TECHNISCHE UNIVERSITÄT WIEN

Sustainable Bioprocess Solutions

Master Thesis

Sustainable production of PHB by utilizing C_1 compounds as carbon source

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Abstract

Environmental concern about conventional plastics regarding their end-of-life management and the rapid depletion of fossil fuels, have sparked the interest in the development of biopolymer alternatives. Among the various groups of biopolymers, polyhydroxyalkanoates (PHAs) have great potential to replace their petrochemical counterparts. The biggest obstacle for PHAs is the high cost associated with their production. The factor contributing most to the overall price are commonly used carbon feedstocks. One-carbon (C_1) compounds like methane and methanol are cheap and abundant potential carbon sources for the microbial production of polyhydroxybutyrate (PHB). Methane can be metabolized by microorganisms called methanotrophs, while reduced C_1 compounds like methanol can be utilized by methylotrophs. Both groups of organisms accumulate PHB under nutrient-insufficient conditions. A limitation in nitrogen was chosen as PHB accumulation strategy in this work.

In the first part, the production of PHB was evaluated in a bubble column reactor (BCR) setup utilizing methane as substrate. A temperature of 37 °C and an ammonium concentration of 8.5 mM were assessed as ideal for the growth of *Methylocystis* sp. GB25 DSMZ 7674 in serum bottles. PEG 400 was determined as suitable antifoam agent for the cultivation of *M.* sp. GB25 DSMZ 7674. However, the PHB accumulation phase could not be reached in the BCR setup. Initial growth was visible in the cultivation of *M.* sp. GB25 DSMZ 7674 and *Methylocystis parvus* OBBP, but both strains showed a sudden stop in microbial activity after 8 to 10 hours. In contrast, a PHB content of 31 % and 11 % of cell dry weight (CDW) could be reached in the cultivation of *M.* sp. GB25 DSMZ 7674 in a stirred-tank reactor (STR) system. The successful production of PHB in the STR system led to the conclusion, that gas-to-liquid mass transfer might not be sufficient in the BCR setup.

In a second approach, the effect of formate as auxiliary carbon source to methanol on methylotrophic growth and PHB accumulation was investigated. A PHB content of 27.4 % per CDW was reached in the cultivation of *Methylorubrum extorquens* DSMZ 1337 with methanol as carbon source, while a PHB content of 16.0 % per CDW could be reached with methanol and formate in combination. Even though yields could not be improved under the given experimental conditions formate as co-substrate is worth being further investigated as it has shown to support increased growth and product yields in multiple organisms and under various conditions.

Zusammenfassung

Umweltbedenken über herkömmliches Plastik in Bezug auf das Management nach ihrer Benutzung und die rasante Erschöpfung von fossilen Energieträgern, haben das Interesse an der Entwicklung von Biopolymeren geweckt. Unter den verschiedenen Gruppen an Biopolymeren haben Polyhydroxyalkanoate (PHAs) großes Potential ihre petrochemischen Gegenstücke zu ersetzen. Die größte Hürde für PHAs sind die hohen Kosten, die aktuell mit ihrer Produktion verbunden sind. Üblich verwendete Kohlenstoffquellen tragen den größten Beitrag zu ihren Kosten. Kohlenstoffverbindungen wie Methan und Methanol sind billige und reichlich vorhandene potenzielle Kohlenstoffquellen für die Produktion von Polyhydroxybutyrate (PHB). Methan kann von methanotrophen Mikroorganismen umgewandelt werden, während reduzierte Kohlenstoffquellen wie Methanol von Methylotrophen verwendet werden können. Beide Gruppen von Organismen akkumulieren PHB unter Nährstoffmangelbedingungen. Eine Limitierung in der Stickstoffquelle wurde als Ansatz für die Akkumulation von PHB gewählt.

