG8 growing on methanol in batch and fed-batch operations. Cultures were harvested in late exponential phase for batch, and at the end of controlled growth in fed-batch. Results showed that the mode of operation largely affected the abundance of metabolites of interest. Both batch and fed-batch operations resulted in bioproduction of commercially valuable metabolites; however, batch cultures led to greater abundance of metabolites linked to starvation and end-of-pathway (valine, leucine, and isoleucine), while fed-batch operation resulted in greater abundance of intermediates of metabolic pathways (e.g. 2,3-dihydroxy-isovalerate, 4-hydroxybenzoate, and phenyllactate).

This work provides a framework for the implementation of the methanotrophic bacterium *M. album* BG8 as an industrial platform organism for the production of industrially relevant bioproducts.

Preface

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

Chapter 3 will be submitted for publication as Bakhtiari Ziabari, F., Stein, L. Y., Sauvageau, D. "Effect of copper on growth of *Methylomicrobium album* BG8 in batch cultures using methanol". As the primary author I was responsible for designing, performing, and analyzing the experiments beside analyzing the data and writing the manuscript. Dr. Dominic Sauvageau (Supervisor) and Dr. Lisa Stein (supervisor) were the supervisory authors who contributed to the all parts of the study including the experimental design and data interpretation, provided advice and guidelines and edited the manuscript.

Chapter 4 will be submitted for publication as Bakhtiari Ziabari, F., Stein, L. Y., Sauvageau, D. "Step-wise fed-batch strategy for enhancing growth of *Methylomicrobium album* BG8 using methanol as sole carbon source". As the author of this chapter I was responsible for designing, troubleshooting, and performing experiments as well as data interpretations and writing the manuscript. Dr. Dominic Sauvageau (Supervisor) and Dr. Lisa Stein (supervisor) were involved in all parts of the study including the experimental design and data interpretation, provided advice and guidelines and edited the manuscript.

Chapter 5 will be submitted for publication as Bakhtiari Ziabari, F., Sugden, S., Stein, L. Y., Sauvageau, D. "Comparative metabomomics of *Methylomicrobium album* BG8 grown on methanol in batch and fed-batch". Scott Sugden, a PhD candidate in the Department of Biological Sciences at the University of Alberta performed statistical analysis on the metabolite abundance data provided by Metabolon, Inc. As the primary author I was responsible for designing and performing pulsing fed-batch operations in bioreactors, analyzing and comparing the data from different experiments and writing the manuscript. Dr. Dominic Sauvageau (Supervisor) and Dr. Lisa Stein (supervisor) were involved in all parts of the study including the experimental design and data interpretation, provided advice and guidelines and edited the manuscript.

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I would like to thank my family for the love and support they gave me through all these years. Thanks for teaching me to be strong and independent and for always reminding me that if I pursue my heart, I can do anything no matter what it is. Thanks for teaching me the value of hard work and for helping me become a better person. I would have not been able to come this far without your kindness and guidance throughout my life. To all my friends here in Canada or in Iran, we have not been able to meet for such a long time due to the coronavirus pandemic. I miss you all so much, and thank you for always calling me and giving me your countless love and support even though from far away and behind the safety of Zoom meetings and WhatsApp calls.

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Table of Contents

1. Introduction **1**

1.1. Motivation and context 1

1.2. Scope of this thesis 3

1.3. Thesis structure 3

1.4. References 4

2. Literature review **7**

2.1. Methanol, a by-product of the pulp and paper industry 7

2.2. Methanotrophic bacteria 8

2.2.1. Methanotrophs for bioconversion of methanol 8

2.2.2. Generalized metabolic steps in methanol bioconversion 10

2.2.3 *Methylomicrobium album* BG8 11

2.2.4. Comparison of *M. album* BG8 with other methanol-converting microorganisms 12

2.3. Industrial application 12

2.3.1. Industrial applications of methanotrophs 12

2.3.2. Valuable products obtained from methanotrophs 13

2.3.3. Challenges of industrialization of methanotrophs 17

2.3.4. Enhancing methanotroph growth through optimization of growth medium 18

2.3.5. Effect of copper on growth of methanotrophic bacteria 19

2.4. Conversion of industrial methanol by methanotrophs 21

2.5. Industrial bioprocessing 21

2.5.1. Bioreactors modes of operation 22

2.6. Metabolomics analysis 26

2.7. Reference 28

3.1. Abstract 44

3.2. Introduction 44

3.3. Materials and Methods 48

3.3.1. Strains and growth conditions. 48

3.3.2. Growth. 48

3.4. Results 48

3.5. Discussion ... 56
3.6. Conclusion ... 59
3.7. Acknowledgements ... 59
3.8. References ... 60
4. Step-wise fed-batch strategy for enhancing growth of *Methylomicrobium album* BG8 using methanol as sole carbon source ... **68**
4.1. Abstract ... 68
4.2. Introduction ... 69
4.3. Materials and methods ... 71
4.3.1. Strains and culture maintenance. ... 71
4.3.2. Bioreactor experiments. ... 72
4.4. Results ... 74
4.5. Discussion ... 84
4.6. Conclusion ... 88
4.7. Acknowledgements ... 89
4.8. References ... 89
5. Comparative metabomomics of *Methylomicrobium album* BG8 grown on methanol in batch and fed-batch ... **95**
5.1. Abstract ... 95
5.2. Introduction ... 95
5.3. Materials and Methods ... 99
5.3.1. Strains and growth conditions. ... 99
5.3.2. Assessing growth. ... 100
5.3.3. Metabolite extraction and analysis. ... 100
5.3.4. Statistical analysis. ... 100
5.4. Results ... 101
5.5. Discussion ... 110
5.6. Conclusion ... 112
5.7. Acknowledgements ... 113
5.8. Reference ... 113
6. Summary, conclusion and future direction ... **120**
6.1. Summary and conclusion ... 120
6.2. Future direction ... 122

6.3. References 123
Unified Bibliography 125

List of Tables

Table 3.1. Yield (ΔOD·mM methanol^{-1}) for the growth of *M. album* BG8 at various concentrations of methanol and copper. 54

Table 4.1. Summary of the conditions and results obtained from various operations for the growth of *Methylomicrobium album* BG8 using methanol as carbon source. NA in this table shows that a condition did not apply to a specific experiment. 83

Table 5.1. Mean abundance of metabolites involved in the Glycolysis, TCA and BCAA pathways for batch and fed-batch operations. 106

Table 5.2. Industrially relevant metabolites displaying greater abundance when *M. album* BG8 was grown under batch or fed-batch operation. 108

List of Figures

Figure 2.1. Overview of the chemical pulping process in a pulping mill (Eregowda, 2019). . 8

Figure 2.2. Generalized pathway for oxidation of methane by methanotrophs (Strong *et al.*, 2017) 10

Figure 2.3. Transmission electron microscopy image of recombinant *Ral*stonia *eutropha* PHB$^-$4 cells containing 90% of the dry cell weight of poly(3HB-co-5 mol% 3-hydroxyhexanoate). Bar represents 0.5 mm (Sudesh *et al.*, 2000). 15

Figure 2.4. Simplified schematic of stirred-tank reactor operated in a continuous mode (Winterbottom and King, 1999) 24

Figure 3.1. Growth of *Methylomicrobium album* BG8 under various concentrations of methanol and copper. (a) 8 mM methanol, (b) 12 mM methanol, (c) 25 mM methanol, (d) 50 mM methanol, (e) 100 mM methanol, (f) 150 mM methanol. 50

Figure 3.2. Variation of final OD of *M. album* BG8 as a function of copper concentration methanol concentration. (a) 8 mM, (b) 12 mM, (c) 25 mM, and (d) 50 mM, (e) 100 mM and (f) 150 mM methanol. Data points sharing the same letter within a graph were found to be statistically equal. 53

Figure 3.3. Variation of growth rate of *M. album* BG8 as a function of copper concentration for methanol concentrations of (a) 8 mM, (b) 12 mM, (c) 25 mM, and (d) 50 mM, (e) 100 mM and (f) 150 mM. Data points sharing the same letter within a graph were found to be statistically equal. 55

Figure 4.1. Optical density at 540 nm of batch cultures of *Methylomicrobium album* BG8 in a bioreactor. 74

Figure 4.2. Experimental and predicted values of optical density at 540 nm of a culture of *Methylomicrobium album* BG8 in a pulsing fed-batch process in a bioreactor. Arrows indicate addition of methanol to a concentration of 25 mM. 75

Figure 4.3. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.0135 h^{-1}. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (µmol/h). 76

Figure 4.4. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.027 h^{-1}. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (µmol/h). 78

Figure 4.5. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.027 h^{-1} and initial methanol concentration of 10 mM. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (µmol/h). 79

Figure 4.6. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.027 h^{-1} and fixed methanol

feeding rate of 2470 µmol/h. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (µmol/h).. 81

Figure 5.1. Heat map of scaled abundance of 324 metabolites detected in either batch or fed-batch cultivation of M*ethylomicrobium album* BG8 grown on methanol. Metabolites are categorized in groups of amino acids, carbohydrates, cofactors and prosthetic and electron carriers, lipids, nucleotides, peptides, hormone metabolism, secondary metabolism, and xenobiotics (the labels of the latter three are not shown in the figure).............................. 103

Figure 5.2. Fold-increase in abundance for metabolites more significantly abundant in *Methylomicrobium album* BG8 grown on methanol through A) batch operation (red), and B) fed-batch operation (blue). * indicates commercially relevant metabolites................. 104

1. Introduction

1.1. Motivation and context

Methanol is a byproduct of the pulp and paper industry in North America, mostly produced in the Kraft process (Bajpai, 2012). Cellulosic fibers, the compound used in paper manufacturing, are separated from lignin, which supports the vascular plant wood. Methanol is produced when methyl groups are separated from lignin (Zhu et al., 2000) and condensed into the evaporator condensate. In most pulp mills, the resulting methanol is usually disposed of as waste. However this methanol can represent a valuable resource for pulp mills by being converted into valuable products.

Methanotrophs – a type of bacteria that can use one-carbon compounds such as methane and methanol as their main source of carbon and energy – can be used to convert methanol into value-added products. In fact, these bacteria have been used for the production of a variety of bioproducts such as single cell proteins (Øverland *et al.*, 2010; Strong *et al.*, 2015), organic acids (Strong *et al.*, 2015), and biopolymers (Kim *et al.*, 1996; Dunfield *et al*., 2003; Pieja *et al*., 2011; Khosravi-Darani *et al.*, 2013) and the outlook of industrializing these bacteria seem very promising.

Methylomicrobium album BG8 is a strain of methanotrophic bacteria showing great potential as an industrial bioproduction platform owing to its stability in various growth conditions, greater growth yields, faster growth rate, and shorter lag phase compared to other methanotrophic strains (Tays et al., 2018). But, in order to enhance the growth and productivity of this strain under industrial conditions, substantial modifications to the current bioprocessing techniques need to be addressed.

The most common industrial bioprocessing technologies currently used for bioproduction using various microorganisms are batch, continuous and fed-batch operations. A batch, in which all the components of a bioprocess are introduced into the bioreactor at the beginning of the operation with no components being harvested until the end of the operation, is the simplest and most conventional mode of operation. The concentrations of nutrients decrease during this

process as the bacteria grow. As a result, the cell densities obtained are limited and are only suitable for the production of low-volume, high-value products (Korovessi and Linninger, 2005).

Continuous operation allows one or more of the nutrients to be continuously fed into the bioreactor while the contents of the reactor are also continuously removed through an effluent streams (Winterbottom and King, 1999). The product concentration obtained in this process is generally lower than in batch operation since the contents of the reactor are continuously diluted by the introduction of fresh medium (Boedeker and Magnus, 2017). Robustness of the process can also be problematic as washout can result from imbalances between the dilution rate and cells growth rate (Yen and Li, 2011)

In fed-batch operation, on the other hand, at least one of the nutrients is periodically or continuously added to the bioreactor through the course of the operation, and the products are harvested at the end of the process. Fed-batch operation typically leads to greater productivity, especially in processes that involve autocatalytic reactions (e.g. bacterial growth) (Lim and Shin, 2013). It is also a versatile mode of operation, as it allows for adjustments in the bioprocess by manipulating various factors such as the feeding rate of nutrients to the reactor.

Although batch, continuous and fed-batch operations have all been previously employed in bioproduction using methanotrophs (Graham *et al.*, 1993; Benstead *et al.*, 1998; Gilman *et al.*, 2015), challenges remain to be addressed in order to industrialize various applications of these bacteria. Some of the most important hurdles are the low cell densities, slow growth and low productivity encountered in methanotrophic bioprocesses. As a result, there is a clear need to improve the modes of operation for methanotroph growth in order to make these processes industrially viable.

In addition, it is important to understand the impact of these modes of operation on the metabolism of the bacteria. Metabolomics enables researchers to study the cells by analyzing a wide array of metabolites present in the biological system. Studying these metabolites when growing the bacteria in various bioprocessing operations (batch, continuous, and fed-batch), provides the opportunity to establish favorable conditions in an industrial operation to produce valuable bioproducts.

1.2. Scope of this thesis

The overall objective of this work was to enhance the growth and productivity of *M. album* BG8 in bioreactors through enhanced medium conditions and tailored processing operation techniques, and establishing the impact of these measures on the strain's metabolome.

The specific aims of this study were: 1) to investigate the effect of copper, a crucial nutrient, on the growth of *M. album* BG8 using methanol as a carbon source; 2) to establish efficient fed-batch strategies to enhance growth and productivity of *M. album* BG8 growing on methanol; and 3) to determine the metabolomic profiles of *M. album* BG8 cultures harvested from batch and fed-batch cultures.

1.3. Thesis structure

Chapter 2 provides a literature review on methanotrophic bacteria and their application in industry. This chapter also elaborates on various available bioprocesses for bioproduction using microorganisms and the challenges faced to take methanotrophic bioproduction schemes to industrial scale. Finally, an overview of how metabolomics analysis are used to study the metabolites produced in bioprocesses is presented.

Chapter 3 presents an investigation on the effect of copper on growth of the methanotrophic bacterium *M. album* BG8 with methanol as the sole source of carbon. Few studies have investigated the appropriate conditions for the growth of *M. album* BG8 for industrial purposes. This work contributes to fill this gap as various conditions of copper and methanol concentrations are tested and both cell densities and growth rates are reported. From these results, the optimal concentrations for the growth of this bacterium under industrial conditions are identified.

Chapter 4 is dedicated to the development of fed-batch strategies to enhance growth and productivity *M. album* BG8 feeding on methanol. Methanol feeding law based on the cell density of the bacteria at different intervals and methanol addition regimes were devised, and final cell density and processing time were monitored. These results were compared to the performance of

batch cultures. From this, the most favorable operation and conditions for the growth of *M. album* BG8 were identified.

In chapter 5, metabolomics analysis was used to investigate the effect of the mode of operation on the metabolites produced by *M. album* BG8 growing on methanol. Batch and pulsing-fed-batch operations were performed and the metabolites produced by the cells during the course of each operation were studied to understand the intracellular processes of the cells under these modes of operation. Based on the type of the metabolites produced, the importance of choice of mode of operation on the industrial bioproduction of commercially significant metabolites was demonstrated.

This thesis concludes with chapter 6, which summarizes the findings and contributions made in this work and the conclusions drawn from the previous chapters. A description of the future directions of the research, based on the results and conclusions of this work, is also presented.

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2. Literature review

2.1. Methanol, a by-product of the pulp and paper industry

Methanol is a common by-product of the forestry and pulping industries. It is usually considered costly to treat it for recovery, and is thus generally disposed of as waste. In the United States alone, the pulp and paper industry processes 108 million tons of pulpwood annually (Bajpai, 2012). In the Kraft process, lignin is separated from cellulose fibers (the compounds used for paper making) using white liquor, a mixture of sodium hydroxide (NaOH) and sodium sulphide (Na_2S). Lignin, which supports the vascular plant wood, is comprised of a phenolic polymeric structure. (Eregowda, 2019). In the Kraft process, methyl groups are removed from lignin and xylan (through de-methylation), which results in the production of methanol (Zhu et al., 2000). Methanol is also released from the wood in the digester during the cooking process in and is then transferred to the weak black liquor and to the flash vapor from the blow tank. Subsequently, blow heat recovery is used to condense this vapor in the form of digester condensate (Velmet technical series, 2018). In the process of evaporation, methanol present in the weak black liquor is evaporated and then condensed into the evaporator condensate. Figure 2.1 shows a general schematic of this pulp mill process (Eregowda, 2019).

Mills employ different methods for removal or disposal of methanol. In North America, the methanol-containing condensate is treated based on the type of contaminant present, and methanol is generally separated out using a stripper. (Bajpai, 2012). The methanol released needs to undergo further separation to remove the high moisture content (35% methanol and 65% water). In a typical Kraft mill, between 10 to 15 kg of methanol is produced per ton of air dry pulp (ADT), correlating to 11 to 16 tonnes of methanol per day for a 1000 air dried tonne-(ADMTP) mill, assuming a 10% pulp yield loss in the process (Valmet technical paper series, 2018). The methanol produced in the pulp mill is a valuable compound and instead of being disposed of as an undesirable waste byproduct, it can be sold as a commodity chemical or transformed into valuable products, creating new revenue streams for pulp mills. For example, Alberta-Pacific Forest Industries (ALPAC) is now selling biomethanol recovered from its pulping activities.

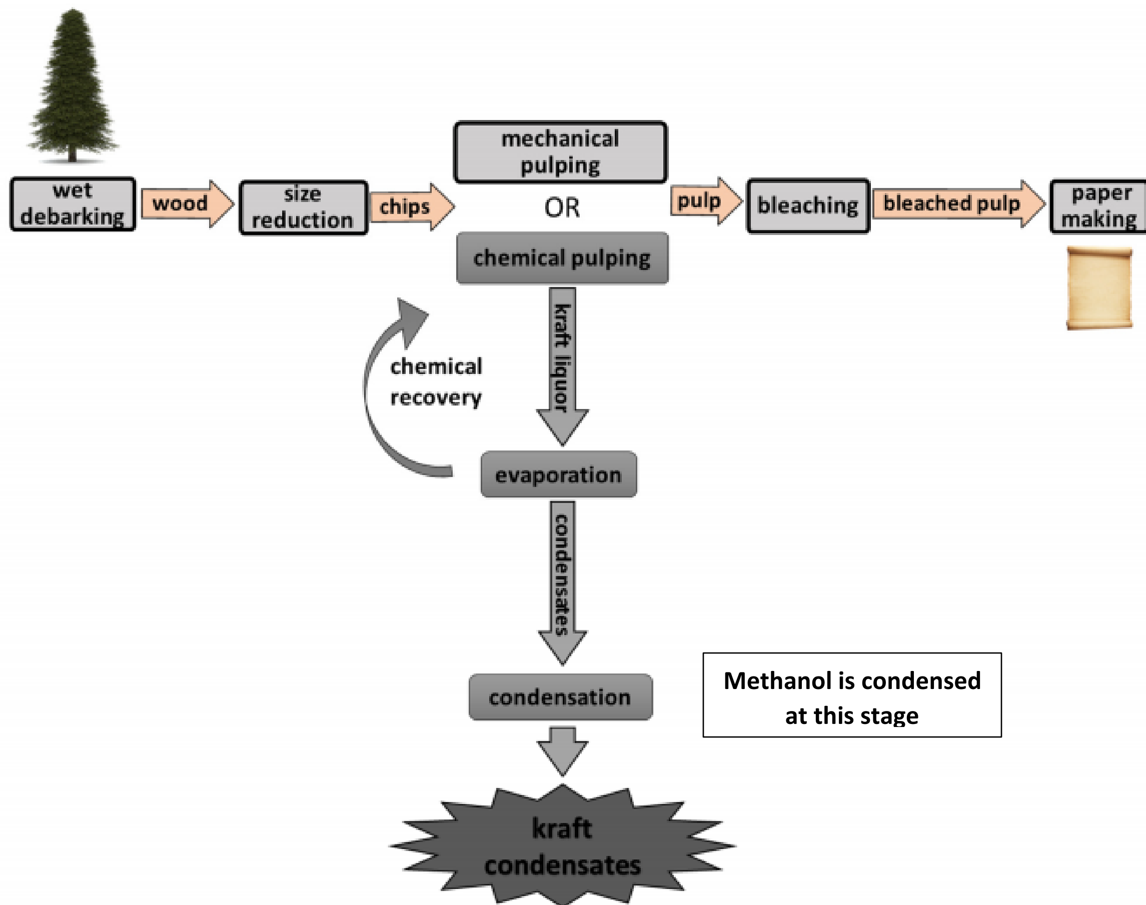

Figure 2.1. Overview of the chemical pulping process in a pulping mill (Eregowda, 2019).

2.2. Methanotrophic bacteria

2.2.1. Methanotrophs for the bioconversion of methanol

Methanotrophic bacteria are a group of methane oxidizing microorganisms that can use methane and methanol as their main source of carbon and energy (Hanson and Hanson, 1996). These bacteria are a subset of methylotrophs, organisms that can grow on one-carbon compounds such as methane, methanol, methylated amines, halomethanes, and methylated compounds containing sulfur (Anthony, 1982; Anthony, 1986; Anthony, 1991; Dijkhuizen *et al.*, 1992; Hanson *et al.*, 1992; Lidstrom, 1992; Lidstrom, 2006). The discovery of methanotrophs is mainly attributed to Söhngen, who isolated a bacterium using methane in 1906 and named it *Bacillus*

methanicus (Söhngen, 1906). These bacteria are present in a wide variety of environments such as soils, peatlands, rice paddies, sediments, freshwater and marine systems, acidic hot springs, mud pots, alkaline soda lakes, cold environments, and tissues of higher organisms (McDonald *et al.*, 2008).

Methanol can be used by many microorganisms including methanotrophic bacteria to produce valuable compounds. As a one-carbon compound, methanol is at least 50% more reduced than traditional sugars such as glucose, resulting in increased bioconversion product titers and yields (Whitaker *et al.*, 2015). Some advantages of using methanol for bioproduction using methanotrophic bacteria are low price, complete water miscibility and moderate requirement of oxygen when used as substrate (Byrom, 1987).

Value-added compounds such as poly(3-hydroxybutyrate) (PHB) can be produced by methanotrophic bacteria using methanol as the main source of carbon. Although the substrates most commonly used for production of PHB by microorganisms are glucose, sucrose and fatty acids (Kim *et al*. 1992; Kim *et al*. 1994; Suzuki *et al*. 1986), cheaper substrates such as methanol can represent an important opportunity for the broad application of PHB (Suzuki *et al*. 1986). Among different strains of methanotrophic bacteria, *Methylobacterium organophilum* was shown to be able to produce PHB using methanol (Kim et al, 1995). It was shown in a study that inducing potassium limitation in a fed-batch culture of *M. organophilum* while keeping methanol concentrations under the inhibitory levels of 3 g/l resulted in production of PHB to a maximum of 56% cell dry weight. In another study it was shown that methanol can be used as a co-substrate for production of PHB using methanotrophic strain *Methylosinus trichosporium* IMV3011 (Zhang *et al.*, 2008). Another study showed that methanol can be used as a co-substrate for production of PHB using *Methylocystis hirsute* (Ghoddosi *et al.*, 2019). Also, L-serine and PHB were produced from methanol using *M. extorquens* (Sirirote *et al.*, 1986; Mokhtari-Hosseini *et al.*, 2009). In a study, serine was produced from *Methylobacterium extorquens* NR-1 (formerly known as *Protomonas extorquens* NR-1) using methanol under controlled conditions at 30°C (Sirirote *et al.*, 1986) and the highest L-serine concentration achieved was 54.5 g/l. In another study, media compoents were optimized using statistical design for the production of PHB from *Methylobacterium extorquens* DSMZ 1340 in a 5-L fermentor (Mokhtari-Hosseini, 2009). It was shown that the optimal medium composition for PHB production was 7.8 (g/l) of $Na_2HPO_4 \cdot 12H_2O$ and no $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, and $MnSO_4$

(Mokhtari-Hosseini, 2009). 9.5 g/L PHB was obtained in this study with a polymer cell content of 62.3% (Mokhtari-Hosseini, 2009).