Im ersten Teil dieser Arbeit wurde die Produktion von PHB in einem Blasensäulenreaktor mit Methan als Substrat evaluiert. Eine Temperatur von 37 °C und eine Ammoniumkonzentration von 8.5 mM konnten als optimal für das Wachstum von *Methylocystis* sp. GB25 DSMZ 7674 in Serumfläschchen bestimmt werden. PEG 400 konnte als passendes Antischaummittel ermittelt werden. Die PHB-Akkumulationsphase konnte im Blasensäulenreaktor jedoch nicht erreicht werden. Anfängliches Wachstum war in der Kultivierung von *M.* sp. GB25 DSMZ 7674 und *Methylocystis parvus* OBBP sichtbar, aber beide Stämme zeigten einen plötzlichen Stopp an Aktivität nach 8 bis 10 Stunden. Im Gegensatz dazu konnte ein PHB-Gehalt von 31 % und 11 % pro Zelltrockengewicht in der Kultivierung von *M.* sp. GB25 DSMZ 7674 in einem Rührkesselreaktorsystem erreicht werden. Die erfolgreiche Produktion von PHB im Rührkesselsystem führte zu dem Schluss, dass der Gas-zu-flüssig Massentransfer im Blasensäulenreaktor vielleicht nicht ausreichend war.

In einem zweiten Ansatz wurde der Effekt von Formiat als zusätzliche Kohlenstoffquelle zu Methanol auf das Wachstum und die PHB-Akkumulation untersucht. Ein PHB-Gehalt von 27.4 % pro Zelltrockengewicht konnte in der Kultivierung von *Methylorubrum extorquens* DSMZ 1337 mit Methanol als Kohlenstoffquelle erzielt werden, während ein PHB-Gehalt von 16.0 % mit Methanol und Formiat in Kombination erreicht wurde. Obwohl der Zusatz von Formiat die Ausbeuten unter den gegebenen experimentellen Bedingungen nicht verbessern konnte, lohnt es sich, Formiat als Co-substrat weiter zu untersuchen, da es bei verschiedenen Organismen und unter verschiedenen Bedingungen erhöhtes Wachstum und Produktausbeuten unterstützte.

Statement of Authorship

I hereby affirm that the master thesis at hand is my own written work and that I have used no other sources and aids other than those indicated.

All passages, which are quoted from publications or paraphrased from these sources, are indicated as such.

This thesis was not submitted in the same or in a substantially similar version, not even partially, to another examination board and was not published elsewhere.

Vienna, 16.12.2022	Andrea Leibetseder
Place, Date	Signature

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List of abbreviations

AMS	Ammonium mineral salts
BCR	Bubble column reactor
C_1 compound	One-carbon compound
CBB cylce	Calvin-Benson-Bassaham cycle
CDW	Cell dry weight
DO	Dissolved oxygen
DoE	Design of Experiment
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
GHG	Green house gas
HPLC	High performance liquid chromatography
k_La	volumetric mass transfer coefficient
Lcl-PHA	Long chain length polyhydroxyalkanoate
Mcl-PHA	Medium chain length polyhydroxyalkanoate
OD_{600}	Optical density at a wavelength of 600 nm
P(3HB-co-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PE	Polyethylene
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PHB	Poly-3-hydroxybutyrate
PVC	Polyvinylchloride
RuMP cylce	Ribulose monophosphate cycle
Scl-PHA	Short chain length polyhydroxyalkanoate
Slpm	Standard litre per minute
TCA cycle	Tricarboxylic acid cycle
TE solution	Trace element solution

Metabolites

ATP	Adenosine triphosphate
H_4F	Tetrahydrofolate
H_4MPT	Tetrahydromethanopterin
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
PQQ	Pyrroloquinoline quinone

Enzymes	
ßD	β-hydroxybutyrate dehydrogenase
ßK	β-ketothiolase
AR	Acetoacetyl-CoA reductase
AST	Acetoacetate succinyl-CoA transferase
E	Enolase
FaDH	Formaldehyde dehydrogenase
FDH	Formate dehydrogenase
FtfL	Formate tetrahydrofolate ligase
GK	Glycerate kinase
HPR	Hydroxypyruvate dehydrogenase
MaDH	Malate dehydrogenase
MDH	Methanol dehydrogenase
ML	Malyl-CoA lyase
MtdB	Methylenetetrahydromethanopterin dehydrogenase
MTK	Malate thiokinase
PD	PHB depolymerase
PEPC	Phosphoenolpyruvate carboxylase
PS	PHB synthetase
pMMO	Particulate methane monooxygenase
SGAT	Serine glyoxylate aminotransferase
SHMT	Serine hydroxymethyltransferse
sMMO	Soluble methane monooxygenase
Strains	
M. extorquens	*Methylorubrum extorquens*
M. parvus OBBP	*Methylocystis parvus* OBBP
M. rockwell	*Methylocystis rockwell*
M. sp. GB25 DSMZ 7674	*Methylocystis* sp. GB25 DSMZ 7674

1. Introduction

1.1. Fossil fuel-based plastics

Fossil fuel-based plastics play an essential role in our modern day-to-day life, providing convenient and inexpensive products. These synthetic polymers have many favourable properties like strength, lightness and durability, thus replacing commodity like glass, metals or paper in packaging[1],[2]. The terms fossil fuel-based, petroleum-based, petrochemical, conventional and synthetic are used synonymously throughout this work to refer to plastics that are derived from fossil fuels.

Global plastics production (production of recycled plastics not included) reached 367 million tonnes in 2020, while plastics production in Europe reached 55 million tonnes. Packaging represents the largest end-use market with 40.5 % in Europe (EU27+3), followed by building and construction and the automotive industry. The rest of the end-use markets include plastics for electronics, household, agriculture, furniture, medical applications, mechanical engineering etc.[3]

While conventional plastics have a lot of beneficial features, environmental concern has been rising in recent years. Like other consumer material, plastic products end up as discarded waste after their use and thus contribute to the growth of municipal waste and urban litter. Plastics are especially persistent in the environment in comparison to other discarded materials like lignocellulosic paper. The lifetime of these polymers depends on their chemical structure and the environment they are disposed in. An especially alarming problem is the amount of plastic litter that ends up in the world's oceans[2].

Recycling of packaging material has increased in recent years, but various processing additives like fillers, colourants and plasticizers, as well as contamination during the use of these products can hinder recycling[4],[5]. Of the 29 million tonnes of plastic waste collected in Europe (EU27+3) in 2020, 42 % went to energy recovery operations, 34.6 % were recycled and still 23.4 % ended up on landfills[3].

Moreover, there is concern over the rapid depletion of fossil fuels and concomitant fluctuations in price[1]. It takes millions of years for fossil fuels to accumulate, while deposits are extracted at a rapid pace. This makes fossil fuels finite and non-renewable natural resources[6].

1.2. Bioplastics

The afore-mentioned concerns over the end-of-life-management of conventional plastics and the depletion of fossil-fuels, sparked the interest in the development of bioplastics[1]. According to European Bioplastics, bioplastics represent around one per cent of the global plastics produced annually. Bioplastics are a diverse family of materials with similar properties as petrochemically derived plastics and offer additional benefits, such as a reduced carbon footprint and further waste management options, such as organic recycling[4].

European Bioplastics defines bioplastics as plastics that are biodegradable, bio-based or both[4]. The degradation of biodegradable materials occurs as a result of the enzymatic action of microorganisms in a limited period of time. The material is broken down into carbon dioxide (CO_2), methane (CH_4), water (H_2O), inorganic compounds or biomass[5]. Bio-based materials are derived from natural origins like polysaccharides (starch, cellulose, lignin and chitin), proteins (gelatine, casein, wheat gluten, silk and wool) and lipids (plant oils and animal fats). Certain polyesters synthesized by microorganisms or plants (polyhydroxyalkanoates) or produced from bio-derived monomers (polylactic acid) as well as a group of miscellaneous polymers (natural rubber) also fall in the category of bio-based[5].