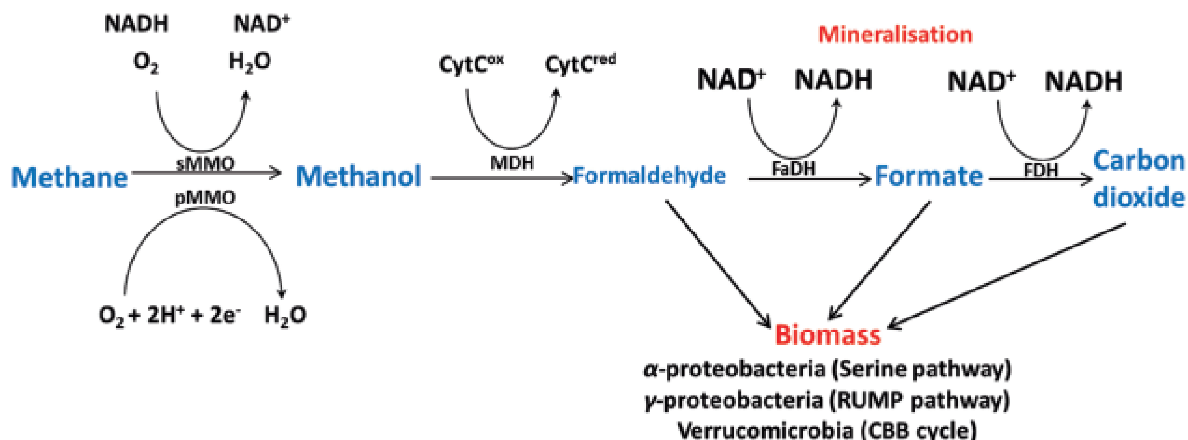

Figure 2.2. Generalized pathway for oxidation of methane by methanotrophs (Strong *et al.*, 2017)

2.2.2. Generalized metabolic steps in methanol bioconversion

Figure 2.2 shows the generalized pathway by which methanotrophs oxidize methane to carbon dioxide or biomass (Strong *et al.*, 2017). Methane is oxidized to carbon dioxide (CO_2) or biomass through intermediates – methanol, formaldehyde and formate (Hanson and Hanson, 1996).

The same pathway, albeit truncated of its first step, is involved when methanol is used as substrate. In this case, uptake is mediated by the enzyme methanol dehydrogenase (MDH), located in the periplasm of the cell, to produce formaldehyde (Smith *et al.*, 2010). In methanotrophs, methanol – the initial oxidation product of methane – is oxidized to formaldehyde using MDH (Smith and Murrell, 2009). MDH is a pyrroloquinoline quinone (PQQ) dependent enzyme consisting of two large (67 kDa) and two small (8.5 kDa) subunits (Smith *et al.*, 2010).

The intermediate metabolites resulting from methane and methanol oxidation – formaldehyde, formate, and to a certain extent carbon dioxide – enter a variety of metabolic pathways involved in biomass and bioproduct formation based on the different types of

methanotrophs. For example, for the alphaproteobacterial methanotrophs the intermediates enter the serine pathway. These bacteria use the serine pathway for formaldehyde assimilation. Gamma-proteobacteria on the other hand, use the ribulose monophosphate cycle (RUMP). As a result of this diversity of pathways, the different types of methanotrophs can be used in industrial settings for the production of different biomolecules of interest using methane or methanol as feedstock.

2.2.3 *Methylomicrobium album* BG8

The gammaproteobacterial methanotroph *Methylomicrobium album* BG8 – formerly called *Methylobacter albus, Methylomonas albus*, and *Methylomonas alba* – was isolated from water and soil samples (Whittenbury *et al.*, 1970). It only contains pMMOs and thus, when growing on methane, responds to variations in copper availability by alterations in cell growth, MMO activity, and intracellular membrane formation (Brantner *et al.*, 1997; Whittenbury *et al.*, 1970). Several features of *M. album* BG8 render it suitable for use in industrial applications: it displays faster growth rates, shorter lag phases, and greater growth yields than many other methanotroph strains (Tays et al., 2018). In addition, it shows more growth consistency under various conditions; for example, in a previous study, growth was not significantly affected when using ammonium or nitrate as the nitrogen source (Tays *et al.*, 2018). When grown on either methane or methanol in the presence of ammonium as the nitrogen source, the bacterial enzymes reduce nitrite to nitric oxide in the media, thereby preventing toxic effects of the former on the cells (Kits *et al.*, 2015; Stein & Klotz, 2011). *M. album* BG8 has been shown to grow better using nitrate as the nitrogen source since it is sensitive to high levels of ammonia, but is resistant to growth inhibition at high concentrations of nitrate (Nyerges *et al.*, 2010).

While methane is the preferred substrate for most strains of methanotrophs, as growth is either inhibited or unreliable when growing on methanol, *M. album* BG8 can be considered as an exception (Tays *et al.*, 2018). In fact, it was shown to have both higher growth and faster growth rates when grown on methanol as the main source of carbon. As a result, when investigating the bioconversion of methanol, *M. album* BG8 presents many advantages over most other methanotrophic strains.

2.2.4. Comparison of *M. album* BG8 with other methanol-converting microorganisms

Aside from methanotrophs, many microorganisms have been used for bioconversion of methanol, some of which have been engineered for bioproduction applications. For example, *M. extorquens* ATCC 55366 and *Pseudomonas* sp. K have been shown to produce poly(3-hydroxybutyrate) (PHB) from methanol (Bourque *et al.*, 1995; Suzuki *et al.*, 1986). Mutants of *Methylobacillus glycogenes* were used for production of L-Glutamate and L-Threonine from methanol (Motoyama *et al.*, 1993) while L-Serine was produced using *M. extorquens* (Sirirote *et al.*, 1986). Also, methylotrophic yeast were reported to have been used to produce pharmaceuticals from methanol (Zhang *et al.*, 2018).

Although all these microorganisms have been used successfully for bioproduction using methanol, methanotrophs have advantages over other microorganisms for these applications. One of these advantages is the ubiquity of these bacteria in the environment (Trotsenko *et al.*, 2005). These bacteria are widespread in nature and inhabit various environments such as soils, wetlands, fresh and marine waters, lakes and sediments and also extreme environments with high and low pH, temperatures or salinities. The fact that methanotrophs are wide-spread in nature, allows for versatile incorporation of these bacteria for in-situ bioconversion and bioremediation applications without the need for relocation and transport of materials (Brigmon, 2001). Other advantages of methanotrophs for methanol bioconversion include the existence of a natural methanol utilization pathway (Strong et al., 2017), which obviates the need for metabolic engineering, inherent tolerance of methanotrophic bacteria to methanol at levels of methanol that are deemed to be toxic to other microorganisms (to a maximum concentrations of 0.01% v/v) (Leadbetter *et al*., 1958; Stocks *et al*., 1964), and the general robustness of these bacteria (Chidambaram Padmavathy *et al.*, 2017; Meruvu *et al.*, 2020).

2.3. Industrial application

2.3.1. Industrial applications of methanotrophs

There is vast interest in using microorganisms for industrial bioproduction and various microorganisms are used to this end. In addition to the more conventional industrial microbial strains (e.g. *Saccharomyces cerevisiae* (Kawamukai, 2009; Steensels *et al.*, 2014; Liu *et al.*, 2020, etc.), *Escherichia coli* (Theisen and Liao, 2017; Chen *et al.,* 2018; Wang *et al.*, 2018,

etc.)), extensive efforts are made to leverage the properties of non-traditional microbes towards as industrial bioconversion platforms.

In this context, methanotrophic bacteria are increasingly used in industry for the production of useful products, as listed above. Like all bioindustrial strategies, bioconversion of methane and methanol by methanotrophic bacteria carries its load of specificity and challenges.

2.3.2. Valuable products obtained from methanotrophs

2.3.2.1. Single cell proteins

Single cell protein can be produced by methanotrophs for animal food consumption (Øverland *et al.*, 2010; Strong *et al.*, 2015). Single cell proteins (SCPs) became of interest due to a lack of protein sources during the 1950s and 1960s, and predictions of a potential global shortage of proteins (Strong *et al.*, 2015). Later, in the 1970s, production of low-cost soya protein slowed down the research in the field of microbial production of SCPs. Many strains of methylotrophs have been used for production of SCPs (Anthony, 1982; Stahl and Esser, 1982; Banat *et al.*, 1989). Commercial production of SCPs from methanotrophs began as a research project in Denmark, later evolving into a company called UniBio A/S (Strong *et al.*, 2015). SCP produced by fermentation of methane using *Methylococcus capsulatus* Bath is a reliable source of protein for animals, such as pigs, broiler chickens, mink, fox, Atlantic salmon, rainbow trout, and Atlantic halibut, based on various factors including amino acid composition, digestibility, and animal performance and health (Øverland *et al.*, 2010). In another study, the feasibility of recovering nutrients from industrial wastewater in form of feed grade SCP was investigated and showed that a protein content of 45% dry weight can be achieved through fermentation using *Methylococcus capsulatus* Bath (Rasouli *et al.*, 2018).

2.3.2.2. Biopolymers

Polyhydroxy-alkanoates (PHA), such as poly(3-hydroxybutyrate) (PHB), are biodegradable alternatives to already existing petroleum derived polymers (Anjum *et al.*, 2016; Meereboer *et al.*, 2020) and are produced as energy storage molecules in bacteria (Moradali and Rehm, 2020). PHB is one of the most developed products of methanotrophs, which has already

been taken to a commercial scale by companies such as Mango Materials that produce PHB from waste methane at an economically viable cost (Mango Materials, 2020). Figure 2.3 is a transmission electron microscopy (TEM) image of *Ralstonia eutropha* bacteria containing poly(3HB-co-5 mol% 3-hydroxyhexanoate), making up about 90% of the bacterial cell dry weight (Sudesh *et al.*, 2000). These biopolymers are formed and stored interacellularly as a result of nutrient limitation and in the presence of excess carbon (Anderson and Dawes, 1990; Lee, 1996). Many alphaproteobacterial methanotrophs have been investigated for PHA production. For example, *Methylosinus trichosporium* was grown on methane accompanied by methanol and citric acid, resulting in a high quality PHB with a yield of 40% (w/w) (Zhang *et al.*, 2008). In another study, *Methylocystis* sp. was utilized in a two-stage process, a growth stage and a PHB accumulation stage, for the production of a high molecular mass PHB (up to 2.5 × 106 Da) with a PHB content of 51% (Wendlandt *et al.*, 2005). Potassium-limiting conditions have also been shown to stimulate the production of polymers, with ultra-high average molecular weight of 3.1 MDa when growing *Methylocystis* sp. in mixed cultures using methane (Helm *et al.*, 2008).

Methanol has also been used as substrate for the production of PHAs. For example, a study showed that PHB could be produced by *Methylobacterium organophilum* using methanol under potassium limitation (Kim *et al.*, 1996). *M. organophilum* was grown using 2-3 g/L methanol and PHB accumulation was induced by lowering the potassium concentration in the culture below 25 mg/L. The concentration of PHB obtained was 130 g/L after 70 h, with a PHB content of 52% to 56% cell dry weight (Kim *et al.*, 1996). Also, *Methylobacterium extorquens* ATCC 55366 was used to produce PHB at high cell densities in a fed-batch fermentation process using methanol (Bourque *et al.*, 1995). In another study, *Methylobacterium extorquens* DSMZ 1340 was used to produce PHB in a process in which media components were optimized by statistical design (Mokhtari-Hosseini *et al.*, 2009). *Methylobacterium* sp. GW2 was used to produce PHB from methanol when with valeric acid supplied as an auxiliary carbon source resulting in a copolymer content of 30 % (w/w) and a PHB to PHV ratio of 1:2 (Yezza *et al.*, 2006).

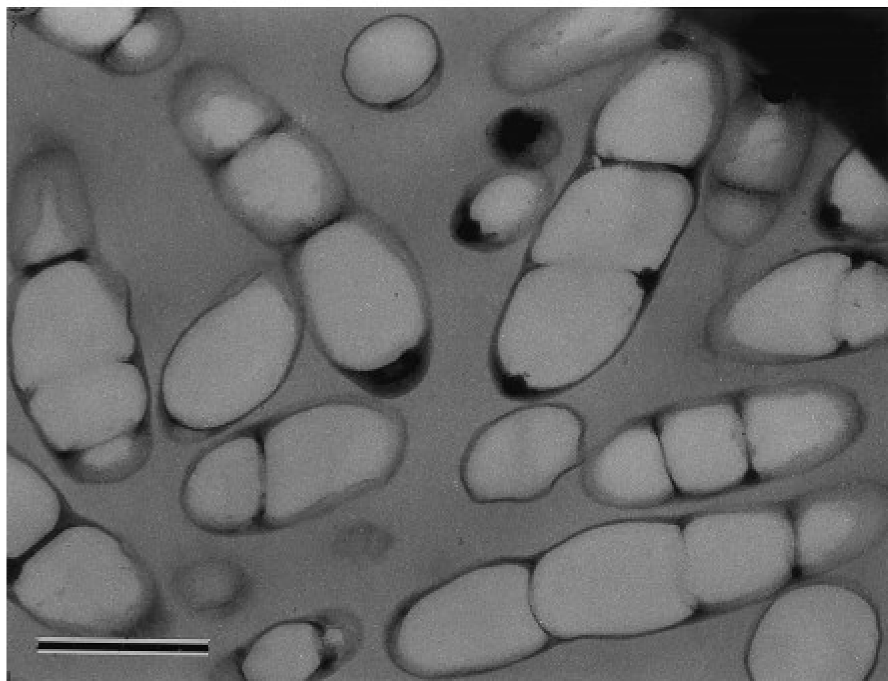

Figure 2.3. Transmission electron microscopy image of recombinant *Ral*stonia *eutropha* PHB$^-$4 cells containing 90% of the dry cell weight of poly(3HB-co-5 mol% 3-hydroxyhexanoate). Bar represents 0.5 mm (Sudesh *et al.*, 2000).

2.3.2.3. Ectoine

Halo-tolerant methanotrophs can produce ectoine (Strong *et al.*, 2015). Ectoine is an osmoprotectant produced and stored intracellularly by bacteria as a means of protection against dehydration (Jiang *et al.*, 2010). Ectoine and its derivatives can be used in the cosmetic industry for their moisturizing and protective effects against UV-induced damage to the DNA of skin cells (Bunger and Driller, 2003). Halotolerant methanotrophs can be used to produce ectoine in processes that could compete with existing expensive ectoine production processes. Moderately halophilic methanotrophs, such as *Methylobacter alcaliphilus* 20Z and *Methylobacter modestohalophilus* 10S, were shown to be able to produce and accumulate ectoine up to 20% of their dry cellular mass using methane as substrate (Khmelenina *et al.*, 1999; Trotsenko *et al.*, 2005).

2.3.2.4. Lipids for biodiesel production

Biodiesel can be used as a transport fuel and has advantages over ethanol as car engines do not need to be modified for its adoption. Production of biodiesel is usually done through extraction and transesterification of animal or plant fats (Muniyappa *et al.*, 1996). Methanotrophs have been reported to produce lipids used in biodiesel production from methane (Fei *et al.*, 2014). Lipid accumulation in methanotrophic bacteria is similar to PHB accumulation: in both cases accumulation can be induced by limitations in oxygen, nitrogen or phosphate. The conversion of methanotrophic/methylotrophic biomass into fuel has also been reported (Silverman *et al.*, 2014). This oil is derived from the cellular membrane of the bacteria.

2.3.2.5. Health supplements

Membrane-derived lipids from methanotrophs have been used in the production of health supplements to reduce plasma cholesterol levels, to lower the ratio of LDL to HDL cholesterol in the plasma, or as an immunoprotectant. (Muller *et al.,* 2007). It has been reported that the decrease in plasma cholesterol occurs because of the presence of phospholipids containing high levels of phosphatidyl-ethanolamine.

2.3.2.6. Valuable soluble metabolites

Soluble metabolites like methanol, formaldehyde, and organic acids are some of the other valuable products produced by methanotrophs (Strong *et al.*, 2015). Although it can be considered a substrate, methanol has also been investigated as a potential product of methane oxidation by methanotrophs. In the metabolic pathway, methanol is rapidly converted to formaldehyde, meaning fermentation techniques or genetic engineering are needed for the production and recovery of methanol before the conversion to formaldehyde by methanotrophic bacteria (Strong *et al.*, 2015). The thermophilic methanotroph *Methylocaludum* was used to produce methanol and the methanol was successfully recovered by constantly removing the methanol in the gas phase from the reactor at 50 °C. High cell density cultures were employed to produce methanol to achieve production of 1 g/L (Duan *et al.*, 2011).

Formaldehyde and formic acid produced by methanotrophs are intermediates while acetate, lactate, and succinate are organic acids that are produced in methanotrophs under oxygen

limitation (Kalyuzhnaya *et al.*, 2013). Although current process yields are low, many companies like Kiverdi, Coskata, CALYSTA Energy, NatureWorks, National Renewable Energy Laboratory and Intrexon are conducting research in this area to commercialize the production of some of these metabolites (Strong *et al.*, 2015).

2.3.3. Challenges of industrialization of methanotrophs

Despite the great interest in the application of methanotrophs for bioproduction, many hurdles still remain, such as scale up and the ability to achieve high cell densities and high productivities. One important issue is that the behavior of cultures in the natural environment or at small scales is not necessarily representative of the behavior of the culture at larger scales (Hanson and Hanson, 1996). When cultivated under laboratory conditions, many microorganisms grow well, but when conditions change (temperature, culture volume, etc.), even under the right nutrient concentrations and supporting growth factors, the growth of microorganisms might not be consistent or optimal.

Another serious issue for methanotroph bioprocesses for the conversion of methane is linked to mass transfer limitations in industrial bioreactors when microorganisms are grown on gaseous substrates. This issue can be addressed using typical engineering solutions, such as increasing the impeller speed or reduction of gas bubble size, but the solutions themselves can cause other issues for the growth of microorganisms (Munasinghe and Khanal, 2010). Growing bacteria at large scales can cause issues such as flocculation which decreases bioconversion efficiency (Gresham and Hong, 2015).

Many studies have focused on different aspects to increase the productivity of methanotrophic bioprocesses. For example, to resolve the problem of poor mass transfer rates of a gaseous substrate in the liquid, technologies such as membrane contactors have been investigated (Meraz *et al*., 2020). One study investigated the influence of pressure on growth of the methanotrophic bacterium *Methylocystis* sp. strain GB 25 in a pilot scale 12-L bioreactor. At pressures up to 0.5 MPa, the authors reported a maximum productivity of 6 g biomass/L·h (Wendlandt *et al.*, 1993). In another study, methanotrophic bacteria were immobilized in a hydrogel used in a bioreactor, resulting in an increase in the mass transfer rate of methane and thus an increase in PHB productivity (Ruelas, 2018).

2.3.4. Enhancing methanotroph growth through optimization of growth medium

The optimization of culturing conditions for methanotrophic bacteria is an ongoing effort. Many studies have focused on optimization of growth media to increase growth and productivity. For instance, the combined effects of carbon and nitrogen sources on the growth of proteobacterial methanotrophs have been investigated (Tays *et al.*, 2018). In this study, optical density (OD), growth rate, and biomass yield of *Methylocystis* sp. Rockwell, *Methylocystis* sp. WRRC1, *Methylosinus trichosporium* OB3b, *Methylomicrobium album* BG8, and *Methylomonas denitrificans* FJG1 grown on methane or methanol as carbon source and ammonium or nitrate as nitrogen source were compared. A general preference for ammonium was observed for the growth of these species (Tays *et al.*, 2018). In another study, the effect of 40 variations on the standard diluted nitrate mineral salts medium on the growth of twelve methanotrophic strains within the genus *Methylomonas*, including the type strains of *Methylomonas methanica* and *Methylomonas koyamae*, was evaluated (Hoefman *et al.*, 2014). It was demonstrated that the design of a customized medium for each strain improved growth by reducing lag phase, leading to faster biomass accumulation. As a result of medium optimization, *Methylomonas* sp. R-45378 displayed a 50 % increase in cell dry weight and had the least nitrogen and oxygen requirements of all strains tested (Hoefman *et al.*, 2014).

Another study was done on the cultivation of type II methanotrophic bacteria from littoral sediments (Bussmann *et al.*, 2004). The most probable number method, using media with varying concentrations of nutrients – phosphate, nitrate, and other mineral salts –, was used to create an optimized medium for growth. The number of cultured methanotrophs declined in the presence of high concentrations of magnesium and sulfate (>1 mM) or high concentrations of nitrate (>500 µM), while phosphate concentrations in the range of 15–1500 µM had no influence on growth. The study further confirmed the presence of both type I and type II methanotrophs in the culture, but showed that the growth of type II methanotrophs was favored even under the improved medium conditions (Bussmann *et al.*, 2004).

The effect of organic chemicals on the growth rate and cell density of *Methylosinus trichosporium* OB3b was investigated (Xing *et al.*, 2006) and the results showed that the presence of riboflavin and organic acids, such as malate, citrate, succinate and maleate, improved cell concentration. Citrate, at an optimal concentration of 0.015 mmol/L, was shown to exhibit

the most significant effect on cell growth with a cell dry weight 3.5-fold greater than that of the control without citrate addition (Xing *et al.*, 2006).

Other studies on optimization of growth media focused more specifically on the optimization of specific components, such as metal ions. One study looked at the effect of growth conditions on the expression of soluble methane monooxygenase (sMMO) on methanotrophic-heterotrophic community enriched from groundwater aquifer solids in Moffett Field Naval Air Base (Mountain View, CA, USA) (Begonja and Hršak, 2001). The results showed that copper deficiency was essential for the detection of sMMO activity, and that substituting potassium nitrate with ammonium chloride also limits growth and sMMO activity of the bacteria. Conversely, increasing iron (II) sulphate concentration from 12 to 52 µM in nitrate mineral salt medium had a positive effect on growth and sMMO activity, suggesting that iron in the form of Fe^{2+} is an essential medium constituent for enhancing the sMMO activity.

2.3.5. Effect of copper on growth of methanotrophic bacteria

Copper has long been known to play an important role in the physiology and metabolic activity of methanotrophic bacteria. As discussed above, two forms of MMOs are found in methanotrophic bacteria, particulate (pMMO) and soluble (sMMO). Copper is a key factor in regulating the expression of the genes encoding sMMO and pMMO, as well as the activity of these enzymes (Takeda and Tanaka, 1980; Stanley *et al.*, 1983). While sMMO is an iron-containing enzyme, pMMO is a copper-containing enzyme and its activity is controlled by the availability of copper. In fact, one of the most important factors in controlling methanotrophic activity is the copper to biomass ratio (Semrau *et al.*, 2010). The effect of copper is so prominent that it is possible to alter the environmental conditions in order to push the cell to produce one of the two forms of MMO more predominantly (Stanley *et al.*, 1983). In cells that contain pMMO, copper was shown to moderate substrate affinity and specificity, and control the expression of the two forms of MMO up to 55-fold (Lontoh, 2000; Choi *et al.*, 2003). The addition of copper to growth media was shown to enhance methane oxidation in both *M. album* BG8 and *M. trichosporium* OB3b, although *M. album* BG8 had higher (~2 ×) pseudo-first-order rates (V_{max}/K_s) of methane oxidation than *M. trichosporium* OB3b (Lontoh and Samrau, 1998, Lintoh, 2000). Large amounts of copper have been found in the active purification of pMMO and could

be involved in the oxidation and/or electron transport to oxygen (Zahn and DiSpirito, 1996; Balasubramanian and Rosenzweig, 2007).

Methanotrophic bacteria expressing pMMO have a high copper demand and respond strongly to variations in copper levels. In subsurface environments specifically, copper bioavailability can be decreased as a result of sorption to the surface of metal oxides and reactions with organic matter available in these environments (Morton *et al.*, 2000). As a result, methanotrophic bacteria must have efficient means of sensing and collecting copper to compete with complexing agents present in the environment that can scavenge copper. One copper-complexing agent is known as methanobactin and was first identified in *M. capsulatus* Bath (Zahn and DiSpirito, 1996). In *M. capsulatus* Bath, methanobactin is the extracellular component of the copper uptake system also involved in the regulation of the two forms of MMO. Growth of *M. capsulatus* Bath under various copper concentrations (0-100 µM) using methane as carbon source has been investigated (Avdeeva, and Gvozdev, 2017). This study showed that when copper was in excess (60-100 µM), methanobactin was secreted into the growth medium and excess copper ions accumulated as granules near the cell surface,within the cytoplasm. When copper was present in the medium at low concentrations (below10 µM), methanobactin was also secreted into the medium (Avdeeva, and Gvozdev, 2017).

In another study, the effect of copper and methane concentrations on the structure and biodegradation kinetics of methanotrophic bacteria was investigated (Cantera *et al.,* 2016). The results showed that an increase in copper concentration from 0.05 to 25 µM increased the maximum specific biodegradation rate and that affinity for target pollutants within the communities enriched by a factor of 3 (Cantera *et al.,* 2016).

The role of copper was also examined in PHB biosynthesis by *Methylosinus trichosporium* IMV 3011 (Dong, 2013). It was shown that the addition of 30 µmol/L $CuSO_4$•5H2O led to a cell dry weight of 0.48 g dry wt/L, which was 2 times that of cultures without copper addition. Also, lag time was shortened and growth rate was increased when compared to the copper-free control. The PHB content increased to 8.3% and pMMO expression increased, potentially contributing to the synthesis of PHB in the cells (Dong, 2013). In another study conducted on twelve strains of methanotrophic bacteria, PHA accumulation reached 25% at a copper concentration of 5 µM (Pieja *et al.*, 2011). In another study, a concentration of 10 µM copper increased the PHA content from 4.22 to 8.07% in mixed consortia of *Methylosinus*

trichosporium OB3b, *M. trichosporium* IMV3011, *Methylococcus capsulatus* HD6T, *Methylomonas* sp. GYJ3 (Zhang *et al.*, 2009). Copper has also been shown to have an effect on the growth of *M. album* BG8 (Collins *et al.*, 1991), where addition of copper increased cell yield and MMO activity. Finally, the formation of intracytoplasmic membranes was observed when cells were grown on copper, affecting the abundance of some membrane proteins (Collins *et al.*, 1991).

Based on these findings, it is clear that the presence of copper can play an important role in the production of biomass and of desirable bioproducts by methanotrophic bacteria. As such, it is important to consider it in the development of bioprocessing strategies using these organisms.

2.4. Conversion of industrial methanol by methanotrophs

Methanotrophs can potentially use methanol produced by pulp and paper mills to convert it to valuable bioproducts. The advantage of using methanol for bioconversion by methanotrophs is that growth on methanol reduces oxygen consumption and energy input demands compared to methane. Also, since methanol is usually handled in liquid form, mass transfer limitations will be reduced compared to a gas phase carbon source like methane. Additionally, methanol, especially waste methanol, is much cheaper than other bioprocess feedstocks.

One of the downsides of using methanol is tied to its toxicity to most bacteria at concentrations as low as 0.01% v/v (Leadbetter *et al*., 1958; Stocks *et al*., 1964). Although many methanotrophs have the ability to grow on methanol, its toxicity of methanol can negatively affect their growth (Hanson & Hanson, 1996; Van Dijken & Harder, 1975), making it difficult to create efficient bioprocesses. Generally, when grown on methane, methanotrophs can reach higher optical densities, and the lag phases and growth yields are more consistent than when grown on methanol. Recently, strains of methanotrophs, such as *Methylomicrobium album* BG8, have been shown to grow faster and reach higher optical densities than other strains, even when grown on methanol (Tays *et al.*, 2018). This study showed that, for *M. album* BG8, toxicity effects were only observed at methanol concentrations above 20 mM (Tays *et al.*, 2018).

2.5. Industrial bioprocessing

Industrial bioprocessing technologies provide the means to take life-science discoveries from the lab to industrial products. Bioprocessing techniques are used to produce an array of

products such as pharmaceuticals, foods, fuels and biochemicals in bioreactors using a biocatalyst such as enzymes, microorganisms, or eukaryotic cells (Singh, 2006). It often involves the genetic engineering of plants, animals, and microorganisms such as yeasts, bacteria and fungi to achieve the desired products. Bioprocessing technologies have provided us with the advantage of using biomass rather than petrochemical-sourced feedstocks for the production of valuable chemicals, thus avoiding the devastating effects of conventional energy sources on the environment. Bioprocess engineering can thus be employed to obtain value-added products using renewable resources, such as agricultural and forestry-based materials for large-scale industrial processes. Using bio-based products is a green alternative to current technologies since it provides benefits such as sustainability of renewable biomass, replacing depleted fossil energy, and reducing GHG emissions (Yang, 2007). This is especially true for methanotrophic bacteria which use GHG as starting material for bioproduction, both decreasing emissions and providing sustainable supply chains.

A variety of industrial bioprocesses require bioreactors. Bioreactors provide optimal conditions for biological reactions to occur and are designed to enhance product titers and the productivity. In order to design an appropriate bioreactor and related strategy suitable for a specific bioprocess, various factors, such as cell growth, metabolism, genetic manipulation, and protein or other product expression levels, must be considered and well understood (Wang and Zhong, 2007). Aside from these factors, an array of conditions need to be considered, such as dissolved oxygen concentration, pH, temperature, mixing, nutrient supplementation, scale-up, and bioproduction costs (Zhong, 2010).

2.5.1. Bioreactors modes of operation

One of the main challenges of scaling-up bioreactor processes is successfully taking a laboratory bioprocess to an industrial scale without major declines in product titer and productivity, which can result from changes in culturing conditions such as shear stress, oxygen supply and substrate composition (Zhong, 2010). To address this issue, bioprocessing techniques need to be further studied and optimized. Microbial cultures are typically cultivated in three classical operational modes: batch, continuous, or fed-batch. The choice of the mode of operation depends on the type of organism being cultivated, the application, and the final goal of the

bioprocess, such as the type of bioproducts targeted. The three classical modes of operation are explained in more detail below.

2.5.1.1. Batch operation

In batch operation all necessary medium components and the inoculum are added at the beginning of the culture, and, aside from gases in aerobic cultures or solutions to adjust pH as required, no components are introduced to or removed from the reactor during processing time. In this mode of operation, the microorganisms use up the nutrients while growing, thereby reducing concentrations over time. In the course of the process, it is common for pH, temperature, dissolved oxygen and foaming to be monitored and controlled. The products of the process are recovered at the end of the operation and, if need be, separated. Since all nutrients are added at the beginning, most approaches to optimization involve the initial medium composition, inoculum load, temperature, and pH (Lim and Shin, 2013). Batch operations are best suited for the manufacture of low-volume, high-value products and are more common in industries such as specialty chemicals (high-purity chemicals and cosmetic pigments), pharmaceuticals, and food (Korovessi and Linninger, 2005). Batch operation has been used for growth of methanotrophic bacteria for long. Stimulation of growth of *Methylomicrobium alcaliphilum* 20Z on both methane and methanol was investigated by addition of tungsten to batch cultures of this bacterium (Cho *et al.*, 2020). Biomass yield and product yield were increased up to 11.50-fold and 1.28-fold, respectively in media containing tungsten. A mixed culture of methanotrophs containing *Methylomonas* (56.26%) and *Methylophilus* (24.60%) were cultivated in batch cultures and tested for valorization of biogas derived from the anaerobic digestion (AD) (Zha *et al.*, 2020). The biomass derived from this experiment had a single cell protein content higher than 41% w/w, which included essential amino acids like histidine, valine, phenylalanine, isoleucine, leucine, threonine and lysine.

2.5.1.2. Continuous operation

In continuous operation (depicted in Figure 2.4), at least one of the nutrients is fed continuously throughout the process and the contents of the reactor, including cells, products, and residuals, are continuously removed from the reactor through an effluent stream (Winterbottom and King, 1999). By keeping the feed and effluent volumetric flow rates equal,

steady state is achieved – the volume of the culture and the nutrient concentrations in the continuous operation is kept constant (Shin and Lim, 2013). In a continuous culture, the residence time of a particle in the reactor is conversely equal to the dilution rate. Dilution rate is defined as the ratio of flow rate to the culture volume in the reactor. For a continuous culture to be at steady state condition, the dilution rate must be equal to the specific growth rate of the bacteria. If the dilution rate is not equal to the specific growth rate, the organisms present in the vessel will be washed out by time (Herbert *et al.*, 1956).

Continuous culture of Methylococcus capsulatus Bath grown using methane was investigated under a turbidostatic and oxystatic fermentation system and The specific growth rate was found to increase from 0.25 h^{-1} to 0.37 h^{-1} (Joergensen and Degn, 1987). In another study *Methylomicrobium buryatense* 5GB1 was grown in continuous culture under methane limitation and O_2 limitation and the bioreactor performance parameters were compared to batch and fed-batch cultures (Gilman *et al.*, 2015).

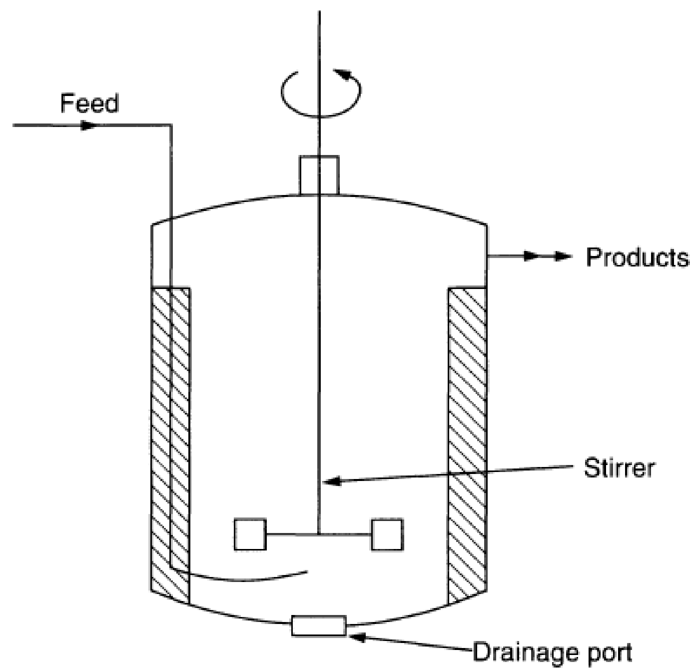

Figure 2.4. Simplified schematic of stirred-tank reactor operated in a continuous mode (Winterbottom and King, 1999)

Continuous cultures are generally not widely used in industries with the exception of single-cell protein production, certain beer production, and municipal waste treatment processes. The reason for this is due to disadvantages such as difficulty in maintaining sterility and lower product yield in continuous operation when compared to batch operation (Boedeker and Magnus, 2017).

2.5.1.3. Fed-batch operation

In fed-batch cultures at least one of the nutrients is either intermittently or continuously added to the bioreactor during operation. The reactor contents are then harvested at the end of the process, similarly to a batch culture (Winterbottom and King, 1999). The addition of nutrients to the culture results in the culture volume being variable during the run. Fed-batch operation is a versatile tool for regulating the concentration of specific compounds that might have a significant effect on growth and/or bioproduction by manipulating the feeding rate of nutrients into the reactor. Fed-batch operation has a greater productivity in processes involving inhibiting enzyme reactions and autocatalytic reactions (reactions in which one of the products acts as a catalyst), certain adiabatic reactions, and parallel reactions (Lim and Shin, 2013). Microbial cultures involve cell reproduction, so in this sense fermentation is an autocatalytic reaction.

Fed-batch cultures are widely used for industrially important fermentation and bioproduction. The oldest well-known process involving fed-batch cultures was for yeast cell production to keep the levels of sugar as low as possible in fermentation to avoid alcohol formation (Reed, 2012). One of the most important factors in running a fed-batch culture is the determination of which nutrients should be fed to the reactor and how they should be fed; answering these questions depends on the characteristics of the organism used and the nature of the target bioproduct. The compounds fed to a fed-batch bioreactor to control the growth rate typically consist of a carbon source, a nitrogen source, a phosphate source, or other limiting nutrients. Feeding laws can be incorporated to regulate the feeding rate and increase the productivity of bioprocesses.

Fed-batch cultures have been used in both laboratories and industrial plants to produce an array of products such as yeast, antibiotics, amino acids, fine organic acids, enzymes, alcoholic solvents, and recombinant DNA products (Lim and Shin, 2013).

Fed-batch operations have been used in bioproduction using methanotrophic bacteria. Fed-batch fermentation consisting of alternating high and low salinity cultivation stages were carried out for the growth of *Methylomicrobium alcaliphilum* 20Z to determine the effect of changes in salinity on ectoine synthesis (Cantera *et al.*, 2017). The results showed that under osmotic shock, *M. alcaliphilum* 20Z responded rapidly and released the accumulated ectoine under hyposmotic shock (Cantera *et al.*, 2017).

In another study, PHB was produced using *Methylosinus trichosporium* IMV3011 growing on methanol in a non-sterilized fed-batch operation (Song *et al.*, 2011). Using this technique, biomass concentrations of 2.91 g dry weight/L and PHB accumulation of 47.6% (w/w) were achieved. To increase the PHB content further, organic acids such as malic acid were added to inhibit the citric acid cycle (TCA) cycle. As a result, the PHB yield increased to 3.32 g dry wt/L and 58.5% (w/w), respectively, by adding 0.2 g/L malic acid and a molecular weight of up to 1.7×10^6 Da was obtained (Song *et al.*, 2011).

Another study investigated enrichment of a mixed culture of methanotrophs from an anaerobic digester process, and their capacity to store PHAs using cyclic fed-batch operation run for 70 days (Fergala *et al.*, 2018). The specific growth rate obtained was 0.078 h^{-1} and the biomass yield was 0.7 mg /mg-methane. The enriched culture was able to accumulate up to 51% PHB. Also, when adding valeric acid to the culture medium, polyhydroxybutyrate-co-valerate (PHBV) was produced up to 52%, with a hydroxyvalerate percentage of 33%, and increasing valeric acid up to 100 mg/L increased the overall PHBV by 60%. The effect of changing the methane to oxygen ratio from 1:1 to 4:1 resulted in a decline of almost 80% in PHB accumulation (Fergala *et al.*, 2018).

2.6. Metabolomics analysis

Metabolomics is an analytical technique for the comprehensive qualitative and quantitative analysis of metabolites present in biological specimens (Clish, 2015; Lajis *et al.*, 2017). Although metabolomics analysis is considered a relatively new field, metabolic studies were performed long ago in ancient China, where diabetes was detected using ants by evaluating the level of glucose in urine samples (van der Greef, and Smilde, 2005). In the late 1940s, Roger Williams and his colleagues first came up with the idea that the metabolic patterns of individuals could be different from each other and could be identified using their biological fluids (Gates and

Sweeley, 1978). The quantification of metabolic profiling was further facilitated in the 1960s and 1970s by the developments of gas chromatography (GC), liquid chromatography (LC), and mass spectrometry (MS). In 1971, GC-MS was successfully used to analyze the metabolites in human urine and tissue extracts (Horning and Horning 1971). In the past decade, high-resolution/sensitivity MS and nuclear magnetic resonance (NMR), accompanied by multivariate statistical analysis, have facilitated the use of metabolomics analysis to solve problems in the field of biology. Today metabolomics contribute greatly to numerous advances in life sciences, food and plant sciences, drug development, toxicology, environmental science, and medicine (Gowda and Djukovic, 2014).

Metabolomics is a powerful tool for bioprocessing industries: it is used to identify engineering targets, to improve the performance of strains during fermentation and bioprocesses, etc. This becomes even more significant when producing chemicals with high value but low yields, such as antibiotics (Choi *et al.*, 2018). Using metabolomics analysis for industrial applications is important because, aside from medium composition and process conditions, the performance of a bioreactor depends on the cells characteristics, based both on genotype and phenotype. During bioreactor operation, the metabolic activity of the cells is very high and the metabolites profiles vary extensively. Identifying the metabolites produced by the cells during the course of operation can shed light on many underlying cell-based activities (Sonntag *et al.*, 2011).

Metabolomics have been used in bioprocesses involving methanotrophic bacteria in many different ways. Methane metabolism in the methanotroph *Methylosinus trichosporium* OB3b has been studied (Kalyuzhanaya *et al.*, 2013). It was shown that similarly to non-methane utilizing methylotrophic alphaproteobacteria, the core metabolism of this microorganism consists of tightly connected metabolic cycles, such as the serine pathway, the ethylmalonyl-CoA (EMC) pathway, and the citric acid (TCA) cycle (Kalyuzhanaya *et al.*, 2013).

In another study, three phylogenetically diverse methanotrophic bacteria were grown using biogas streams derived from anaerobic digestion (AD) and were analyzed by metabolic profiling (Henard *et al.*, 2018). These strains were shown to maintain a comparable central metabolic activity and did not show growth inhibition under AD biogas stream or pure methane. However, metabolite analysis showed that the cells demonstrated oxidative stress when grown under biogas streams (Henard *et al.*, 2018).

In a study on the growth of *Methylomicrobium buryatense* 5GB1 on methanol and methane, a targeted metabolomics approach was used to investigate the core metabolism shift as a result of the change in carbon source. The results showed that when grown on methanol as opposed to methane, there is a shift in the active core metabolism and in both the Entner-Doudoroff pathway and the partial serine cycle; there was an increased flux while the TCA cycle was incomplete.

In other studies, comparative metabolomics analysis were used to compare on ethanol production by *Saccharomyces cerevisiae* growing in industrial batch and continuous fermentation processes (Ding *et al.*, 2009). Results showed that three main phases could be recognized in both modes of operation using principal components analysis in which the levels of metabolites involved in central carbon metabolism varied significantly. Also, the abundance of specific metabolites, such as glycerol and phosphoric acid, was an indicator of transition between the three phases in continuous operation, while lactic acid and glycerol contributed to various phases of the batch operation (Ding *et al.*, 2009).

Metabolites are the products of cellular regulatory processes. Their presence and abundance can thus be indicators of the response of the microorganisms to various growth conditions (Fiehn, 2002). Metabolomics analysis is a great way to investigate the response of an industrial strain to various conditions and modes of operations, and to establish conditions that can be favorable to specific bioproducts.

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3. Effect of copper on growth of *Methylomicrobium album* BG8 in batch cultures using methanol

3.1. Abstract

Methanotrophic bacteria are a group of microorganisms able to use single-carbon compounds as their main source of carbon and energy. Methane, a potent greenhouse gas, and methanol, a byproduct of pulp processing, can both be used by these bacteria as feedstock for conversion to valuable bioproducts. To improve the economic viability of using such bioconversion strategies at industrial scale, it is crucial to enhance growth of the bacteria and improve the productivity of the bioprocesses. Copper has been known to influence the growth of methanotrophs not only through regulation of expression of the two forms of the enzyme methane monooxygenase (MMO), but also through interfering with the effect of some rare earth elements on regulation of the enzyme methanol dehydrogenase (MeDH). This study investigates the effect of copper on growth of the methanotrophic bacteria *Methylomicrobium album* BG8 with methanol as the main source of carbon. The results show that the highest optical densities (0.52) were achieved when no copper was supplemented to the culture. While addition of copper did not significantly affect the final optical densities, it negatively impacted growth rates. The results suggest that, for a methanol concentration of 25 mM, limiting copper concentration below 0.01 mM renders optimal conditions in terms of both final optical density (0.4052) and growth rates (0.0355).

3.2. Introduction

Methanotrophs are aerobic bacteria that use methane as their sole source of carbon and energy (Hanson et al., 1996). These bacteria are in fact a subset of methylotrophs, microbes that can grow on reduced single- (C1) or multi-carbon compounds lacking carbon-carbon bonds (Chistoserdova et al., 2003). Some methanotrophs are also able to grow on other carbon sources, such as methanol, methylamines, and acetate (Bowman, 2006). Methanotrophs can be found in a wide range of environments such as soils, wetlands, marshes, lakes, oceans, and rice paddies (Smith. et al., 2010). This diversity translates into versatility towards processing many different

compounds (Vorobev et al.; 2013; Eswayah et al., 2017; Lai et al, 2016b). Hence, methanotroph-based biotechnological applications have been developed, including the degradation of organic pollutants (DiSpirito et al., 1992; Oldenhuis et al., 1989), metals detoxification (Al Hasin et al., 2009; Lai et al., 2016a; Vorobev et al.; 2013; Baral et al., 2014) and control of methane emissions (Oswald et al., 2016; Khmelenina et al., 2018)

Methanol is one of the byproducts of the forestry industry in North America, which is usually considered a waste product and is disposed. In a Kraft process, lignin is separated from cellulose fibers, which are used for paper-making. Lignin then goes through a de-methylation process, which results in the production of methanol (Zhu et al., 2000). Approximately, 10 to 15 Kg of methanol is produced per ton of air dried pulp (ADT) in a typical Kraft mill. About 108 million tons pulpwood is annually processed in the pulp and paper industry in the US alone (Bajpai, 2012). Although the methanol produced in this process is usually disposed as an undesirable byproduct, it can be used by methanotrophs to be transformed into valuable products such as 3-polyhydroxybutyric acid (PHB). Growth of methanotrophs on methanol can be advantageous relative to growth on other one-carbon compounds since growth on methanol reduces oxygen consumption and energy input demands (McDonald, 2019). Also, mass transfer limitations using a liquid carbon source such as methanol is reduced significantly when compared to a gaseous feedstock such as methane.

The advantage of using methanol for growth of methanotrophs is that growth on methanol reduces oxygen consumption and energy input demands. Also, since methanol is usually handled in liquid form, mass transfer limitations will be reduced in comparison to a gas phase source of carbon like methane.

The ability of methanotrophs to utilize C1 compounds can be used towards the production of valuable products, such as biopolymers (Asenjo *et al*., 1986; Valappil *et al*., 2007; Khosravi-Darani *et al*., 2013), soluble metabolites (Kalyuzhnaya *et al*., 2013), formaldehyde and organic acids (Strong *et al*., 2015), lipids for biofuels (Knothe *et al*, 2010; Sunde *et al*., 2011), and single-cell proteins (Øverland *et al*., 2010). Since methanol is a common by-product of many industrial activities, waste methanol effluents can be considered a cheap and abundant feedstock for the production of value-added products.

There have been many efforts to scale methanotroph-based processes up to industrial scales. Researchers have tried to enhance growth to optimize the production of different compounds (Jeong *et al*., 2014; Sundstrom *et al*., 2015), and increase the uptake of methane or even compounds such as trichloroethylene (TCE) (Soni *et al*., 1998; Reddy *et al*., 2014). Many studies have focused on the effects of addition of chemicals (Cai *et al*., 1999; Xing *et al*., 2006), media composition (Hoefman *et al*., 2014), pressure (Wendlandt *et al*., 1993), etc. on the growth of the bacteria. For example, addition of riboflavin and organic acids such as malate, succinate, maleate and citrate have been shown to improve the cell concentration of *Methylosinus trichosporium* OB3b in the presence of methane. Dry cell weights increased 3.5-fold when citrate was added to the growth media compared to controls without citrate (Xing *et al*., 2006). Addition of the co-metabolic substrate, chloromethane, enhanced the growth of *Methylomicrobium album* BG8 on methanol, although it could not be used as the sole growth substrate (Han *et al*., 2000). This suggests that the reactions previously thought to be cometabolic can be beneficial to the growth of methanotrophs (Han *et al*., 2000). The presence of other bacterial species can also stimulate both methanotrophic growth and substrate utilization. One of these bacteria, *Rhizobium* sp. Rb122, has been shown to stimulate the growth of *Methylovulum miyakonense* HT12 through the production of cobalamin (Iguchi *et al*., 2011). Finally, applying moderate atmospheric pressure on methanotrophic bacteria has also been shown to increase biomass concentration by increasing the methane and oxygen mass transfer in cultures of *Methylocystis* sp. GB 25 (Wendlandt *et al*., 1993).

Copper has long been considered central to aerobic bacterial methane oxidation. Methanotrophs oxidize methane to carbon dioxide through the intermediates methanol, formaldehyde, and formate (Hanson *et al*. 1996). Two forms of methane monooxygenase (MMO) – the enzyme that converts methane to methanol – exist: an iron-containing soluble form (sMMO) and a copper-containing membrane-bound particulate form (pMMO). For methanotrophs containing both sMMO and pMMO, copper availability plays a key role in the expression of either type of the enzyme (Dalton *et al*., 1984; Stanley *et al*., 1983; Murrell *et al*., 2000). This unique metabolic phenomenon, known as a “copper switch”, has been investigated in methanotrophs such as *Methylococcus capsulatus* (Bath) (Nielsen *et al*., 1996; Nielsen *et al*., 1997) and *Methylosinus trichosporium* OB3b (Stafford *et al*., 2003), which possess both types of MMO, growing on methane. Studies showed that at lower copper concentrations, these

methanotrophs express sMMO, whereas at higher copper concentrations pMMO is expressed more significantly (Nielsen *et al*., 1996; Nielsen *et al*., 1997; Stafford *et al*., 2003). While pMMO has a higher affinity for methane, sMMO has a greater turnover rate. Thus, copper availability can be used as a tool to control the activity of methanotrophs and to devise better bioremediation strategies (Semrau *et al*., 2018).