However, the production of bioplastics poses some challenges. The area of arable land to grow renewable raw materials like wheat, corn, and sugar cane may come into competition with the production of food and feed. Also, the use of fertilizers and chemicals in the cultivation of renewable resources can lead to acidification of the soil and eutrophication. Moreover, the cost of bioplastics is generally still a lot higher than the cost of their petrochemical counterparts, as renewable feedstocks are more expensive[7].

Nevertheless, the bioplastics industry is expected to grow continuously, and has the potential to decouple economic growth from resource depletion and environmental impact[4]. Among the various groups of biopolymers, polyhydroxyalkanoates (PHAs) are promising candidates to replace synthetic plastics.

1.3. Polyhydroxyalkanoates

1.3.1. Historical view and structure

PHAs are polyesters of hydroxyalkanoates, synthesized by various microorganisms and stored in the form of water-insoluble granules during environmental stress. These PHA granules serve as carbon and energy storage material for the producing cells[1]. The stress conditions during

which PHAs are formed constitute in a limitation of essential macro-nutrients such as nitrogen and phosphate or micro-nutrients such as magnesium, sulphate, iron, potassium, manganese, copper, sodium, cobalt, tin and calcium while carbon is available in sufficient amounts[8].

The observation of PHAs as granules in bacterial cells under the microscope dates back to Beijerincka in 1888, although their role and structure remained unresolved. The composition of a PHA was first described by Lemoigne in 1926. He obtained poly-3-hydroxybutyric acid (PHB) from *Bacillus megaterium*. The functional role of PHB was discovered by Macrae and Wilkinson in 1958 who showed that it serves as carbon and energy storage material in bacterial cells and is produced only in an increased carbon-to-nitrogen ratio. Extensive research followed in subsequent years[1],[9].

Industrial interest in PHAs as alternative to petrochemical plastics was sparked in response to the 'oil crisis' in the 1970s, when fears about the price of fossil fuels no longer being reliable began to rise[9]. In the 1980s Imperial Chemical Industries developed poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)), later marketed under the trade name 'Biopol' by Monsanto, followed by Metabolix[10].

1.3.2. Chemical structure

PHAs are linear polyesters consisting of hydroxy acid monomers that are connected through ester bonds. The composition of the side chain or atom R and value of x determine the identity of a monomer unit (Figure 1). PHB, the most prominent representative of PHAs, is composed of monomers with a methyl group as side chain and a value of x=1 (Figure 2)[9].

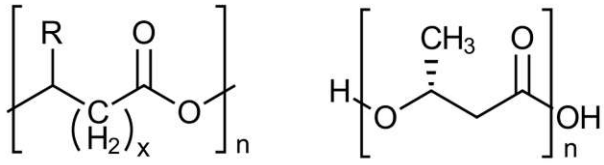


Figure 1 and Figure 2: General formula of PHAs (left) and PHB (right)

PHAs are distinguished into three main groups according to the number of carbon atoms in the monomers: short chain length (scl, < 5 carbon atoms), medium chain length (mcl, 5-14 carbon atoms), and long chain length (lcl, > 14 carbon atoms)[11].

Also, copolymers like (P(3HB-co-3HV)) can be produced when mixed substrates are used during microbial cultivation[1].

1.3.3. Physical properties and applications

About 150 types of PHAs are reported to date. Their properties differ according to structural variations in the constituting monomers[11].

Some general characteristics of PHAs are that they are water-insoluble and relatively resistant to hydrolytic attack. They are soluble in chloroform and other chlorinated hydrocarbons and show good ultra-violet resistance but poor resistance to acids and bases. They are biocompatible and thus suitable for medical applications as well as nontoxic[10].