The effect of copper on the growth of methanotrophic bacteria is not limited to MMO expression. Methanotrophs use methanol dehydrogenase (MeDH) to oxidize the methanol generated from methane oxidation to formaldehyde. Many methanotrophs possess multiple MeDH systems. One is the calcium-containing Mxa-MeDH and the other is Xox-MeDH, which contains rare-earth elements (e.g. lanthanum) in its active site (Chu *et al*., 2016). Although cerium has been shown to affect the expression of Mxa-MeDH and Xox-MeDH in some strains of methanotrophic bacteria, the presence of copper in the culture medium was shown to significantly impede on this effect (Haque *et al*., 2015; Gu *et al*., 2016; Gu *et al.*, 2017). Cerium is a rare earth element belonging to the lanthanide group, the presence and type of which has been shown to influence the enzymatic activity and the growth rate of methanotrophic bacteria (Picone *et al.*, 2019). The presence of these rare earth elements regulates the gene expression between calcium and the Ln-dependent MeDH genes known as ‘lanthanide switch’ (Picone *et al.*, 2019). Studies showed that growth of the methanotrophic bacterium *Methylacidiphilum fumariolicum* SolV was enhanced when rare earth elements such as cerium, lanthanum, praseodymium, and neodymium were present (Pol *et al.*, 2014). From these findings it can be speculated that, under different copper concentrations, the expression of genes encoding for sMMO and pMMO could vary and that the expression of the two alternative forms of MeDH could also be affected.. As such, it is important to establish whether copper has an effect on the growth of methanotrophic bacteria when methanol is the sole source of carbon, and if so, the scale of this effect. In this study, we thus investigated the effects of copper concentration on the growth rate and yield of the methanotrophic bacterium *Methylomicrobium album* BG8 in the presence of various concentrations of methanol as the sole carbon source.

3.3. Materials and Methods

3.3.1. Strains and growth conditions.

The bacterium *Methylomicrobium album* BG8 (ATCC 33003) was grown at 30°C and 150 rpm in 250-ml Wheaton glass media bottles (capped with butyl-rubber septa cap to avoid headspace losses) and containing 100 ml of modified nitrate mineral salts (NMS) medium (Whittenbury *et al*., 1970) with various concentrations of copper ($CuSO_4$; 0-0.3 mM). For growth without added copper, no $CuSO_4$ was added to the medium. The pH of the cultures was kept at 6.8 by addition of 1.5 ml of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4 in distilled water) to 100 ml of culture. High performance liquid chromatography-grade methanol was used as the sole carbon source at concentrations ranging from 8-150 mM. A previously grown culture no older than one week was used for inoculation at 1% v/v. The experiments were conducted at atmospheric pressure and all conditions were performed in triplicates.

3.3.2. Growth.

Growth of the bacteria was monitored by determining the optical density (OD) at 540 nm of 500-µl samples using a microplate reader (Multiskan Spectrum, Thermo Scientific). Growth rate (µ) was determined based on values of OD obtained within the exponential phase of the growth curve (linear section of a plot of ln X vs time) for each experiment using the equation:

$$\mu = \frac{ln\left(\frac{X_2}{X_1}\right)}{(t_2 - t_1)} \quad (1)$$

3.4. Results

Preliminary growth experiments were performed at 25 mM methanol and copper concentrations ranging from 0 to 0.075 mM to establish whether there was an optimal copper to methanol ratio for growth. The assumption for these preliminary experiments was that there could be an optimal copper to methanol ratio for which the growth of *M. album* BG8 is maximized. Based on the results of these preliminary experiments, the hypothetical optimum

copper to methanol ratio was estimated to be 2×10^{-4}. The concentrations of copper at six different methanol concentrations shown in the experiments in Figure 3.1 were determined based on this hypothetically optimal ratio; however, further results from this study dispel the existence of an actual optimal copper to methanol ratio for the growth of *M. album* BG8 using methanol as the main source of carbon.

Figure 3.1 shows the growth of *M. album* BG8 at six concentrations of methanol (8 mM, 12 mM, 25 mM, 50 mM, 100 mM and 150 mM) and varying concentrations of copper (0-0.3 mM). As seen in Figure 3.1, at concentrations of methanol lower than 25 mM, the growth was lower and the final ODs observed were lower than 0.4. On the other hand, at methanol concentrations higher or equal to 25 mM, the ODs were higher except when copper concentrations were increased beyond 0.2 mM (which results in reduced OD). The negative effect of excessive copper concentrations on growth can be observed in Figure 3.1, where no growth was observed at a copper concentration of 0.3 mM and a methanol concentration of 150 mM. Another effect that can be seen in Figure 3.1 is the impact of higher copper concentrations on the growth rate. In Figure 3.1d, e and f, at copper concentrations greater than 0.05 mM, the growth rate of the bacteria declined notably in comparison to lower copper concentrations.

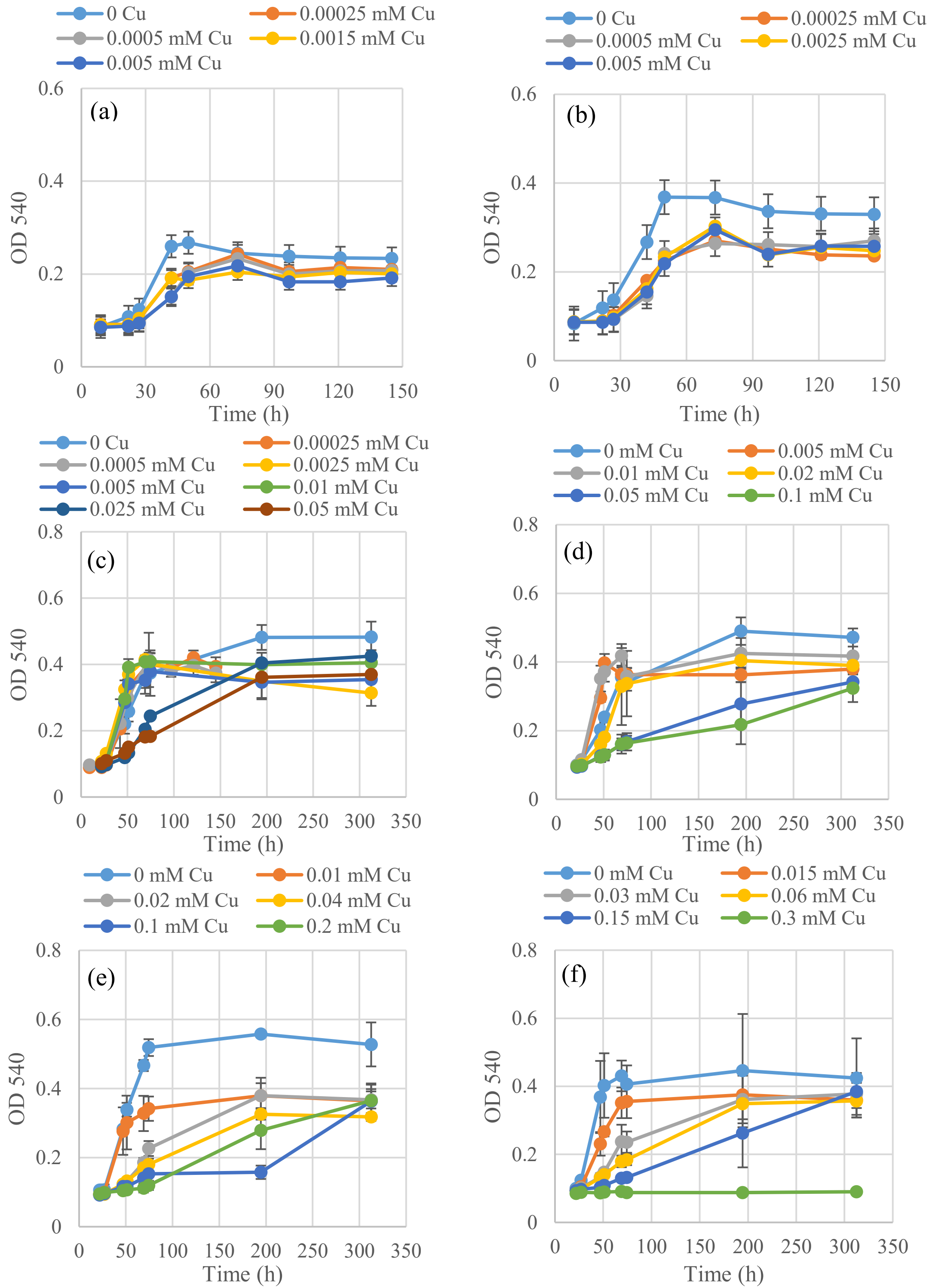

Figure 3.1. Growth of *Methylomicrobium album* BG8 under various concentrations of methanol and copper. (a) 8 mM methanol, (b) 12 mM methanol, (c) 25 mM methanol, (d) 50 mM methanol, (e) 100 mM methanol, (f) 150 mM methanol.

One-way ANOVA was conducted to compare the combined effects of copper and methanol concentrations on the final OD achieved between the experiments reported in Figure 3.1(a-f). Based on this analysis, a significant effect of methanol and copper treatments on the final OD was observed for all six conditions tested ($p = 0.000174$). Post hoc comparisons using the Tukey HSD tests indicated that the mean value of the final OD achieved for cultures grown on 8 mM methanol (0.2013 ± 0.0076) was significantly different from those measured for cultures grown on 25 mM (0.3895 ± 0.0504), 50 mM (0.3863 ± 0.0558) and 100 mM (0.3846 ± 0.0722) methanol. The OD obtained from the 12 mM methanol treatment (0.2546 ± 0.0131) was also significantly different from those measured at 50 mM, 100 mM and 150 mM (0.3279 ± 0.1329). The final OD for all other treatments did not significantly differ from one another.

The final ODs of cultures grown at different methanol concentrations (from Figure 3.1) are reported as a function of copper in Figure 3.2. As can be seen, at methanol concentrations of 8, 12 and 25 mM, increasing copper concentration did not have a significant effect on growth except for the presence of a minimum in OD observed for low copper concentrations when growing on 25 mM methanol. At concentrations of 50, 100 and 150 mM methanol, increasing the copper concentration generally resulted in a decrease in final OD. In addition, for all concentrations of methanol tested, the highest OD was achieved when cultures were grown when no copper was added; addition of any concentration of copper led to lower OD.

To further investigate the effect of addition of copper on the final OD, ANOVA tests were performed for each amount of copper tested at each concentration of methanol. There was a significant effect of copper concentration on the final OD ($p < 0.05$) for the cultures grown on methanol concentrations of 8 mM ($p = 0.0190$), 12 mM ($p = 0.0351$), 25 mM ($p = 0.0010$), 50 mM ($p = 0.0119$), 100 mM ($p = 0.0010$) and 150 mM ($p = 0.0002$).

Table 3.1 shows the yield of the growth of *M. album* BG8 at the conditions tested in Figure 3.1. This yield is calculated by dividing the OD obtained in each experiment over the concentration of methanol used as the source of carbon in that culture. As shown in Table 3.1, the yield was higher in lower concentrations of methanol. The highest yields were obtained for 8 mM methanol ($0.0262\ mM^{-1}$), although in general the ODs for 8mM methanol cultures were lower when compared to cultures supplied with greater methanol concentrations. As the

concentration of both methanol and copper increased, the yield decreased to values as low as 0.0023 mM^{-1}.

Further analysis was done on the cultures shown in Figure 3.1 to investigate the effect of copper and methanol concentrations on the growth rate of the bacteria. Figure 3.3 shows the variation of growth rate as a function of copper concentration for different concentrations of methanol. ANOVA test followed by post hoc Tukey HSD tests were performed to identify the data points that were significantly different for a given methanol concentration. As can be seen, at lower concentrations of methanol (8 and 12 mM), variations in copper concentration did not have a significant effect on the growth rate of the cultures except in cases for which no copper was added, which had the fastest growth rate observed. At higher concentrations of methanol (25, 50, 100 and 150 mM), the growth rate had a decreasing trend with increase in copper concentration. The slowest growth rate ($<0.01\ h^{-1}$) was observed for cultures containing more than 0.2 mM copper.

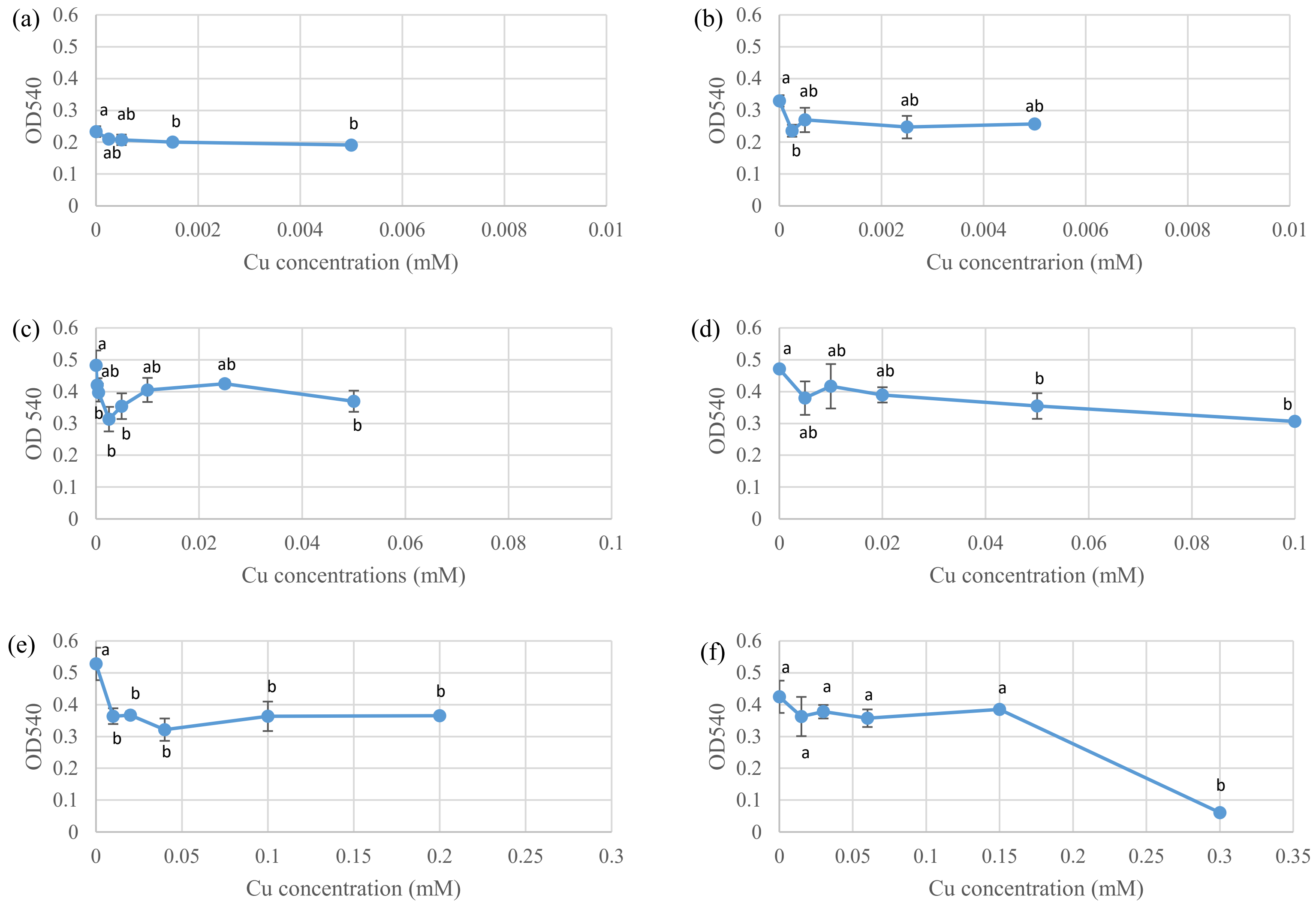

Figure 3.2. Variation of final OD of *M. album* BG8 as a function of copper concentration methanol concentration. (a) 8 mM, (b) 12 mM, (c) 25 mM, and (d) 50 mM, (e) 100 mM and (f) 150 mM methanol. Data points sharing the same letter within a graph were found to be statistically equal.

Table 3.1. Yield (ΔOD·mM methanol^{-1}) for the growth of *M. album* BG8 at various concentrations of methanol and copper.

	Methanol concentration (mM)					
Copper supplementation (mM)	8 mM	12 mM	25 mM	50 mM	100 mM	150 mM
0	0.0246	0.0218	0.0192	0.0094	0.0052	0.0028
0.00025	0.0262	0.0196	0.0157			
0.0005	0.0259	0.0224	0.0149			
0.0015	0.0250					
0.005	0.0239	0.0214	0.0141	0.0075		
0.0025		0.0206	0.0125			
0.01			0.0162	0.0083	0.0036	
0.015						0.0024
0.025			0.0170			
0.03						0.0025
0.04					0.0032	
0.05			0.0147	0.0070		
0.06						0.0023
0.02				0.0077	0.0036	
0.1				0.0061	0.0036	
0.15						0.0025
0.2					0.0036	
0.3						0.0025

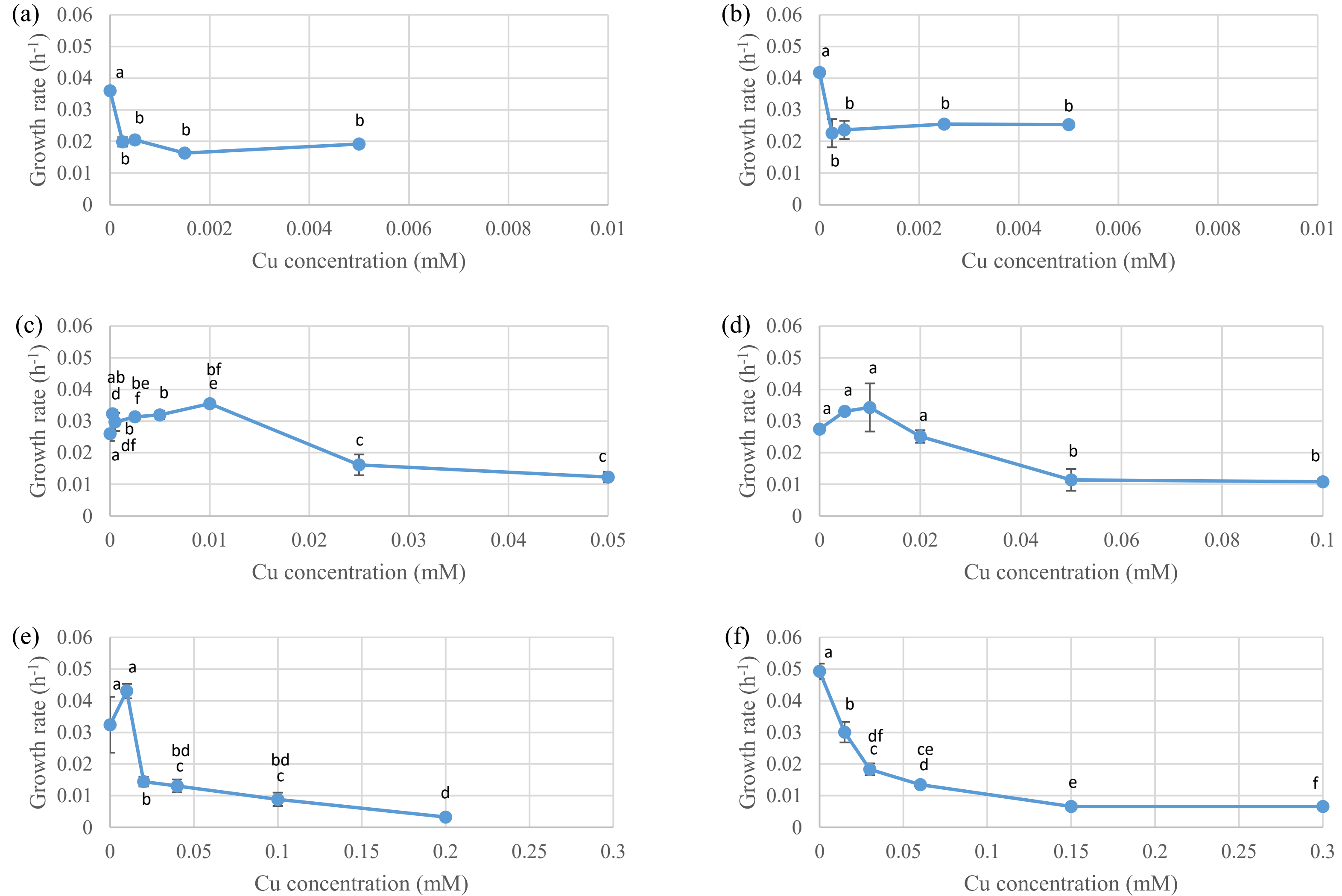

Figure 3.3. Variation of growth rate of *M. album* BG8 as a function of copper concentration for methanol concentrations of (a) 8 mM, (b) 12 mM, (c) 25 mM, and (d) 50 mM, (e) 100 mM and (f) 150 mM. Data points sharing the same letter within a graph were found to be statistically equal.

3.5. Discussion

In evaluating the impact of copper and methanol concentrations, analysis of the final OD provides important information. The final OD obtained at various methanol concentrations (Figure 3.1) shows that increasing the concentration of methanol from 12 to 25 mM increased the final OD significantly (from about 0.2 to about 0.4). But at methanol concentrations above 25 mM, the final OD did not increase significantly and, in fact, started to decrease at the highest methanol concentrations tested (100 and 150 mM). This is reflected in the values of yields of OD to methanol, which were not constant for the conditions tested (Table 3.1). In fact the yield ranged from 0.0262 OD mM^{-1} for 8 mM methanol and 0.00025 mM copper conditions to 0.0023 OD mM^{-1} for 150 mM methanol and 0.06 mM copper condition. The decrease in yield at higher methanol concentrations (0.0023 OD mM^{-1} at 150 mM methanol) was likely the result of methanol toxicity, which has been demonstrated in most methanotrophs at concentrations as low as 0.01 % v/v (Leadbetter *et al*., 1958; Stocks *et al*., 1964; Whittenbury *et al*., 1970). However, based on the results shown in Figure 3.1d, e and f, lower yields could not solely be explained by the effect of methanol toxicity, since growth rates (Figures 1d and e) and final OD (Figure 3.1f) were also affected at higher copper concentrations. In fact, it appears there was no significant growth at 0.3 mM copper and 150 mM methanol, while final ODs were not impacted at the same methanol concentration with lower copper. Comparing cultures grown on 150 mM methanol and 0.3 mM copper with cultures grown on less copper, it can be presumed that the observed decline in the final OD and yield of the copper-containing cultures would also be the result of inhibitory effects of copper at these concentrations. Previous studies have shown that high copper concentrations can be toxic to microorganisms (Lerch, 1980; Rosen, 1986; Trevors *et al*., 1990).

Also, based on the statistical analysis at methanol concentrations of 8 mM and 12 mM, the OD was generally lower than at concentrations of 25 mM, 50 mM, 100 mM, and 150 mM. Whereas, increasing the methanol concentration from 25 mM to 150 mM did not significantly increase the optical density. This could mean that at concentration of 8 mM and 12 mM, methanol is the limiting nutrient for the growth of *M. album* BG8.

Figure 3.2 presents a better demonstration of the effect of copper concentration on the final ODs at various levels of methanol. As statistical analysis reveals, for cultures grown in all concentrations of methanol other than 150 mM, the final OD of the cultures with no copper

addition was significantly different from the cultures that were grown in the presence of copper (Figure 3.2). As seen in Figure 3.2a-e, contrary to expectations, cultures containing no additional copper had a higher final OD than cultures grown in copper-amended media. This is in disagreement to previous findings in which copper availability was shown to increase biomass yields in methanotrophic bacteria (Leak *et al*., 1986; Collins *et al*., 1991; Peltola *et al*., 1993; Nielsen *et al*., 1997 Murrell *et al*., 2000). Although it is important to note that, unlike in the present work, most studies on the effect of copper on growth of methanotrophic bacteria have been done with methane as the main source of carbon. Furthermore, copper remaining in the water and glassware was not chelated prior to these experiments, such that trace copper was continuously available to the cultures even when copper was not added.

The highest OD and yields were obtained in cultures in which there was no copper addition, regardless of the methanol concentration used. However, looking only at experiments conducted with media for which copper was added (Figure 3.2), Tukey analysis showed there was no significant difference between the final ODs obtained for a given methanol concentration. This could mean that increasing the concentration of copper does not affect the achieved final OD. The results of the Tukey HSD test shows that at each concentration of methanol, addition of more copper to the culture did not lead to an increase in the final OD achieved compared to the cultures with no added copper. This is in contrast with previous findings on the effect of copper in growth of methanotrophic bacteria growing on methane (Leak *et al*., 1986; Collins *et al*., 1991; Peltola *et al*., 1993; Nielsen *et al*., 1997 Murrell *et al*., 2000). Methanotrophic bacteria use the enzyme methane monooxygenase to oxidize methane to methanol, which is available in the forms of sMMO and pMMO (Dalton et al., 1984; Stanley *et al*., 1983; Murrell *et al*., 2000; Sazinsky *et al*., 2015). When sufficient copper is present, sMMO is down-regulated and pMMO is the preferred enzyme to oxidize the carbon source (Nielsen *et al*., 1997; Stafford *et al*., 2003; Semrau *et al*., 2010). *M. album* BG8 contains only pMMO as a form of methane monooxygenase (Lloyd *et al*., 1999; Semrau *et al*., 2018) and hence there is no copper switch involved in this organism. But, since in this study methanol was used as the substrate, the bacteria do not require MMO to oxidize the substrate, explaining the lack of effect of the concentration of copper on yield. It is also important to note that even the cultures that were not supplied with copper could possibly have some copper carried over from the overnight culture and from the glassware, which was used as the inoculum for these experiments. This implies that higher ODs achieved in

the cultures without additional copper does not invalidate the importance of copper for the growth of *M. album* BG8 under methanol. But, it could mean that lower copper concentrations might be more favorable with methanol as the sole carbon source rather than methane.

Also, the results of the Tukey HSD tests in Figure 3.2f show that, although addition of copper to the culture did not enhance the final OD, high concentrations of methanol (150 mM) and copper (0.3 mM) actually resulted in a decrease in the final optical density of the culture.

On the other hand, copper concentration clearly affected growth rate more substantially at all concentrations of methanol (Figure 3.3). Statistical analysis of growth rates showed that at all methanol concentrations except 25 mM, the growth rate of the culture without copper amendment was significantly greater than that of cultures grown in media with copper addition. Based on the statistical analysis, at 8 and 12 mM methanol the growth rate of the bacteria grown in copper-amended media did not differ from each other significantly; and increasing the copper concentration had no effect on the growth rate. However, analysis of the significance of the data in cultures grown on 50, 100 and 150 mM methanol showed that increasing the copper concentration resulted in a decline in growth rate. This effect was seen at copper concentrations greater than ~0.02 mM in 50 and 100 mM methanol. However, for the 150 mM methanol cultures, any addition of copper to the media led to significant decreases in growth rate. Also, based on the statistical analysis, at 50 mM methanol the growth rate for cultures with no added copper was not significantly different to that of cultures with copper concentrations up to 0.02 mM; contrasting with cultures containing 8 mM, 12 mM, 100 mM and 150 mM methanol, for which faster growth rates were observed for cultures with no added copper. However, addition of copper at concentrations above 0.02 mM led to decreases in growth rates similar to those observed at 8 mM, 12 mM, 100 mM and 150 mM methanol.

In contrast, for the experiments conducted at 25 mM methanol, addition of up to 0.01 mM copper to the media resulted in increased growth rate; beyond this concentration growth rate declined with increasing copper concentration. It seems that when methanol was not impeding growth – e.g. at concentration of 25, 50 and 100 mM methanol –, low concentrations of copper had a positive effect on the growth rate.

These results suggest that the selection of methanol and copper conditions towards methanotroph-based industrial applications depends more on the goal of the bioprocess at hand.

From Figure 3.2 it is evident that to achieve the highest growth of *M. album* BG8 the process may benefit from a lower copper concentration. But, if the goal is to rapidly reach a specific level of biomass, conditions favoring a process with higher growth rates without impeding the OD should be selected. As mentioned above, in the conditions tested, 25 mM methanol led to high yields and faster growth rates of *M. album* BG8. Also, based on results of final OD and growth rate (Figures 1 and 3), media at 25 mM methanol allowed a threshold at copper concentrations between 0.00025 and 0.01 mM that rendered a satisfactory combination of OD, growth rate and copper tolerance.

3.6. Conclusion

This work shows that the highest OD acquired for the growth of methanotrophic bacteria *M. album* BG8 under methanol as the substrate can be achieved in low concentrations of copper. On the other hand, variations in copper concentration did not seem to have a significant effect on the resulting final OD and yield. Rather, the variation in methanol concentration had the most significant effect on these parameters. 25 mM methanol was found to be the most favorable condition for the growth of the bacteria. Although adding copper to the media did not significantly affect the final OD – except when both methanol and copper were at higher concentrations –, it did have an important impact on growth rate. It seems that at optimum methanol concentration like 25 mM, addition of copper at very low concentrations of less than 0.01 mM can improve the growth rate. But at the lower and higher concentrations of methanol tested (8 and 12 mM, and 100 and 150 mM), addition of copper led to gradual decreases in growth rate. To find a suitable process for the growth of *M. album* BG8, concentrations of copper and methanol should be carefully adjusted to the goal of the bioprocess.

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3.8. References

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4. Step-wise fed-batch strategy for enhancing growth of *Methylomicrobium album* BG8 using methanol as sole carbon source

4.1. Abstract

The interest in methanotrophic bacteria has increased over the recent years, particularly in the context of climate change. These bacteria can utilize methane and other single carbon compounds, such as methanol, as their main source of carbon and energy. Methane is a potent greenhouse gas with a global warming potential of approximately 34 times greater than carbon dioxide on a 100-year basis. Methanol, on the other hand, is largely produced as an industrial by-product of, among others, the forestry and pulping industries. Methanotrophic bacteria are capable of converting methane and methanol to value-added products. This conversion has long been studied in laboratories but much effort is still needed to enhance productivity for implementation at industrial scale. In this study, different methanol feeding strategies (pulse and step-wise addition) were used to improve cell density of the methanotrophic bacterium *Methylomicrobium album* BG8 through fed-batch operation. In step-wise feeding, the rate of methanol addition was set based on the cell density of the culture present in the bioreactor and the desired growth rate. This approach allowed improvement of growth and minimized accumulation of methanol in the system. By controlling the specific growth rate to 0.0135 h^{-1} and 0.027 h^{-1}, the maximum optical density obtained was respectively ~18.8- and 17.6-fold greater than that obtained in a conventional batch process. Although the final optical density was greater at a specific growth rate set-point of 0.0135 h^{-1}, the duration of the experiment was greater than for 0.027 h^{-1}; this was mostly due to slower growth rate and longer lag phases. In comparison, fed-batch operation based on pulsing methanol addition achieved a ~13.4 fold increase compared to batch operation. The present study establishes a basis for the improvement of growth and productivity towards the industrial application of *M. album* BG8 as a methanol bioconversion platform.

4.2. Introduction

Methanotrophic bacteria are a group of aerobic bacteria that can use a number of single-carbon compounds as their main source of carbon and energy (Hanson *et al*., 1996). These bacteria are ubiquitous in nature and can be found in various environments such as soils, wetlands, rice paddies, landfills and aquatic systems (Smith *et al*., 2010). Methanotrophs have a significant environmental role but have also great potential to be used in numerous applications, including assimilation and mitigation of methane emissions (Hanson *et al*., 1996; Wendlandt *et al*., 2010), remediation of toxic organic compounds (Sullivan et al., 1998; Ho *et al*., 2019), and production of valuable compounds (Bothe *et al*., 2002; Valverde-Pérez *et al*., 2020; Asenjo *et al*., 1986; Karthikeyan *et al*., 2014).

Many methanotrophic bacteria have the capacity to grow on methanol – a compound toxic to most bacteria at concentrations of 0.1% or higher. This compound is a common by-product of forestry and pulping industries, and is produced during the cooking process in Kraft pulp mills (Kuosa *et al.*, 2012). Methanol is released from the wood in the digester, transferred to the weak black liquor and to the flash vapor from the blow tank before being condensed in the form of digester condensate. The methanol in weak black liquor is further flashed in an evaporation process and then condensed as evaporator condensate. In a typical Kraft mill, between 10 to 15 kg of methanol are produced per ton of air dry pulp (ADT), which corresponds to about 11 to 16 ton methanol per day for a 1000-air dried tonnes (ADMTP) mill, assuming a 10% pulp yield loss in the process (Valmet technical paper series, 2018). In the US alone, the pulp and paper industry processes about 98 million tonnes pulpwood annually (Bajpai, 2012). Methanol produced in the pulp and paper industry used to be regarded as a waste requiring treatment before disposal, but it can be purified and sold as a commodity to produce valuable products (Yoneda *et al.*, 1994; Liu *et al.*, 2018; Zhang *et al.*, 2018).

Many methanotrophs can convert methanol to a wide range of products, such as organic acids, biopolymers and single cell proteins. For example, lactate, an industrial platform chemical and a precursor to the bioplastic polylactic acid, can be produced by *Methylomicrobium alcaliphylum* (Kalyuzhnaya et al., 2013) and *Methylomicrobium buryatense* (Henard *et al*., 2016). Polyhydroxyalkanoates, environmentally friendly alternatives to conventional non-biodegradable plastics, are produced by many alphaproteobacterial methanotrophs. In fact,

various strains of methanotrophs have been shown to produce polyhydroxybutyrate (PHB) under conditions of nutrient limitation and/or carbon excess (Whittenbury *et al*., 1970; Pieja *et al*., 2011) and have been implemented in industrial settings. *Methylocella* and *Methylobacterium extorquens* grown on methanol have been shown to produce PHB (Bourque *et al.*, 1992; Dunfield *et al*., 2003; Mokhtari-Hosseini *et al.*, 2009; Höfer *et al.*, 2010; Rohde *et al.*, 2017). Using methanol as an alternative substrate for PHB production was shown to be advantageous for various reasons such as of low price, moderate oxygen requirements, and complete water miscibility (Khosravi-Darani *et al.*, 2013).

There is extensive interest in utilizing methanotrophs in industrial settings; however, despite much progress, many obstacles must still be overcome in order to scale up laboratory experiments to industrial scales. Many studies have sought to increase the productivity of methylotrophs to make bioprocesses more economically viable at commercial scale. These tackled a range of strategies such as adjusting the carbon and nitrogen source (Tays *et al*., 2018), feeding strategies (Bourque *et al*., 1995; Mokhtari-Hosseini *et al*., 2009) and physical modifications of bioreactor setups to increase mass transfer of methane to the media (Meraz *et al*., 2020). In one study, improving feed composition and feeding strategies resulted in higher biomass density and productivity for the production of PHB from methanol by *M. extorquens* (DSMZ 1340) (Mokhtari-Hosseini *et al*., 2009). In a similar study on high-cell-density production of PHB from methanol by *Methylobacterium extorquens* ATCC 55366, cell biomass levels reached between 100 g/L and 115 g/L (dry weight) with PHB contents between 40% and 46% (dry-weight basis) (Bourque *et al*., 1995). This was achieved through maintaining low methanol concentrations through addition in proportion to the amount of available dissolved oxygen (DO), thus preventing oxygen limitation. This experiments was done in a two-phase process consisting of a biomass production phase with no limitation on nitrogen and a PHB production phase stimulated by a nitrogen limitation period.

Multiple studies have used bioreactor systems with the aim to scale up and industrialize applications of methanotrophs. Two examples include the use of gas-phase bioreactors to demonstrate the feasibility of using *Methylomonas methanica* to remove methane from coal mine atmosphere (Apel *et al*., 1991), and nitrate removal in a membrane biofilm reactor (Modin *et al*., 2010). A significant portion of the research in this area has focused on developing feeding

strategies to increase the efficiency of processes. In a study on TCE degradation by methanotrophic enrichment, intermittent TCE feeding accompanied by continuous methane addition led to sustained TCE degradation (Walter *et al*., 1997). Also, fed-batch and continuous feeding strategies have been employed to grow *Methylomicrobium alcaliphilum* 20Z under high and low salinity for bioconversion of methane to ectoine (Cantera *et al*., 2017). Some studies have targeted the effect of mineral nutrients on growth and methane utilization. Customized media were designed to increase the growth of 12 different *Methylomonas* strains – in some cases leading to 50% increase in cell dry weight (Hoefman *et al.*, 2014). In another study, the effects of nutrients such as ammonia, iron, copper, manganese, phosphate, and sulphide on the kinetics of methane utilization by a mixed culture of methanotophic bacteria were investigated (Boiesen *et al*., 1993). Iron was shown to increase the maximum methane utilization rate and yield, whereas relatively high levels of ammonia (70 mg/l) and copper (300 µg/l) inhibited methane uptake (Boiesen *et al*., 1993).

In this study we investigated a dynamic step-wise fed-batch strategy regulating the addition of methanol in bioreactor cultures of the gammaproteobacterial methanotroph *Methylomicrobium album* BG8 to increase cell density and productivity. The performance was compared to batch and pulse-input fed-batch operation. Establishing a target growth rate at the onset of each feeding step enabled significant increases in cell density and improvements in productivity – mostly through a reduction of fermentation time. This study opens the door to towards the implementation of *M. album* BG8 in industrial contexts.

4.3. Materials and methods

4.3.1. Strains and culture maintenance.

The methanotrophic bacterium *Methylomicrobium album* BG8 ATCC 33003 was grown in 100-ml batches in 250-ml Wheaton glass bottles fastened by butyl-rubber septa caps under constant shaking at 150 rpm and 30°C. Cultures were grown in nitrate mineral salt (NMS) medium (Whittenbury *et al.*, 1970). 2.5 mmol of air were extracted from the bottle head space (in order to keep the pressure at 1 atm) and the same volume of methane was injected using 0.22-µm filter-fitted syringes. 1.5 ml of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) were also

added to keep the media at pH 6.8. The bottles were then inoculated with 1 ml of a culture of *M. album* BG8 previously grown under identical conditions. These cultures were grown and used as the inoculating cultures in bioreactor batch and fed-batch experiments.

4.3.2. Bioreactor experiments.

The experiments were performed in a 3-L bioreactor controlled by a New Brunswick BioFlo® Benchtop unit. The bacteria were grown at 30°C, and DO levels were monitored using an Inpro 6800 polarographic oxygen sensor (Mettler Toledo) and kept above 30% by controlling agitation (220-1000 rpm). Air was introduced into the bioreactor through a sparger and dispersed by two impellers. Air flow into the reactor was kept at 0.75 VVM and passed through a 0.3-μm Whatman Hepa-Vent^{TM} in-line filter to ensure aseptic air addition. Nitrate mineral salt (NMS) medium (Whittenbury *et al.*, 1970) was used as the growth medium. 30 ml of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) were added to the 2-L working volume to buffer pH at ~6.8. 20 ml (1% v/v) of a previously grown overnight culture were added to the reactor as inoculum. Pure high performance liquid chromatography grade methanol was used as the carbon source.

The optical density (OD) of culture samples (500 μl) was measured at 540 nm using a Multiskan Spectrum 48-well microplate reader (Thermo Scientific).

In batch experiments, the initial methanol concentration was 25 mmol/L. For the pulsing fed-batch experiments, after the initial batch growth, methanol was added to the reactor every 24 h using a syringe to reach a concentration of 25 mmol/L. For the step-wise fed-batch experiments, after the initial batch growth, methanol was constantly added to the culture using a syringe-pump (NE-1000 Multi-Phaser^{TM}, New Era). The flow rate of methanol was adjusted every 12 h using the following equations and a desired target specific growth rate:

$$\frac{\Delta S}{\Delta t} = \mu \cdot X \cdot \frac{V_2}{Y_{\frac{X}{s}}} \quad (1)$$

$$Y_{\frac{x}{s}} = \frac{\Delta X}{\Delta S_p} \quad (2)$$

where ΔS is the total amount of methanol (mol) to be injected in the upcoming time interval, Δt is the time interval between two subsequent samples (h), μ is the target specific growth rate (h^{-1}), X is the biomass concentration (as measured by OD) at the latest sample time, V_2 is the volume of the bacterial culture at the latest sample time (L), $Y_{X/S}$ is the yield of biomass per amount of methanol used (ΔOD·L/mmol) over the last time interval, ΔX is the change in biomass over the last time interval (ΔOD), ΔS_p is the amount of methanol consumed during that same last time interval per volume of culture (mol/L).

The experimental results were compared to the results from a model that shows the expected value of OD at the end of a time interval derived from solving the following equations simultaneously using the software R.

$$\frac{dx}{dt} = \frac{\mu_{max} * x * s}{K_s + s} \tag{3}$$

$$\frac{ds}{dt} = \dot{N} - \frac{dx}{Y * dt} \tag{4}$$

Where μ_{max} is the maximum specific growth rate equal to 0.49 h^{-1}, x is the expected cell density (OD), s is the expected substrate concentration (mol/L), K_s is the Monod constant equal to 1.0 mol/L, $\dot{N}$ is the flow rate of substrate (mol/L.h), and Y is the growth yield (L/mol). The values of the constants μ_{max} and K_s were calculated based on the data from multiple growth curves obtained from a triplicate of batch cultivations of *M. album* BG8 using methanol as the source of carbon. In order to calculate the predicted cell density, equations 3 and 4 were solved simultaneously for each step of methanol addition in the fed-batch experiments. The initial conditions used for each step of the experiments involved s and x for both pulsing fed-batch and step-wise fed-batch operations. At each step, the initial condition for x in both types of operation was the measured biomass at the end of the preceding step. The initial condition of s for the pulsing fed-batch operation was the actual amount of methanol added at each step, while for the step-wise fed-batch operation it was zero. Also, while the value of $\dot{N}$ for the pulsing fed-batch process was always 0, it varied for the step-wise fed-batch operation and was pre-determined for each step based on equation 1.

4.4. Results

Figure 4.1 shows growth of *M. album* BG8 in a batch process, using methanol as the carbon source. The figure shows that the final OD achieved was ~0.35. It can also be observed that it took approximately 50 h for the bacterial culture to reach stationary phase.

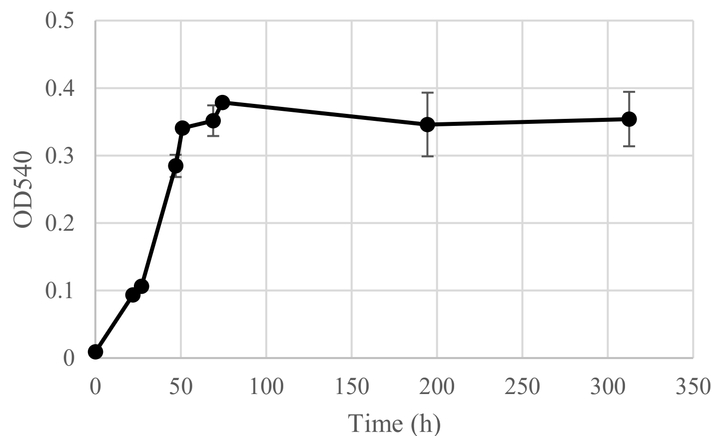

Figure 4.1. Optical density at 540 nm of batch cultures of *Methylomicrobium album* BG8 in a bioreactor.

For the pulsing fed-batch experiment, DO was monitored using a polarized DO probe to infer the depletion of methanol, observed through an increase in DO. Upon depletion of methanol, a pulse of substrate was added to the reactor to reach a concentration of 25 mM. Figure 4.2 shows the trends of OD during pulsing fed-batch operation and the predicted values of OD for each pulse addition of methanol based on the OD at the time of the addition As the fed-batch reactor is initiated in the same manner as the batch operation, the early stages of operation (0 to 54 h) thus correspond to the patterns observed in batch operation. For each growth period following a pulse input, bacteria consumed the methanol leading to increased population density until methanol was depleted and growth slowed down, at which point another pulse of methanol was injected into the culture. Looking at Figure 4.2, the growth of the bacteria can be categorized into three phases. In the first phase, up to approximately 100 h, the bacteria displayed slower growth. The second phase from 100 h to about 200 h consisted of relatively constant rapid growth. Finally, in the third phase, from 200 h onward, a significant reduction of growth rate was observed, even upon several additions of methanol. The final OD obtained in this operation is

~4.7. Also, as shown in Figure 4.2, the predicted values of OD obtained from equations 3 and 4 are very similar to the experimental values for the whole experiment, except at 125 and 200 h where experimental results underwent a sharp increase in OD.

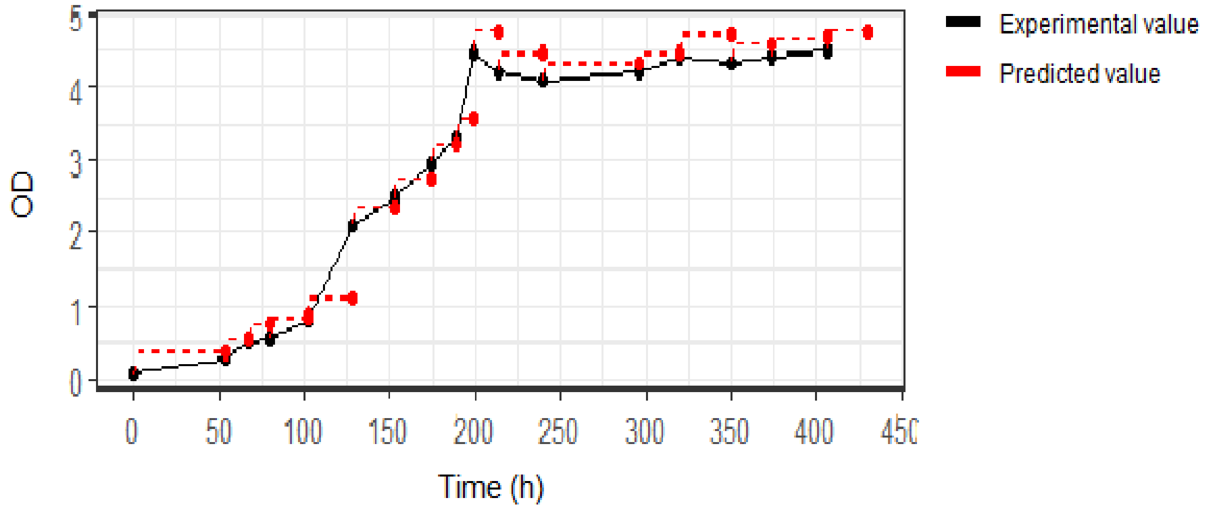

Figure 4.2. Experimental and predicted values of optical density at 540 nm of a culture of *Methylomicrobium album* BG8 in a pulsing fed-batch process in a bioreactor. Arrows indicate addition of methanol to a concentration of 25 mM.

Step-wise addition of substrate can improve the growth in fed-batch culture by minimizing the amount of methanol present in the reactor at all times. This mode of operation was initially performed with a target growth rate of 0.0135 h^{-1}. Variations on the feeding in the early stages of the cultures were attempted. Figure 4.3 shows the first step-wise fed-batch experiment with a target growth rate of 0.0135 h^{-1} in which the initial methanol concentration was 25 mM and additional feeding (based on the feeding law) started at 50 h (which corresponded to an OD_{540} of 0.273). Figure 4.3 also demonstrates predicted values of OD obtained from the solution of equations 3 and 4.