Scl-PHAs are highly crystalline and therefore rather stiff and brittle. Their melting point ranges from 173 to 180 °C whereas the glass transition temperature is between 5 and 9 °C. Scl-copolymers such as P(3HB-co-3HV) have much lower melting points, are less crystalline, easier to mold and tougher than scl-homopolymers, which makes them more attractive for application. The melting points of mcl-PHAs range from 39 to 61 °C and their glass transition temperature is usually below room temperature between −43 to −25 °C. Mcl-PHAs are less crystalline and thus more flexible and elastic than scl-PHAs[1].

PHAs show good barrier properties like polyvinylchloride (PVC) and polyethylene terephthalate (PET) and thus can be used as biodegradable plastics in the packaging industry. PHB is compatible with the blood and tissue of mammals and is therefore considered as material for surgical implants like heart valves, stents and vascular grafts or as seam threads for the healing of wounds and blood vessels in medicine. In pharmacology, PHAs can be used for drug delivery systems such as microcapsules or as materials for cell and tablet packaging. They can be used as carriers for long term release of herbicides or insecticides. Ultra-high molecular weight PHAs can be useful to produce especially strong fibres for the fisheries industry like fishnets. Other possible applications include flowerpots, sanitary goods, disposable cups, agricultural foils and fibres in textiles[1],[10].

1.3.4. Feedstocks and lowering the cost of PHA production

The main drawback of PHAs is the high cost associated with their production, which limits their industrial application. The prices of PHAs are estimated to be 5–10 times higher than the price of conventional plastics such as polyethylene (PE). The main factor contributing up to 50 % to the overall production cost are commonly used carbon substrates like pure sugars, fatty acids, or noble oils. Also, running cost of fermentation, process productivity and downstream processing add to the overall price. Therefore, research interest has been focused on utilizing a

broad range of waste and surplus materials as a means of reducing the overall production cost of PHAs[11].

Table 1: Overview of (semi)industrial PHA production[12]

Company	Country	Production period	Trade-mark	Strain	Substrate	Type of PHA
Chemie Linz	Austria	Late 1980ies to early 1990ies	-	*Alcaligenes latus* DSM 1124	Glucose from carbohydrate feedstocks	PHB
Biomer	Germany	1993	Biomer™	*Cupriavidus necator*	Glucose from corn starch	PHB
Imperial Chemical Industries (Later: Zeneca, Monsanto)	United Kingdom	1976-1998	BIOPOL®	*Cupriavidus necator*	Glucose from carbohydrate feedstocks	PHB, PHBHV
Metabolix (with Monsanto technology)	USA	1980-ongoing	-	n.r.	Carbohydrates	PHBHV and others
Tepha Inc.	USA	2007-ongoing	TephaFLEX™	n.r.	Sugars, 4HB-precursors	P4HB, P(3HB-co-4HB), mcl-PHA
Polyferm Canada	Canada	ongoing	VersaMer™	Wild type bacteria	Vegetable oils, sugars	mcl-PHA
Tianjin Green Bioscience& DSM	PR China	2004-ongoing	GreenBio™	n.r.	Starch-based sugars, 4HB-precursors	P(3HB-co-4HB)
TianAn	PR China	ongoing	Enmat™	*Cupriavidus necator*	Starch-based sugars, 3HV-precursors	PHBHV
PHB Industrial SA/Copersucar	Brazil	1995-ongoing	BIOCYCLE™	*Cupriavidus necator, Burkholderia sacchari*	Cane sugar	PHB, PHBHV

n.r. (information not provided by manufacturer), PHB (poly-3-hydroxybutyrate), PHBHV (Poly(3-hydroxy-butyrate-co-3-hydroxyvalerate)), P4HB (poly-4-hydroxybutyrate), P(3HB-co-4HB) (poly(3-hydroxybutyrate-co-4-hydroxybutyrate))

In Europe and North America, surplus whey from the dairy industry is available in large amounts, providing lactose as feedstock[8]. Additionally, the increasing production of biofuels generates massive quantities of its major by-product glycerol. Other raw materials include non-wood lignocellulosic materials from rice, corn, and sugar cane as well as starch, sugar-cane

molasses, waste vegetable oils and plant oils. Lignocellulosic and cellulosic material is also generated in major shares by wood-processing, paper and agriculture industries[8],[11]. An overview of several commercialized PHAs and the respective carbon sources is given in Table 1: Overview of (semi)industrial PHA production[12Table 1.