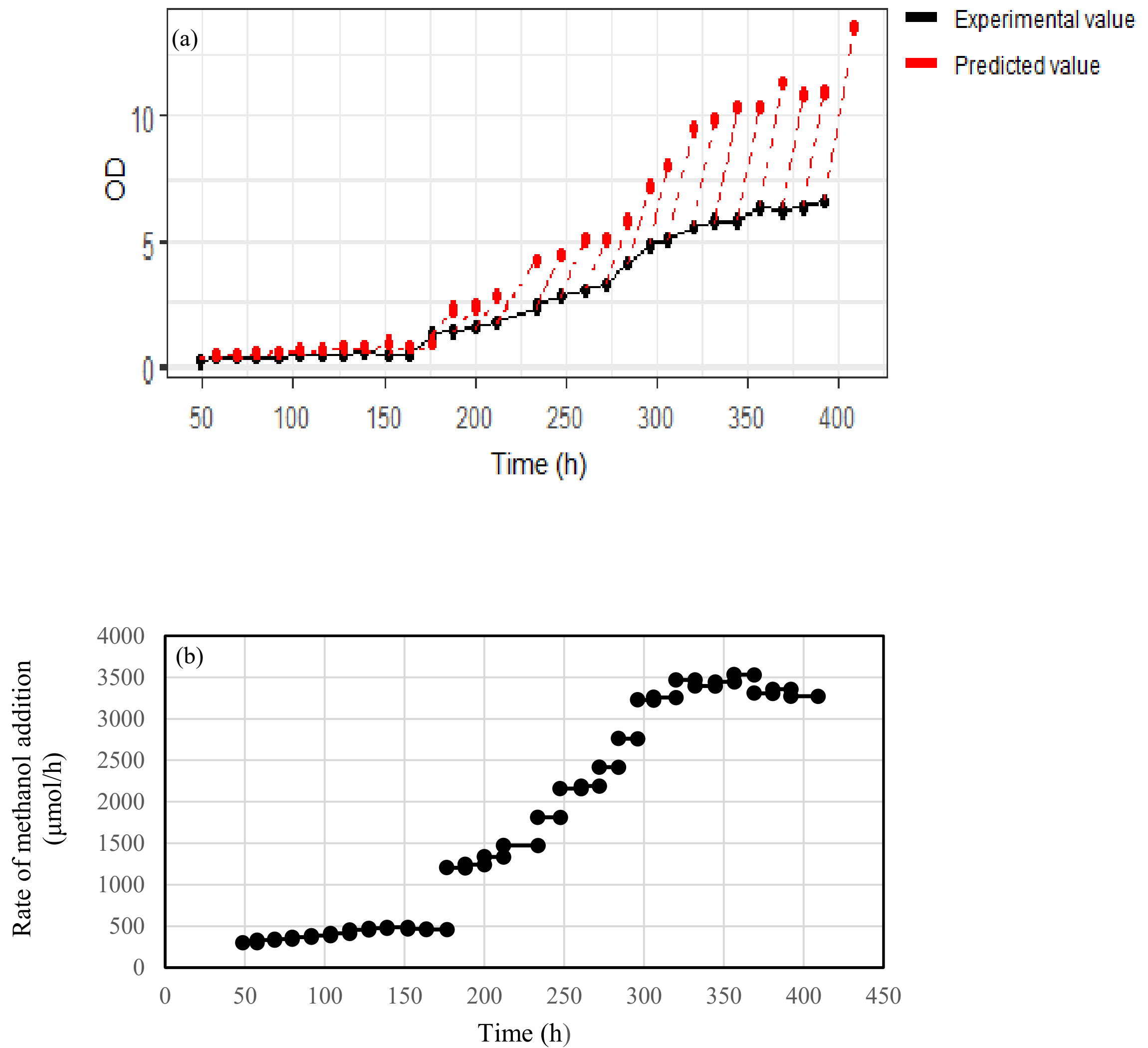

Figure 4.3. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.0135 h^{-1}. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (µmol/h).

As can be seen in Figure 4.3a, the first phase of the growth curve which displays slow growth and low OD_{540} (below 0.6) lasted approximately 160 h. From this point, faster sustained growth was observed until approximately 350 h, reaching an OD_{540} of ~6.3. As can be seen in Figure 4.3b, the rate of methanol addition based on the feeding law remained low, below 500 µmol/h, in the early stages of operation. Starting at ~175 h, an increase in OD_{540} translated in

increased addition rate, a trend that continued until ~350h when the addition rate reached ~3500 μmol/h. This level was then sustained until the end of the fed-batch at approximately 400 h. The final OD obtained in this experiment was ~6.6. Also, as can be observed in this figure, after 175 h of operation, the predicted values of OD were significantly greater than the experimental values and the difference increased with longer operation. The modeling predicted a final OD greater than 12 which was ~2 fold greater than that obtained from the experiments.

In an attempt to reduce the processing time while obtaining higher final OD, a target growth rate of 0.027 h^{-1} was used in a new set of experiments shown in Figure 4.4. As shown in Figure 4.4b, methanol addition rate remained lower than 1000 μmol/h until 100 h into the experiment. Although methanol addition rate increased to 1000 μmol/h from the previous experiment, the OD still remained less than 0.5 (Figure 4.4a). It can be seen from Figure 4.4a that the slow growth phase can be also be observed in this experiment and it last ~100 h. From this point, OD increased until 200 h where the methanol addition rate reached a plateau at ~9200 μmol/h (OD = 5.3). The final OD obtained in this experiment was ~6.1. Figure 4.4 also demonstrates the predicted values of OD obtained using equations (3) and (4). As can be seen in this figure, after ~120 h the experimental OD was consistently lower than the predicted values. In addition, the final optical density predicted for this experiment was ~17, which is approximately 3 times greater than the value obtained experimentally.

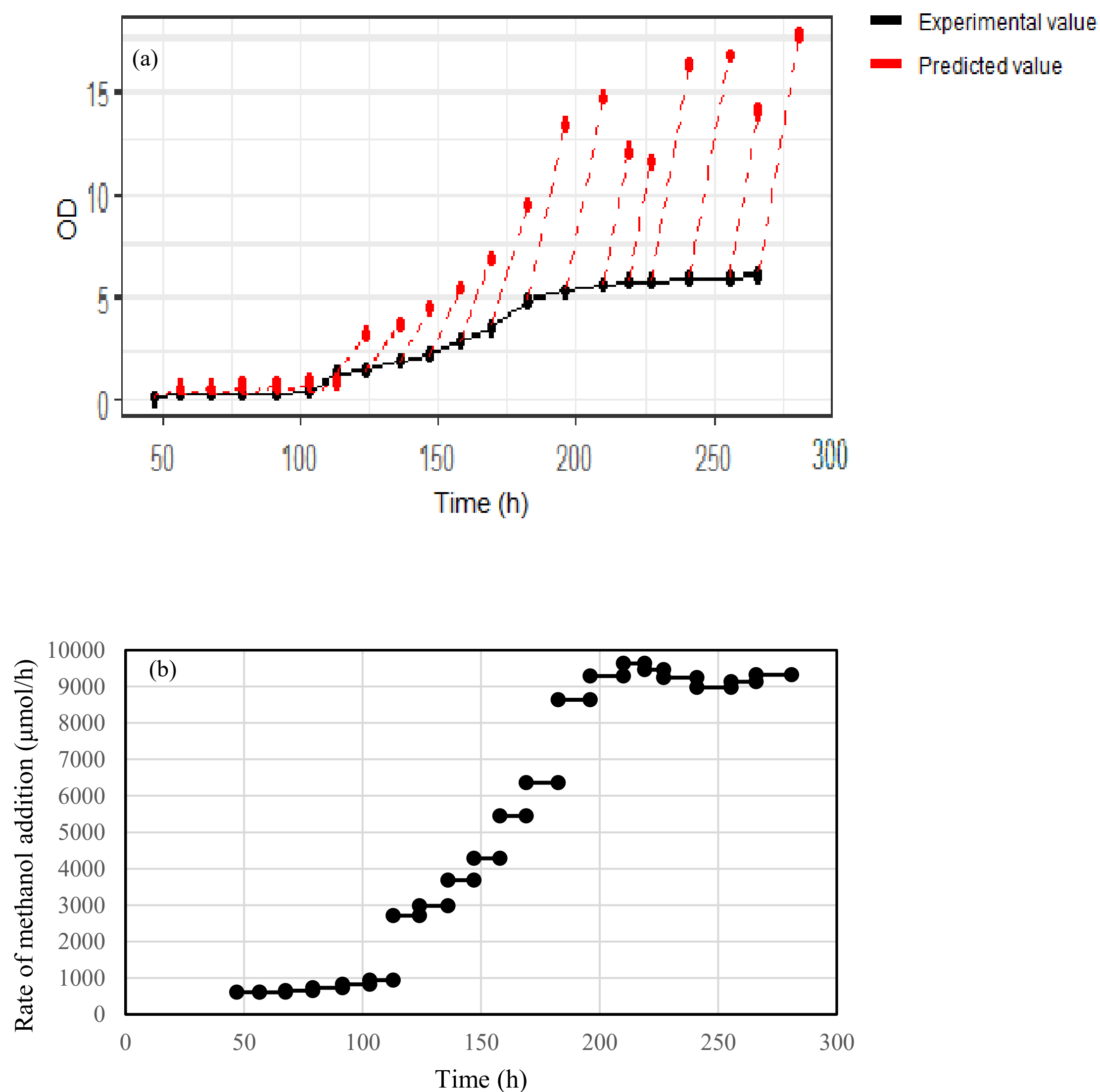

Figure 4.4. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.027 h^{-1}. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (μmol/h).

In both previous step-wise fed-batch experiments, injection of methanol into the culture based on the feeding law started right after an initial batch-like growth (leading to an OD of ~0.3). In an attempt to reduce the lag phase, a lower initial methanol concentration was used (10 mM) and the target growth rate was set to 0.027 h^{-1} (Figure 4.5). As can be seen in Figure 4.5a,

the initial growth reached a value of 0.193 at ~50 h, at which feeding based on the feeding law was started. As a result of this adjustment, not only was the initial phase of slow growth still observed, but growth halted after approximately 70 h, reaching an OD slightly lower than 0.35. Similarly to previous experiments, the model (equations (3) and (4)) consistently predicted greater OD values than expected, and the final value of OD (~0.32) was approximately 3-fold lower than predicted (~0.8).

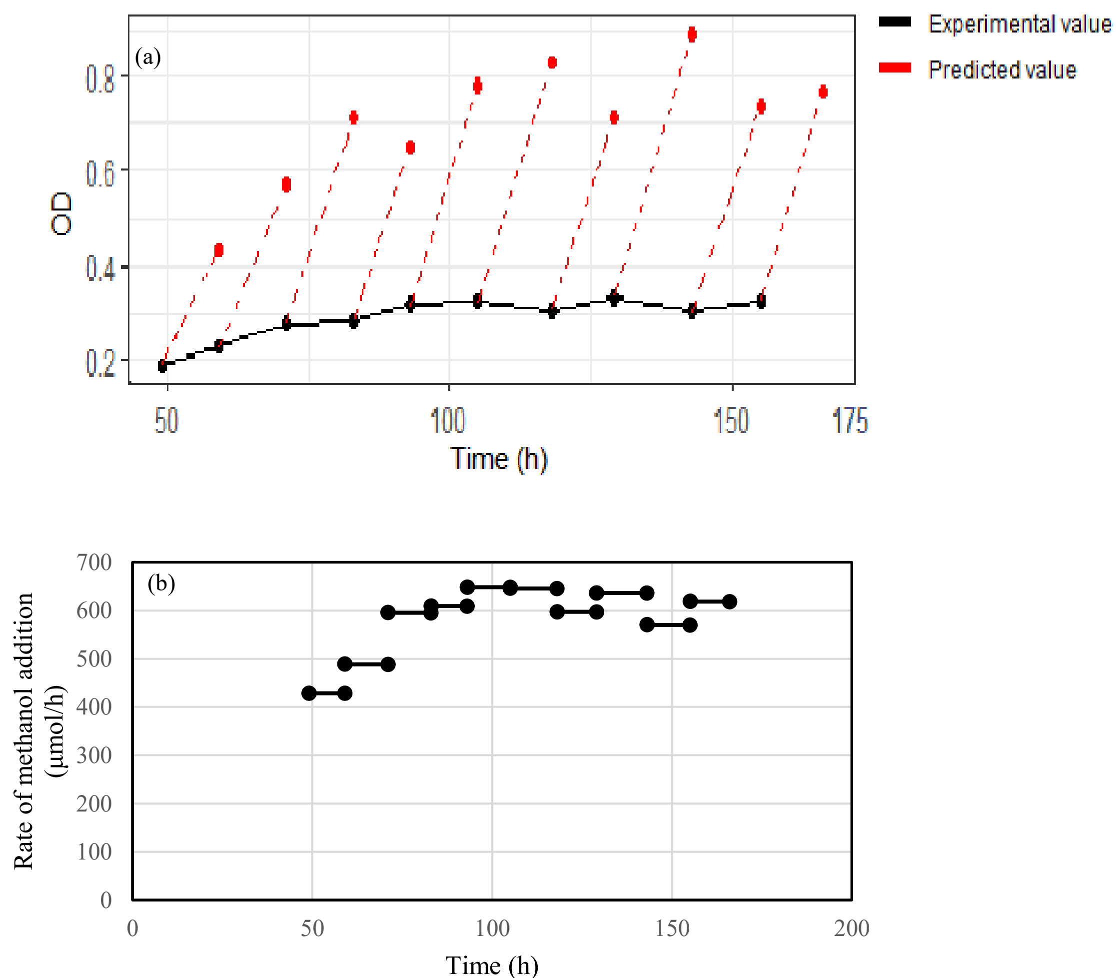

Figure 4.5. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.027 h^{-1} and initial methanol concentration of 10 mM. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (µmol/h).

Aiming at reducing the initial slow growth phase, the strategy of using an elevated fixed methanol feeding rate of 2470 μmol/h at ODs lower than 1 was tested (Figure 4.6). As it can be seen, the duration of the early slow growth phase was not reduced using this approach (lasting 140 h to reach OD ~1.27). A stage of rapid growth, which lasted until ~220 h, was then observed to reach a final OD of ~4.7 – lower than in some previous experiments. As can be anticipated from previous experiments, in this experiment the predicted values of OD (~17) were more than ~3 times greater than the final OD obtained experimentally.

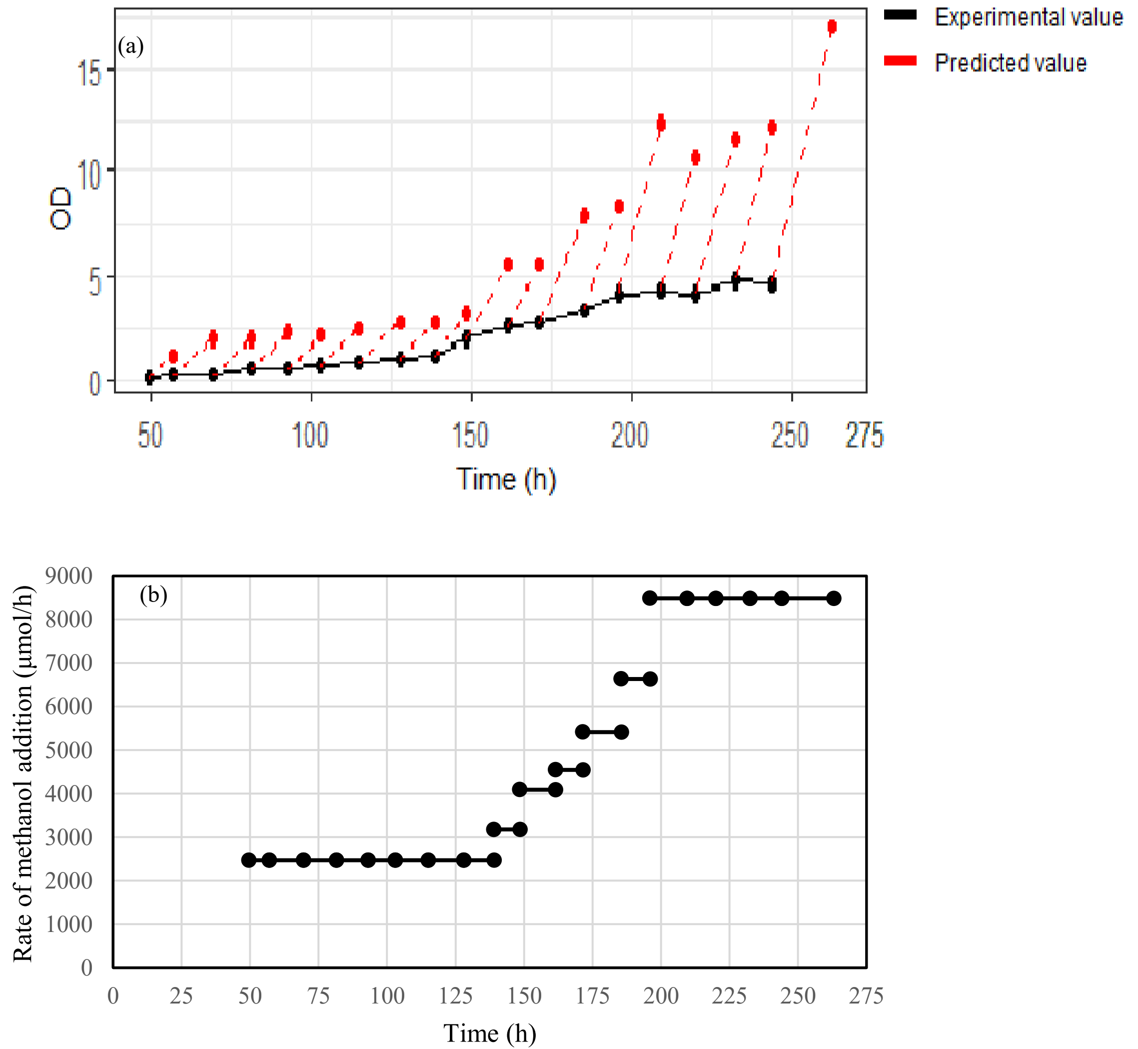

Figure 4.6. Experimental and predicted values of step-wise fed-batch culture of *Methylomicrobium album* BG8 with a target growth rate of 0.027 h^{-1} and fixed methanol feeding rate of 2470 µmol/h. (a) Growth reported as OD_{540}, and (b) rate of methanol addition (µmol/h).

Table 4.1 summarizes all the conditions used in the above experiments with the results obtained for the growth of *Methylomicrobium album* BG8 using methanol as carbon source. This table also shows biomass productivity for each of these experiments which is calculated as the difference between the OD at the end of the fast growth phase and initial OD divided by the time needed for the process to reach a plateau (end of the fast growth phase). As seen in Table 4.1, the highest biomass productivity was achieved in the experiment in Figure 4.4 (biomass productivity = ~0.027 h^{-1}) which had an initial methanol concentration of 25 mM and a target growth rate of 0.027 h^{-1}. The lowest biomass productivity (0.003 h^{-1}) was observed in the experiment in Figure 4.5 which had an initial methanol concentration of 10 mM and a target growth rate of 0.027 h^{-1} which is almost the same value as the batch experiment in Figure 4.1 (biomass productivity = 0.007 h^{-1}). Also, biomass productivity is almost the same in experiments in Figures 2 (biomass productivity = 0.022 h^{-1}) and Figure 4.3 (biomass productivity = 0.018 h^{-1}) and Figure 4.6 (biomass productivity = 0.02 h^{-1}). Among these three figures though, Figure 4.2 and 6 have the same final OD (~4.7) which is lower than OD in Figure 4.3 (~6.6).

Table 4.1. Summary of the conditions and results obtained from various operations for the growth of *Methylomicrobium album* BG8 using methanol as carbon source. NA in this table shows that a condition did not apply to a specific experiment.

Figure #	Initial methanol concentration (mM)	Target growth rate (h^{-1})	OD at the end of first feeding step	Duration of slow growth phase (h)	OD at the end of slow growth phase	Duration of fast growth phase (h)	Final OD	Biomass productivity (h^{-1})
1	25	NA	NA	~22	0.09	~25	0.35	0.007
2	25	NA	0.3	~100	0.825	~100	4.7	0.022
3	25	0.0135	0.3	~160	0.5	~190	6.6	0.018
4	25	0.027	0.3	~100	0.46	~100	6.1	0.027
5	10	0.027	0.19	NA	NA	~20	0.3	0.003
6	25	0.027	0.23	~140	1.27	~80	4.7	0.020

4.5. Discussion

As the performance of bioproduction schemes greatly depends on biomass density for high product titers, an important aspect of the present study was establishing the influence of using various processing and feeding strategies on final OD. In this case, batch operation served as a baseline for the comparison of performance of the various feeding strategies. The first observation was that a pulsing fed-batch approach could greatly increase cell density – as much as ~13.4-fold greater than batch operation (Figure 4.2 compared to Figure 4.1 as shown in Table 4.1). When implementing step-wise feeding initiated upon depletion of the initial methanol, the increase in cell density obtained was even more marked, reaching ~18.8- and 17.6- fold the cell density of the batch operation for target growth rates of 0.0135 h^{-1} and 0.027 h^{-1}, respectively (Figures 3 and 4 compared to Figure 4.1 as summarized in Table 4.1). Also, the data in Table 4.1 shows that the step wise feeding strategy increased the final ODs ~1.4 and 1.3 fold for 0.0135 h^{-1} and 0.027 h^{-1} target growth rates, respectively in compared to the pulsing fed-batch operation.

The greater final cell density (as measured by OD) observed when using a step-wise feeding approach is likely linked to this method limiting the amount of methanol in the reactor at any given time. Previous research shows that some strains of methanotrophs such as *Methylomicrobium buryatense* 5B produce elevated concentrations of metabolites like formate and formaldehyde when growing on methanol (Eshinimaev *et al.,* 2001; Costa *et al.*, 2001). Also, some metabolic products like formaldehyde can have an inhibitory effect on the growth of the bacteria (Costa *et al.*, 2001). Accumulation of inhibitory metabolites during the course of experiment can lead to early termination of growth. In comparison, the pulsing fed batch approach implies periodical increases in methanol concentration to 25 mM, a condition that can stress the cultures and impede on their full development to high cell densities. This can limit growth, as methanol is known to exhibit toxicity in most methanotrophs at concentrations as low as 0.01 % v/v (Leadbetter *et al.*, 1958; Stocks *et al.*, 1964). Considering this, the step-wise feeding strategy, in which the rate of methanol supplementation dictates the rate of methanol uptake by the cells and thus minimizes the concentration of methanol in the reactor, avoids methanol-induced responses/stresses throughout the culture.

Increasing the target growth rate in step-wise fed-batch experiments proved to be a double edged sword. By increasing the target growth rate, the final OD achieved was decreased

while the processing time was reduced. As the data in Table 4.1 shows, by increasing the target growth rate from 0.0135 h^{-1} (Figure 4.3) to 0.027 h^{-1} (Figure 4.4) OD decreased from ~6.6 to ~6.1. But, the processing time in target growth rate of 0.027 h^{-1} (~250 h) was dramatically lower than that of 0.0135 h^{-1} (380 h). This all could mean that doubling the growth rate in general had a positive effect on reducing the processing time but a negative effect on the final OD obtained. The same trend can be observed comparing the results from the step wise fed-batch (Figure 4.3 and 4) to the pulsing fed-batch experiment (Figure 4.2) showing that the pulsing fed-batch had the lowest processing time among these three experiments (200 h) but also the lowest final OD (~4.7).

The cessation of growth these experiments could have happened due to various reasons such as depletion of nutrients and accumulation of increased concentrations of growth inhibitory metabolites in the culture. Accumulation of inhibitory metabolites during the course of experiment due to increased methanol feeding rates could result in early termination of the experiment as seen in the pulsing fed-batch operation or reduction in the achieved final OD at elevated target growth rates as seen in the step wise fed-batch operation. Controlling the growth dynamics through growth rate aids to reduce the production of secondary metabolites (Chen *et al.*, 1997; Åkesson *et al.*, 2001; Kim *et al.*, 2004; Habegger *et al.*, 2018) and thus increase the final optical density obtained in the bioprocess. Although, this might be achieved at the cost of longer processing times as observed in the above experiments.

In all the experiments summarized in Table 4.1, an initial slow growth phase can be observed which contributes to increasing the processing time of the experiments. This reason for the slow growth phase having happened could be that the overnight culture used to start the experiment was grown under methane as the main carbon source. When this inoculum was injected to a batch of fresh media to start the experiment, the bacteria that had previously grown under methane needed to adapt themselves to methanol as the carbon source. Therefore, it takes a while for the bacteria to start growing at a higher rate causing the early stages of the growth process to be slower. Early feeding strategies tested to reduce this slow growth phase include increasing the target growth rate (Figure 4.4), lowering the concentration of methanol to 10 mM at the initiating step of the experiment (Figure 4.5), and using an elevated fixed methanol feeding rate of 2470 μmol/h at ODs lower than 1 (Figure 4.6). Among these strategies, lowering the

concentration of methanol in the initial batch-like stage of the experiment to 10 mM (Figure 4.5) resulted in precipitous termination of the experiment. Increasing the target growth rate to 0.027 h^{-1} in step wise fed-batch experiment (Figure 4.4) reduced the duration of slow growth phase to 100 h in compared to 160 h in the target growth rate of 0.0135 h^{-1} (Figure 4.3). Also, the operation employing a preliminary fixed methanol feeding rate (Figure 4.6) resulted in a longer slow growth phase (140h) accompanied by a lower final OD (~4.7) in compared to the experiment in Figure 4.4.

Initiating the step-wise fed-batch experiments with a lower methanol concentration of 10 mM instead of 25 mM (Figure 4.5) was performed to start feeding methanol using the feeding law at lower ODs and thus reduce the period of slow growth. But, it seems that for the bacteria to reach a population appropriate for operating a step-wise fed-batch process, the concentration of methanol for initiating the experiment should be at least as high as in a batch process. Increasing the target growth rate (Figure 4.4) on the other hand, could have supplied the population of the bacteria with a higher methanol concentration, which was still at a level not inhibitory to the growth of the bacteria which resulted in faster growth and reduction in the duration of the slow growth phase. The reason for the slow growth phase being longer and leading to lower ODs for the experiment in Figure 4.6 in compared to Figure 4.4, could be that keeping higher concentrations of methanol at the initiating stage of the experiment when there is still a lower number of bacteria present in the culture, could be disadvantageous to the growth of the bacteria resulting in lower final ODs while demanding a longer time to reach that OD.

Biomass productivity is an important aspect for the industrialization of a bioprocess (Table 4.1). The step wise fed-batch with a target growth rate of 0.027 h^{-1} (Figure 4.4) had the highest biomass productivity (~0.027 h^{-1}) meaning that in this operation the obtained cell density was maximized in proportion to the processing time needed to achieve this cell density. In an industrial setting this could be a favorable processing technique as it would render the highest possible cell density over the processing time. The step-wise fed-batch operation with a target growth rate of 0.0135 h^{-1} (Figure 4.3) on the other hand, resulted in lower biomass productivity (~0.018 h^{-1}) but a higher final OD (~6.6) in compared to the final OD in the experiment in Figure 4.4 (~6.1). This confirms the fact that increasing the target growth rate in step-wise fed-batch operation can increase the final OD at the cost of increasing the processing time, thus lowering

the biomass productivity. It can be seen in Table 4.1 that although the biomass productivities in the pulsing fed-batch experiment seen in Figure 4.2 (~0.022 h^{-1}) and the experiment with the fixed initial methanol feeding rate in Figure 4.6 (~0.020 h^{-1}) and the step wise fed batch operation with target growth rate of 0.0135 seen in Figure 4.3 (~0.018 h^{-1}) are very close to each other. But, the final ODs in Figures 2 and 6 (~4.7) is much less than OD in Figure 4.3 (~6.6). This means that both very low and very high methanol feeding rates will result in lower biomass productivities but the advantage of lower methanol feeding rates is achieving increased cell densities. Among the experiments summarized in Table 4.1 the step-wise fed batch with a target growth rate of 0.027 h^{-1} renders the most promising combination of both higher biomass productivities and final ODs for the growth of *M. album* BG8 using methanol. From these results, it is evident that the choice of the process operation technique depends on the goal of the bioprocess and on whether maximum cell densities obtained in the lowest processing times are of interest or whether only the highest optical densities need to be achieved regardless of the processing times.

Comparing the values of OD obtained experimentally to those predicted from the model (equations (3) and (4)), it can be seen that, in all cases other than the pulsing fed-batch experiment, the model overestimated OD. In the case of the pulsing fed-batch, predicted values were only slightly higher than the experimental values. Also, comparing the values from model and experiments, it can be seen that in most cases the predicted and experimental values are very close at the onset of the experiments, more specifically in the slow growth phase of the experiment. The difference between the experimental and predicted values were actually more significant for the experiments with a higher target growth rate, as can be observed by comparing the results in Figure 4.3 (target growth rate of 0.0135 h^{-1}) and Figure 4.4 (target growth rate of 0.027 h^{-1}). The fact that the predicted and experimental values differed significantly as step-wise fed-batch experiments proceeded could be due to various reasons such as increased methanol concentrations (resulting from slower methanol uptake than expected) and methanol toxicity at higher target growth rates, production of inhibitory metabolites (e.g. formate) at higher target growth rates, and/or quorum sensing as cultures reach higher cell densities. To improve the accuracy of the model, factors such as the effect of methanol toxicity at higher methanol injection rates could be taken into account in the developed equations in the form of cell death rate or substrate inhibition (Doona *et al.*, 2005; Taub *et al.*, 2003; Flynn *et al.*, 2014).

Although in this study optical density was used as a proxy for biomass concentration, there are limitations associated with this application. One of these limitations is the fact that the relationship between OD and cell measurement parameters such as cell dry weight can be non-linear. A calibration curve would therefore help establish a relationship between OD readings and cell concentrations in the system at hand (Moo-Young, 2019). Also, since measurements of OD are performed based on optical absorbance and scattering over a narrow slit, this measurement can vary based on the width of the instrument's slit, and the condition of the filter and detector. Although, the variation in the measurement will not affect the results when the Beer-Lambert law is taken into account.

4.6. Conclusion

We investigated the impact of different methanol-feeding strategies on the growth of *Methylomicrobium album* BG8 in bioreactors. Pulsing fed-batch and step-wise fed-batch (for which two target growth rates and various approaches to the initiation of feeding were tested) were compared to batch operation as a reference. Although all conditions tested improved on the cell density observed in batch culture, the operation that led to the highest cell density was the step-wise fed-batch with a target growth rate of 0.0135 h^{-1} and the initiation of feeding law once the culture reached an OD ~03. Using this approach, which is assumed to minimize the production of inhibitory metabolites while keeping methanol at a low level in the bio-reactor, led to a final OD of 6.6 (a ~18.8-fold increase on the batch culture) within ~350 h, for a biomass productivity of ~0.018 ΔOD/h. On the other hand, using a faster target growth rate (0.027 h^{-1}) led to slightly lower cell density (OD ~6.1) but a faster culture time (200 h to reach the plateau) These results suggest there is a trade-off between the process duration and the maximum cell density obtained. Further experiments showed that increasing the concentration of methanol, especially in the early stages of the cultures, has a negative effect on the growth of the bacteria; in order to achieve higher final OD, slower supplementation of methanol is required. As increased cell density is a crucial factor in the economic viability of microbial bioconversion processes, this work opens the door to the implementation of *M. album* BG8 as a production platform in industrial setting.

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4.8. References

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5. Comparative metabomomics of *Methylomicrobium album* BG8 grown on methanol in batch and fed-batch

5.1. Abstract

Methanotrophic bacteria are microorganisms, ubiquitous in nature, which play an important role in the global methane cycle and in mitigating the effect of this potent greenhouse gas. Methanotrophs are also of interest for their industrial applications mostly based on their ability to produce valuable products using affordable substrates such as methanol, an important industrial byproduct. Industrial bioproduction using methanotrophs can be done through a variety of techniques including batch and fed-batch processes. The study of the influence of these modes of operation on the metabolome of methanotrophs provides information on metabolism, physiology and energetic requirements of cultures, and can help identify industrially relevant prospective bioproducts. In this work, the metabolomes of batch (late exponential) and fed-batch cultures of the gammaproteobacterial methanotroph *Methylomicrobium album* BG8 growing on methanol were obtained and compared. Comparative metabolomics highlight the fact that, in each process, the abundance of different industrially relevant metabolites was favored. Under fed-batch operation, an increase in the prevalence of intermediates of metabolic pathways (e.g. 2,3-dihydroxy-isovalerate, 4-hydroxybenzoate, and phenyllactate) was observed, which is consistent with this mode of operation promoting an active metabolic state in the cultures. It also led to lower levels of branched chain amino acids (BCAAs). In contrast, late exponential batch cultures led to greater abundance of molecules linked to starvation (valine, isoleucine, and leucine), suggesting early starvation behavior. The results show that the mode of operation can significantly change the type and abundance of metabolites accrued during bioprocessing. This information is especially valuable for industrial bioproduction applications since it can shed light on the type of process that will result in production of commercially relevant metabolites.

5.2. Introduction

Methanotrophs are bacteria that can use methane and methanol, among other one-carbon compounds, as their main source of carbon and energy (Hanson *et al*., 1996). They are widely

present in the environment and play an important role in global carbon cycling (Hanson *et al.*, 1980; Lenhart *et al*., 2012; Gougoulias *et al.*, 2014). Interest in methanotrophs stems from their capacity to be used in their numerous potential industrial applications, such as production of single cell protein (Øverland *et al*., 2010), biopolymers (Asenjo *et al*., 1986; Khosravi-Darani *et al*., 2013), formaldehyde and organic acids (Strong *et al*., 2015), lipids as precursors of biofuels (Knothe *et al*, 2010; Sunde *et al*., 2011), etc. Methanol, a byproduct of forestry industry, can also be converted to bioproducts such as biofuels and biopolymers using methanotrophic bacteria (Khosravi-Darani *et al.*, 2013; Bennett *et al.*, 2018).

Although various culturing techniques can be employed to manufacture valuable products using bacteria at industrial scale, batch and fed-batch operations are amongst the most common. In batch culture the concentration of substrate varies as the bacteria grows, and the products of the growth (intracellular or extracellular) are collected at the end of the bioprocess. In these cases, the main optimization parameters are the initial concentration of the growth medium, inoculum load, temperature, aeration rate, agitation, and batch time (Lim and Shin, 2013). A continuous process, on the other hand, generally leads to growing cultures at sub-maximal growth rates by allowing a steady flow rate for both feed and effluent streams, resulting in steady state growth conditions (Schaechter, 2009). Continuous operation is not widely used in the bioprocessing industry as it is can be difficult to keep the cultures stable and sterile – since the continuous process is an open culture system (Lim and Shin, 2013). In fed-batch processes, nutrients are continuously added to the culture medium and harvesting the culture only occurs at the end of the run (Yamanè and Shimizu, 1984; Lim and Shin, 2013). The feeding rate of a limiting nutrient can be controlled; therefore the concentration of the desired product can be maximized (Minihane and brow, 1986; Modak and Lim, 1987; Lim and Shin, 2013). Fed-batch operation is more suitable for bioprocesses that are more sensitive to the concentration of a specific nutrient or metabolite. For example, it is suitable for bioprocesses in which the growth rate enhances with the increase in concentration of a specific nutrient but decays as the concentration of that nutrient exceeds a specific limit (Lim and Shin, 2013). This makes fed-batch operation particularly well-suited for the growth of methanotrophic bacteria on methanol, an inherently toxic substrate.

Batch, continuous and fed-batch processes have all been used for the production of valuable components from methanotrophic bacteria. For instance, methanol was produced from methane using *Methylosinus trichosporium* IMV 3011 in a continuous ultrafiltration reactor (Xin *et al.*, 2004). In another study both batch and continuous cultivations were employed for the growth of methanotrophic bacteria feeding on biogas derived from anaerobic digestion of a series of energy crop residues (Henard *et al.*, 2018). This study showed that growth arrest occurred in continuous operation while the cultures showed minimal growth inhibition when growing under batch conditions. In another study, ectoine, a bioproduct from *Methylomicrobium alcaliphilum 20Z*, was produced from methane in a continuous process (Cantera *et al.*, 2017). Among various other bioproducts, poly-3-hydroxybutyrate (PHB) is a common product of alphaproteobacterial methanotrophs, which has already been commercially developed (Cantera *et al.*, 2018). Production of polyhydroxyalkanoates (PHA) using methanotrophic bacteria has also been achieved in batch (Myung *et al.*, 2015), continuous and fed-batch processes (Asenjo*et al.*, 1995; Fergala *et al.*, 2018). Two-step fed-batch operation – in which a first step focused on biomass growth, followed by a nitrogen-limiting second step to optimize the production of PHA – has been particularly well-suited for this aim (Zúñiga *et al.*, 2011; Pieja *et al.*, 2012; Rahnama *et al.*, 2012). Fed-batch strategies have also been used in bioproduction using methanol as the feedstock. A feeding strategy based on sorbitol addition as a co-substrate in fed-batch fermentation of methanol by *Pichia pastoris* was developed, resulting in 1.8-fold increase in recombinant protein production (Celik *et al.*, 2009). In another study, independent glycerol and methanol exponential feeding strategies were developed for the production of human epidermal growth factor (hEGF) using recombinant *Hansenula polymorpha* DL-1 (Moon *et al.*, 2003).

In order to improve the performance of bioproduction processes, it is important to understand how the process conditions affect the metabolism of the bacteria. Metabolomics is the study of a wide array of metabolites present in a biological system under a given set of conditions (Shulaev, 2006; Dettmer *et al.*, 2007). Understanding the production and abundance of metabolites present in an organism growing under different modes of operation can help answer specific biological questions and establish favorable processing conditions towards the production of valuable bioproducts. For example, previous studies showed that a repeated fed-batch strategy for the production of docosahexaenoic acid by *Crypthecodinium cohnii* results in decreased stability of the culture system. Metabolome analysis revealed that the decreased

nitrogen utilization capacity and the down-regulation of glycolysis and TCA cycle led to this decreased stability (Liu *et al.*, 2020). In another study, metabolomics were used to study the effect of batch fermentation, yeast extract brand and fermentation time on cultures of five *Streptoccoccus thermophilus* strains (Khakimov *et al.*, 2017). Results showed that the batch process, fermentation time, and their interaction had the most significant effect on variations in metabolome. Yet another study used comparative metabolomics to investigate the effects of inoculum load on stress response and metabolism during fermentation of *Saccharomyces cerevisiae* (Ding *et al.*, 2009). Significantly higher levels of glycerol and proline were found in fermentation processes initiated with greater inoculum loads, showing the importance of these two metabolites in protecting yeast from stresses in high cell density cultures.

Study of metabolic pathways of bacteria can also identify the differences between the methanotrophs type I and type II, and metabolic products of these two groups. For instance, it is known that production of PHB is limited to type II methanotrophs and type I methanotrophs do not produce this biopolymer. Studies have shown that PHB production is linked to the serine cycle. As a result, type I methanotrophic bacteria, which employ the RUMP-pathway for assimilation of formaldehyde into biomass and carbon dioxide, are not readily capable of PHB production (Pieja *et al.*, 2011). Based on the studies performed on PHB production using various strains of methanotrophs, it is hypothesized that under unbalanced growth conditions PHB is produced by type II methylotrophs through the serine pathway as a carbon storage polymer while type I methylotrophs, which use the RUMP pathway, produce exopolysaccharides instead (Babel *et al.*, 1992). As an example, various RUMP-pathway methylotrophs were investigated for PHB production and none of those produced a measurable amount of PHB or possessed key enzymes for PHB production (Föllner *et al.*, 1993).

Knowledge of metabolites produced in a bioreactor provides the quantitative information for understanding the cellular processes and intracellular reactions taking place under the given process conditions. On the other hand, comparative metabolomics of different bioreactor operations, such as batch and fed-batch, sheds light on how these modes of operation affect cellular activity, metabolic pathways, the presence of inhibiting factors, and conditions under which a specific metabolite is accumulating in the cells. In this study, we use metabolomics to compare *Methylomicrobium album* BG8 cultures using methanol as substrate under batch and

fed-batch operation. We demonstrate how the modes of operation impact metabolic pathways and the prevalence of industrially relevant metabolites.

5.3. Materials and Methods

5.3.1. Strains and growth conditions.

For batch experiments: the gammaproteobacteria M*ethylomicrobium album* BG8 (*M. album* BG8) was grown at 30°C and 150 rpm in 250-ml Wheaton glass media bottles (capped with butyl-rubber septa cap to avoid headspace losses) containing 100 ml of modified nitrate mineral salts (NMS) medium (Whittenbury *et al.*, 1970). 1.5 ml of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4 in distilled water) was added to 100 ml of culture to keep the pH of the cultures 6.8. Methanol (high performance liquid chromatography (HPLC) grade) at concentration of 25 mM was used as the source of carbon. The cultures were inoculated with a previously grown culture of not older than one week was at 1% v/v. All conditions were performed in triplicates and at atmospheric pressure.

For fed-batch experiments: The pulsing fed-batch shown in Figure 4.2 was used for metabolomics analysis. The experiments were done in a 3-L bioreactor controlled with a New Brunswick BioFlo® /CelliGen® 115 Benchtop unit. The culture volume was 2 L and nitrate mineral salt (NMS) was used as growth medium (Whittenbury *et al.*, 1970). The bacteria were grown at 30 °C and an integrated temperature sensor was used along with a heating jacket to monitor and sustain the temperature. Dissolved oxygen levels were monitored using an Inpro 6800 polarographic oxygen sensor from Mettler Toledo. The level of oxygen in the liquid was kept above 30% at all times by controlling agitation (performed using two Rushton impellers). Air was introduced into the bioreactor at a flow rate of (1500 mL/min) through a sparger. A 0.3 μm Whatman Hepa-Vent™ in-line filter was used on the inlet air to avoid contamination to the reactor. Nitrate mineral salt (NMS) media containing potassium and nitrate was used as the growth medium. 30 ml of phosphate buffer (26 g/L KH_2PO_4, 33 g/L Na_2HPO_4) was added to the 2-L volume of culture to buffer the pH to 6.8. 20 ml (1% volumetric ratio to the culture) of a previously grown overnight culture were added to the reactor as inoculum. HPLC-grade methanol was used as the carbon source. Methanol was fed to the reactor every 24 h using a

syringe; the amount of methanol added was adjusted so that its concentration in the bioreactor reached 25 mM for each pulse.

5.3.2. Assessing growth.

Cell density was monitored by measuring optical density (OD) at 540 nm of culture samples using a UV-vis spectrometer (Multiskan Spectrum, Thermo Scientific). All the measurements were repeated in triplicates.

5.3.3. Metabolite extraction and analysis.

100 µg of biomass was retrieved from cultures in the late exponential phase for batch (OD = 0.35) and high cell density cultures (OD = 4.6) of fed-batch. Frozen cell pellets were sent to Metabolon (Morrisville, NC) for global metabolite profiling. Standard sample preparation techniques were employed according to the guidelines provided by Metabolon. Samples were prepared using the automated MicroLab STAR® system (Hamilton Company) and analyzed using positive and negative ion mode electrospray ionization (ESI) by Reverse Phase Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (RP/UPLC-MS/MS; Waters Corp.) and also using negative ion mode ESI by hydrophilic interaction (HILIC) UPLC-MS/MS. A fixed amount of sample pellet was processed per volume of extraction solvent in order to maintain internal consistency. Chromatographic properties and mass spectra were used to identify compounds present in the samples using library entries of purified standards. Abundance of the compounds was quantified by assessing the area under the peak of LC-MS curves for each metabolite. For each treatment (batch and fed-batch), the metabolomics analyses were the results of four replicates (n = 4).

5.3.4. Statistical analysis.

324 metabolites were detected between the two treatments (batch and fed-batch). Metabolites not detected in at least one of the four replicates were excluded from the analysis. The metabolite abundances in samples were divided by the median abundance of that metabolite across all samples (median-scaled). The missing values for each metabolite were replaced with one-half the minimum abundance of that metabolite (scaled-imputed) and then transformed to natural-log scale to reduce the effect of outliers. Principal component analysis (PCA; R package vegan (Jari Oksanen *et al.*, 2018)) and orthogonal partial least squares discriminant analysis

(OPLS-DA; R package ropls (Thevenot *et al.*, 2015)) were performed using natural log-transformed metabolite abundances to assess broad-scale differences between treatments and to identify the metabolites driving those differences. Differential abundance testing was performed for each metabolite using Welch's t-test to identify metabolites that were significantly more (or less) abundant in a particular treatment. False discovery rate (FDR) correction was used to correct p-values to reduce the likelihood of identifying false positives. Significance was defined as having an FDR-corrected $p<0.01$ and a $\log_2$-transformed fold change > |1|.

5.4. Results

In establishing the significance of the modes of operation tested, the principal component analysis showed 91.4% variation in the metabolomes between the two treatments (batch and fed-batch operation). In addition, the variation within each treatment was lower than 5.4%, showing consistency of the replicates of each treatment.

Differential abundance was used to show metabolites that were significantly more abundant in each treatment. 324 metabolites were detected between the two treatments (batch and fed-batch). Of these, 92 were present in batch samples only (not detected in fed-batch). 23 were present in the fed-batch samples only (not detected in the batch cultures) and 209 were detected in both treatments – of which 136 were significantly differentially abundant between treatments ($|\log_2$ fold-change$| > 1$ and FDR-adjusted p-value < 0.01).

Figure 5.1 shows a heat map of the metabolites that were more abundant in each treatment, sorted in nine different categories (amino acids, carbohydrate, cofactors and prosthetic groups and electron carriers, lipids, nucleotides, peptides, hormone metabolism, secondary metabolism, and xenobiotics). Treatments were compared based on the fold-change between metabolite abundances in each treatment. In general, the abundance of most metabolites appeared to be lower in the fed-batch culture; although a few metabolites, mostly lipids, had much greater abundance in this mode of operation. Looking at this figure, more blue appears in the fed-batch abundance heat map compared to batch, showing that the fed-batch process was the treatment with the lower overall metabolite response.

Metabolites that were significantly more abundant in batch are shown in Figure 5.2A, whereas the metabolites more abundant in fed-batch are shown in Figure 5.2B. For each metabolite in these figures, abundance is shown as the natural logarithm of the fold change between treatments. It can be seen from Figure 5.2 that 100 metabolites were more abundant in batch cultures, whereas only 36 metabolites were more abundant in fed-batch. The most significantly more abundant metabolite in batch was gamma-glutamylvaline with a fold change of ~1840. In fed-batch operation on the other hand, 2,3-dihydroxyisovalerate with a fold change of ~57 times was the most significantly more abundant metabolite compared to batch. It is evident that, not only is the number of more abundant metabolites is greater in batch cultures, but the level of abundance of these metabolites is also generally higher in batch.

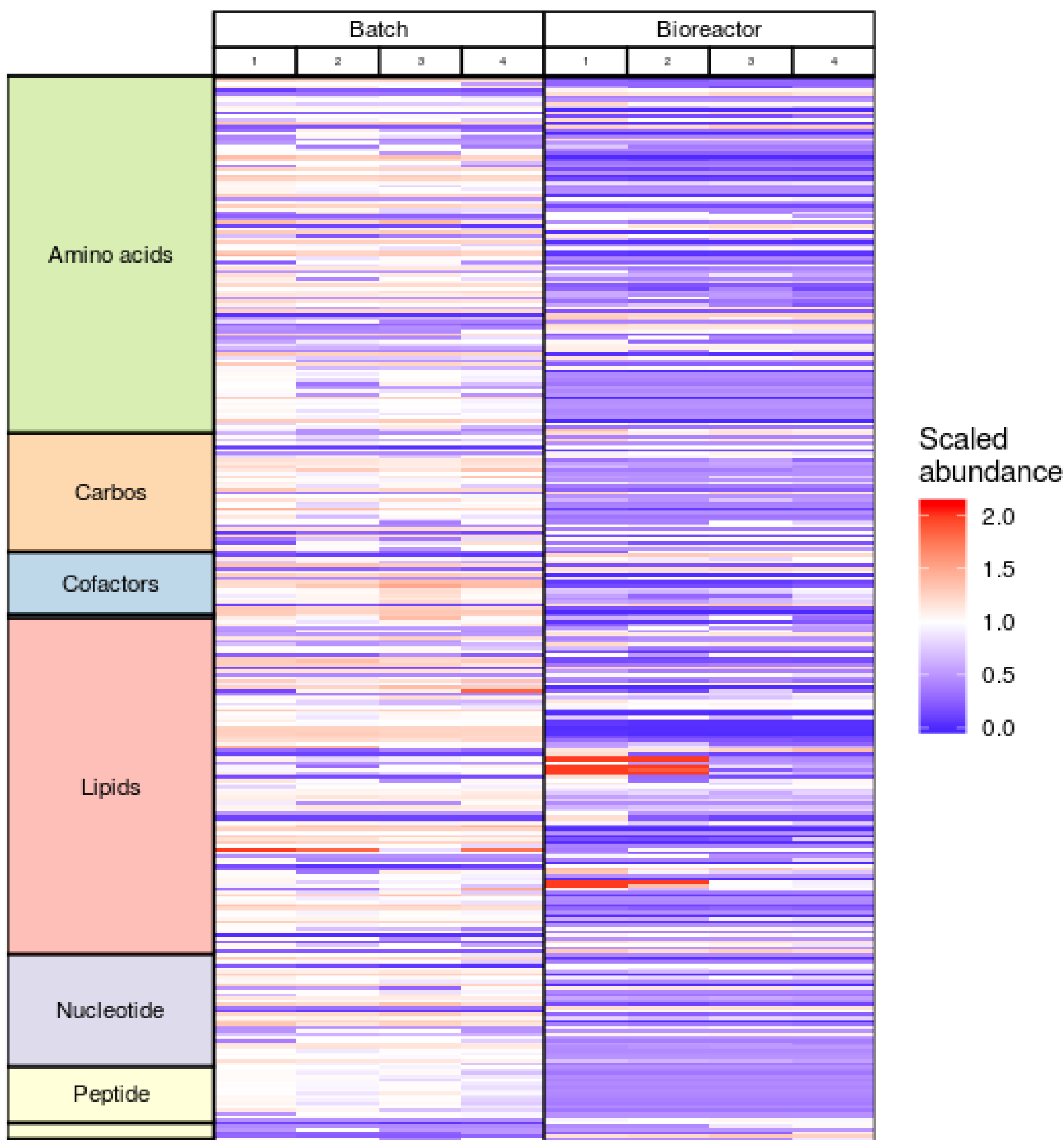

Figure 5.1. Heat map of scaled abundance of 324 metabolites detected in either batch or fed-batch cultivation of M*ethylomicrobium album* BG8 grown on methanol. Metabolites are categorized in groups of amino acids, carbohydrates, cofactors and prosthetic and electron carriers, lipids, nucleotides, peptides, hormone metabolism, secondary metabolism, and xenobiotics (the labels of the latter three are not shown in the figure).

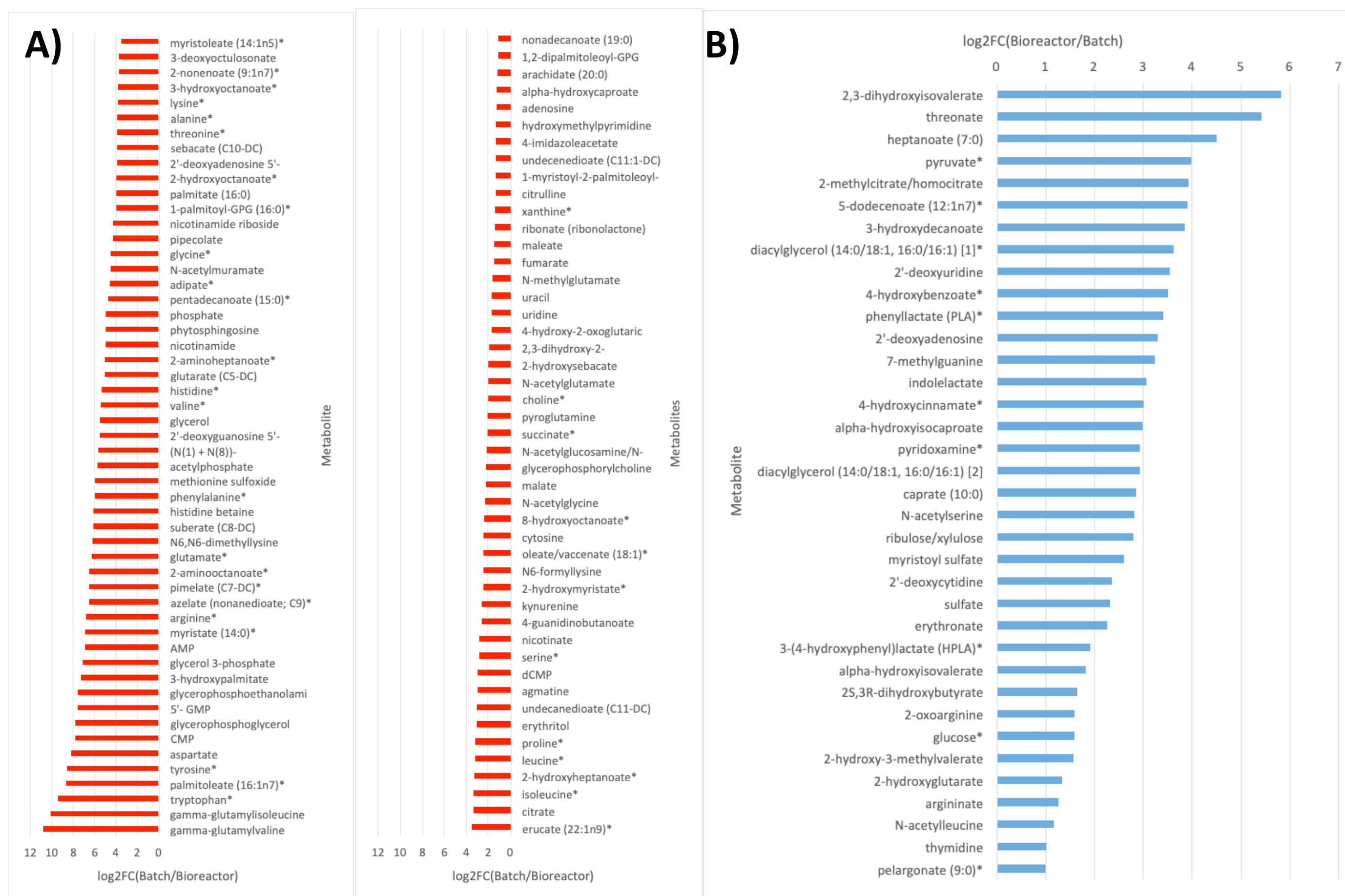

Figure 5.2. Fold-increase in abundance for metabolites more significantly abundant in *Methylomicrobium album* BG8 grown on methanol through A) batch operation (red), and B) fed-batch operation (blue). * indicates commercially relevant metabolites.

Table 5.1 shows the metabolites being more significantly abundant in glycolysis, the citrate cycle and the branched chain amino acid (BCAAs) pathway for batch and fed-batch operation, respectively. This table quickly demonstrates the effect of the culturing method on some of the important metabolic pathways. As can be seen for the glycolysis pathway, phosphoenolpyruvate and glucose 6-phosphate were abundant in batch (and not detected in fed-batch), whereas pyruvate and glucose were more abundant in the fed-batch operation. Inspection of the citrate cycle (TCA) in Table 5.1 shows that components of TCA such as citrate, succinate, fumarate, maleate, and malate were all detected in both batch and fed-batch operations, although they were all significantly more abundant in batch. Data for the abundance of branched chain amino acids (BCAAs) in Table 5.1 show that these amino acids were detected in both treatments, but the type of abundant metabolites differed for the two treatments. In batch, valine, leucine, isoleucine, 4-methyl-2-oxopentanoate, and isovalerylcarnitine (C5) were more abundant. In fed-batch, on the other hand, mostly intermediate metabolites – such as 2,3-dihydroxyisovalerate, 2-hydroxy-3-methylvalerate, alpha-hydroxyisocaproate, alpha-hydroxyisovalerate, n-acetylleucine, n-acetylvaline – were more abundant.

Table 5.1. Mean abundance of metabolites involved in the Glycolysis, TCA and BCAA pathways for batch and fed-batch operations.

Pathway	Metabolite	Mean abundance in treatments	
		Batch	Fed-batch
Glycolysis	Pyruvate	0.06	1.02
	Glucose	0.53	1.58
	Phosphoenolpyruvate	0.99	Not detected
	Glucose 6-phosphate	0.98	Not detected
TCA	Citrate	1.08	0.10
	Succinate	1.65	0.38
	Fumarate	1.55	0.54
	Maleate	1.52	0.54
	Malate	1.91	0.40
BCAA	Valine	2.08	0.04
	Leucine	1.99	0.21
	Isoleucine	1.91	0.19
	2,3-dihydroxyisovalerate	0.03	2.04
	2-hydroxy-3-methylvalerate	0.59	1.7
	4-methyl-2-oxopentanoate	0.7	Not detected
	Alpha-hydroxyisocaproate	0.23	1.87
	Alpha-hydroxyisovalerate	0.4	1.56
	N-acetylleucine	0.61	1.38
	N-acetylvaline	Not detected	0.91
	Isovalerylcarnitine (C5)	0.62	Not detected

Some of the metabolites detected in these processes can be seen as industrially relevant or can be used as precursors for the production of pharmaceutical substances and consumer goods. Table 5.2 shows a list of these metabolites produced in abundance in either batch or fed-batch operation, and whether these compounds are terminal (end products of pathways) or intermediate metabolites. These metabolites were selected among the list of abundant metabolites shown in Figure 5.2 and were identified as intermediate or late metabolite by looking at their respective metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG). As can be seen, the number of commercially relevant metabolites detected in the treatments was higher for batch (30) compared to fed-batch (10) cultures, owing to the fact that the batch operation contained a greater number of highly abundant metabolites in general.

Table 5.2. Industrially relevant metabolites displaying greater abundance when *M. album* BG8 was grown under batch or fed-batch operation.

Process	Metabolite	Type of metabolite
Batch	Tryptophan	Late metabolite
	Palmitoleate	Late metabolite
	Tyrosine	Late metabolite
	Myristate	Late metabolite
	Arginine*	Late metabolite
	Azelate	
	Pimelate	Intermediate
	2-aminooctanoate	
	Glutamate	Late metabolite
	Phenylalanine	Intermediate
	Valine	Late metabolite
	Histidine	Late metabolite
	2-aminoheptanoate	
	Pentadecanoate	
	Adipate	Intermediate
	Glycine	Late metabolite
	1-palmitoyl-GPG	
	Palmitate	Late metabolite
	2-hydroxyoctanoate	
	Threonine	Late metabolite
	Alanine	Late metabolite
	Lysine	Late metabolite
	3-hydroxyoctanoate	
	2-nonenoate	
	Myristoleate	Late metabolite
	Erucate	Late metabolite
	Citrate	Intermediate
	Isoleucine	Late metabolite
	2-hydroxyheptanoate	

	Leucine	Late metabolite
	Proline	Late metabolite
	Serine	Late metabolite
	2-hydroxymyristate	
	Oleate/vaccenate	
	8-hydroxyoctanoate	
	Succinate	Intermediate
	Choline	Late metabolite
	Xanthine	Late metabolite
	Cystine	Late metabolite
	Gluconate	Late metabolite
	Inosine	Late metabolite
	Phenylpyruvate	Intermediate
Fed-batch	Pyruvate	Intermediate
	5-dodecenoate	
	Diacylglycerol	
	4-hydroxybenzoate	Intermediate
	Phenyllactate (PLA)	Late metabolite
	4-hydroxycinnamate	Intermediate
	Pyridoxamine	Intermediate
	Caprate	Late metabolite
	3-(4-hydroxyphenyl)lactate (HPLA)	
	Pelargonate	
	Glucose	
	Arabitol/Xylitol	Intermediate
	Benzoate	Intermediate
	Linolenate	Intermediate
	alpha-hydroxyisocaproate	Intermediate
	N-acetylleucine	

5.5. Discussion

In this study, cultures from batch operation in the late exponential phase were compared to cultures from fed-batch operation growing at high cell density. These represent common harvesting conditions in the respective modes of operation. To account for the variation in the biomass density, metabolite abundance was normalized according to cell pellet amount per sample. This enabled appropriate and valid comparison between treatments.

One of the most notable effects of moving from batch process to fed-batch was the general decrease in abundance detected in fed-batch operation (Figure 5.1). In fact, with the exception of some lipids and a few scattered metabolites, fed-batch operation resulted in lower metabolite abundance for all classes of compounds. This is likely explained by the stresses caused by higher cell densities and the possible accumulation of metabolites in batch cultures collected in the late exponential phase versus fed-batch cultures harvested in their active growth state. In addition, the fed-batch experiments were operated in a bioreactor, which was a scale-up from the Wheaton bottles used in the batch operation. Studies on the change of metabolic profiles at lab and production scales (Chrysanthopoulos *et al.*, 2010; Gao *et al.*, 2016) confirm the occurrence of scale-up discrepancies, which could cause differences in process performance at different operational scales. Using combined metabolomics and proteomics in Chinese hamster ovary (CHO) bioprocess (Gao *et al.*, 2016), showed that the reduction in productivity in a scale-up from a 20-L to a 5000-L bioreactor was caused by excess generation of reactive oxygen species (ROS). Addition of copper to the 5000-L reactor resulted in reduction of ROS, showing that the excess ROS was due to hypoxia evidenced by the reduction of fibronectin with increased copper concentrations. Another study on mammalian cell cultures in bioreactors (Vernardis *et al.*, 2013) showed a metabolic signature of phase transitions. This study showed that the vast majority of intracellular metabolites had lower concentrations in the middle phase of the growth compared to late phase. This could explain why metabolomics data collected from the late growth phase of the batch culture differed from the fed-batch culture, as the latter was continuously supplied with the substrate and kept in an active state of growth, similar to a middle phase resulting in lower overall metabolite concentrations in the study by Vernardis et al. (2013).

When looking at differential abundances in specific pathways, some clear patterns appear. For example, there are clear differences in the abundance of metabolites linked to the

BCAA pathway between batch and fed-batch cultures (Table 5.1). As can be seen in Figure 3, the BCAAs valine, isoleucine and leucine were 42-, 10- and 9-fold more abundant, respectively, in batch in compared to fed-batch. These compounds are some of the end metabolites of the BCAA pathway. On the other hand, the intermediates to these BCAAs were found to be more abundant in fed-batch cultures, suggesting a bottleneck in the pathway. For example, 2,3-dihydroxy-isovalerate, a precursor of valine, leucine and isoleucine biosynthesis, was 56 times more abundant in cells grown in fed-batch. In fact, 2,3-dihydroxy-isovalerate was the most abundant metabolite in fed-batch in compared to batch cultures (Figure 5.2). These results suggest that, at the time of harvest, the batch culture could have initiated metabolism associated with starvation, resulting in increased production of BCAAs (Holeček *et al.*, 2001). In the fed-batch culture on the other hand, it seems that valine, leucine and isoleucine were not yet synthesized at high abundances, with cells accumulating their precursors n-acetylvaline and alpha-hydroxyisovalerate (precursors of valine), alpha-hydroxyisocaproate and n-acetylleucine (precursors of leucine) and 2-hydroxy-3-methylvalerate (precursor of isoleucine).

A similar pattern was observed in other pathways. Pyruvate, one of the intermediate metabolites of glycolysis, was more abundant in cells harvested from fed-batch than from batch operation (Table 5.1). In fact, pyruvate was found to be 16 times more abundant in fed-batch (Figure 5.2), even being the fourth-most abundant metabolite in this mode of operation. This can be further confirmed by looking at metabolites from the citrate cycle – citrate, succinate, fumarate, maleate and malate (Table 5.1) – which were all detected in both treatments but were also more abundant in batch. This suggests the energy-generating citrate cycle was predominantly employed in harvested batch compared to fed-batch cultures.

Pyruvate is known to breakdown to produce acetyl coenzyme A (acetyl CoA), which is then converted to oxaloacetate to enter the citrate cycle for energy production. The fact that pyruvate was accumulating in the fed-batch process, combined with the pattern observed for the BCAAs pathway, suggests that, under fed-batch operation, acetyl CoA could be mostly produced from BCAAs rather than by the breakdown of pyruvate.

The evaluation of the metabolome of *M. album* BG8 growing under batch and fed-batch operation also allowed the identification of high-abundance metabolites (Table 5.2) with industrial relevance for the food, pharmaceutical, cosmetic, and dietary supplement industries.

For instance, among the metabolites displaying high abundance in the batch process, citrate is used in detergents (Hoyt *et al*., 1992; Wood and Calton, 1996) and also as an anticoagulant agent (Oudemans-van Straaten, 2009; Buturovic-Ponikvar, 2016). Derivatives of myristate are used as surfactants (Fauzi *et al*., 2013), as phase change materials (Sari *et al*., 2013) and in cosmetic products (Garcia *et al*., 1996). Tyrosine is used as a food supplement to enhance cognitive performance (Colzato *et al*., 2015; Kühn *et al*., 2019). On the other hand, as discussed above, the fed-batch process led to high abundance of pyruvate; a compound widely used as a food additive, in cosmetics, as a precursor for biosynthesis of pharmaceuticals, and in the production of agrochemicals (Ogawa *et al*., 2001; Li *et al*., 2001; Zhu *et al*., 2008). Derivatives of benzoate are known to have antimicrobial properties (Park *et al*., 2001; Kumar *et al*., 2009) and are also used as preservatives in the production of carbonated beverages (Kalpana *et al*., 2019).

A significant difference between the metabolomic patterns for cultures harvested from batch (at late-exponential stage) and fed-batch processes can be seen from data in Table 3. In batch cultures, for which the nutrients are provided once at the onset of operation and growth takes place until limiting nutrients run out, the metabolites produced were mostly "late metabolites" with larger skeletal backbones, resulting from the later stages of metabolic pathways. In fact, few intermediate metabolites – except phenylalanine, adipate, citrate, succinate and phenylpyruvate – had elevated relative abundance in batch cultures. Conversely, most of the high-abundance metabolites resulting from fed-batch operation were early or intermediate metabolites, with only a few – e.g. phenyl lactate and caprate – resulting from the final stages of a metabolic pathway. As discussed previously, this could be happening due to the continuous introduction of the substrate in fed-batch operation, which results in the bacteria remaining in an active state in which the metabolic activity is high and the substances necessary for growth are being continuously generated.

5.6. Conclusion

The results of this study show that although the abundance of many metabolites was lower in fed-batch fermentation of *M. album* BG8 compared to batch, but both processes yielded commercially valuable metabolites. The comparison of metabolomes shows that fed-batch fermentation of *M. album* BG8 leads to high abundance of intermediate compounds, probably as

a result of the microorganism being maintained in an active metabolic state. In batch cultures harvested in late exponential phase, on the other and, most of the accumulating compounds were late metabolites of pathways. In batch cultures, BCCAs were more significantly abundant whereas in fed-batch they were converted to derivatives of BCAAs. Further, pyruvate was found to accumulate in fed-batch while most metabolites of the citrate cycle accumulated in cells harvested from batch. This study shows that the culturing process can have a significant effect on the metabolome of *M. album* BG8. This demonstrates that metabolomics analysis accompanied with bioprocessing strategies can become a powerful tool for the design and improvement of industrial production of valuable compounds using microorganisms.

5.7. Acknowledgements

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5.8. Reference

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6. Summary, conclusion and future direction

6.1. Summary and conclusion

Methanotrophic bacteria have shown promising capacity to be used in bioproduction of commercially relevant compounds (Asenjo *et al*., 1986; Valappil *et al*., 2007; Khosravi-Darani *et al*., 2013). In this work, we investigated strategies to increase the growth and biomass productivity of the methanotrophic bacterium *Methylomicrobium album* BG8 using methanol as carbon source. Second, various process operation techniques were compared and a feeding strategy was developed to increase the productivity of growth of *M. album* BG8. Lastly, metabolomics analysis were performed on batch and fed-batch processes to evaluate the effect of various modes of operation on the metabolite response of *M. album* BG8.

Firstly, the effect of copper, an essential component for the growth of methanotrophic bacteria, on cell density and growth rate of *M. album* BG8 growing on methane was studied and presented in Chapter 3. Copper is known to be central to aerobic bacterial methane oxidation and methanotrophs have shown great sensitivity to its availability (Fru, 2011; Semrau *et al.*, 2018). Results showed that copper concentrations did not have a significant effect on the final optical density and yield but could substantially affect the growth rate of cultures. It was found that the optimal conditions for the growth of *M. album* BG8 was methanol at a concentration of 25 mM and copper concentrations below 0.01 mM. At methanol concentrations other than 25 mM (8, 12, 100 and 150 mM) addition of copper resulted in gradual decreases in growth rate. The results of Chapter 3 provided conditions of methanol and copper concentrations promoting favorable growth of *M. album* BG8.

In the process of taking methanotrophic bioproduction from laboratory to industrial scales, attention must be paid to improving growth and biomass productivity. To accomplish this goal, as presented in Chapter 4, *M. album* BG8 was grown using conventional batch operation, a pulsing fed-batch operation, and a step-wise fed-batch operation. To enhance the performance of the developed methanol feeding strategy used in step-wise fed-batch, various adjustments were tested when performing this strategy, including two different target growth rates, early initiation of the feeding law, and using a fixed initial methanol feeding rate. The results showed that all the conditions tested led to improved cell densities compared to batch culture. The operation through

which the highest cell densities were achieved was the step-wise fed-batch with a target growth rate of 0.0135 h^{-1}, leading to a final OD of 6.6 after ~350 h. Increasing the target growth rate to 0.027 h^{-1} resulted in to slightly lower cell density (OD ~6.1) although the process time was reduced to 200 h, improving the biomass productivity. The results suggested a trade-off between final cell density and the duration of the process. Slower methanol feeding rates in the early stages of the bioprocess also led to higher final cell densities. From these experiments, it could be established that the mode of operation and the feeding regimen could be tailored for different process objectives: whether high cell densities or productivity.

For the aim of using methanotrophic bacteria for bioproduction at industrial scale, it is also important to know the effect of the mode of operation on the intracellular processes themselves, and the abundance of commercially relevant metabolites. The effect of batch and fed-batch modes of operation on the metabolic response of *M. album* BG8 was investigated in Chapter 5. The results showed that, although the abundance of many metabolites was lower in fed-batch compared to the batch, both modes of operation yielded commercially relevant compounds. In fed-batch operation, higher abundance was mostly observed for intermediate compounds, whereas in cultures harvested from batch, most of the compounds detected were late, "end-of-pathway" metabolites. This could be happening because in fed-batch methanol is being continuously fed and the bacteria are constantly kept in an active growth state, whereas the batch cultures were harvested in the late exponential phase. Also, BCCAs were more significantly abundant in batch cultures, and were converted to BCAA derivatives in fed-batch cultures. Pyruvate was found to accumulated in the fed-batch culture, while most metabolites of the citrate cycle accumulated in the batch process. The study in Chapter 5 showed that the mode of operation can have a significant effect on the metabolome of *M. album* BG8, and that metabolomics is an important tool used to assess bioprocessing strategies and design bioprocesses with enhanced performance towards the production of targets bioproducts.

Overall, the results of this work led to improved knowledge on favorable conditions for the growth of *M. album* BG8 and the bioprocessing techniques leading to improved biomass productivity for *M. album* BG8 growing on methanol. It also identified conditions promoting the abundance of target metabolites with industrial relevance. These results open the door towards industrialization of methanotrophic bioprocesses.

6.2. Future direction

This thesis set out to explore process operation techniques for taking methanotrophic bioprocesses from laboratory to industrial scale. To fully understand various aspect of the developed techniques and improve the performance of the bioprocess, and also target the production of valuable bioproducts many interesting studies can be carried out in future research projects. These include:

- **Metabolomics analysis of the developed step-wise fed-batch strategy**
  In Chapter 6, batch and pulsing fed-batch operations for growth of *M. album* BG8 were investigated using metabolomics analysis to see the differences in the metabolome using these two modes of operation. Metabolomics analysis could also be performed on the step-wise fed-batch strategy developed in Chapter 4 of this study to shed light on the abundance of metabolites produced using this technique. This could be very interesting since the developed strategy proved to be the ideal operation in compared to batch and pulsing fed-batch operations in terms of the achieved cell density and the duration of the process. It is important to understand whether this process could also produce commercially relevant compounds while showing better overall performance in compared to the previous techniques.
- **Genetic engineering of *M. album* BG8 for bioproduction**
  Another interesting aspect of future studies could be production of compounds using genetically modified methanotrophs using the bioprocesses developed in Chapter 4. For instance, biopolymer polyhydroxybutyrate (PHB) is a biodegradable polymer that can be produced by some methanotrophs as an energy storage molecule (Pieja *et al.*, 2012; Rostkowski *et al.*, 2013). But, *M. album* BG8 is not able to produce the PHB. Genetic modification of *M. album* BG8 and employing the modified strain in the bioprocesses developed in Chapter 4 can provide means to not only achieve higher growth productivities but also produce valuable compounds that could not be normally produced using *M. album* BG8.
- **Incorporation of nitrogen limitation in step-wise fed-batch operation using the strains that produce PHB**

Some strains of methanotrophic bacteria can inherently produce PHB without the need for genetic modifications. These strains include but are not limited to *Methylocystis parvus* OBBP and *Methylosinus trichosporium* OB3b (Pieja *et al.*, 2012; Rostkowski *et al.*, 2013). An interesting study would be to use these bacteria in the developed step-wise fed-batch operation accompanied by periods of nitrogen limitation to induce PHB accumulation. Also, the samples from this operation can be extracted and compared to batch and pulsing fed-batch operation to investigate the PHB content and PHB quality of the strains grown using various modes of operation.

- **Techno-economic analysis of step-wise fed-batch operation**

  The bioprocesses developed in this study were performed either in 250-ml shake flasks (batch) or in 3-L reactors (pulsing fed-batch and step-wise fed-batch operations). As the long-term aim of the field of study is to take bioprocesses from laboratory to industrial scale, it would be very useful to design the batch and pulsing fed-batch and step-wise fed-batch processes in an industrial scales and perform techno-economic analysis to compare the feasibility of taking this bioprocess to an industrial level in terms of the production cost.

6.3. References

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