1.3.5. One-carbon compounds as carbon sources for PHB production

An alternative group of substrates for the production of PHB are one-carbon (C_1) compounds such as methane, methanol and formate. In contrast to sugars and agricultural products, these compounds do not compromise food security[13].

Methane is cheap and naturally abundant. It is considered the second major greenhouse gas (GHG) with a global warming potential 25 times higher than that of carbon dioxide[14]. The two main sources of methane are non-renewable natural gas and renewable biogas. Natural gas contains 80–95 % CH_4 and according to the U.S. Energy Information Administration projection there is more than 7.200 trillion cubic feet of reserves globally. Biogas contains 50–70 % CH_4 and can be produced through anaerobic digestion of organic matter such as agriculture waste, municipal organic waste and sewage sludge within a short period of time[15]. Biogas production has been growing significantly in Europe in recent years, mainly encouraged by favourable support schemes[16]. Biologically, methane can be utilized by a group of microorganisms called methanotrophs, which are discussed in detail in chapter 1.4. Methanotrophs can generate several valuable products from methane like single-cell protein, lipids for biodiesel production, soluble metabolites like methanol, formaldehyde, organic acids and ectoine, as well as the biopolymer of interest PHB[17].

Methanol is already an important carbon feedstock for the chemical industry and can be produced from various carbon-containing feedstocks. Currently, about 90 % of methanol is produced from non-renewable natural gas[18]. On the other hand, methanol can be sustainably produced from CO_2 and renewable energy by catalytic hydrogenation. As CO_2 is a very stable molecule, considerable energy input, optimized reaction conditions and a highly active catalyst are required for its conversion into value-added products. When the required hydrogen is derived from water dissociation by electrolysis using a renewable source of electricity such as wind or solar, the catalytic hydrogenation of CO_2 can be regarded as a completely green process[18]. The C_1 compound formate can also be produced from CO_2 by electrochemical reduction.

The utilization of GHG like CH_4 and CO_2 for the production of value-added products is crucial for the establishment of a circular carbon-economy[13].

Biologically, methanol and formate can be utilized by microorganisms called methylotrophs, which are discussed in Chapter 1.5.

1.4. Methanotrophs

Methanotrophs represent a subset of the methylotrophs. They have the ability to assimilate methane through an enzyme called methane monooxygenase (MMO)[19],[20]. According to nomenclature, methanotrophs include both the methane oxidizing bacteria and anaerobic methane oxidizing archaea, but the ability to produce biopolymers from methane is exclusive to the methane oxidizing bacteria[14]. Therefore, only the methane oxidizing bacteria are discussed in this work. Aerobic methane oxidizing bacteria can be distinguished into three major types primarily based on the metabolic pathway they use to assimilate carbon and related attributes such as cell membrane composition and arrangement. Type I methanotrophs or *gamma-proteobacteria* utilize the ribulose monophosphate (RuMP) cycle for carbon assimilation, while Type II methanotrophs or *alpha-proteobacteria* use the serine cycle. In Type III or *Verrucomicrobia* methanotrophs, carbon is assimilated in the form of carbon dioxide via the Calvin-Benson-Bassaham (CBB) cycle[14],[21],[22]. Only one anaerobic methanotrophic bacterium is known, which is Candidatus *Methylomirabilis oxyfera*[14],[23]. The accumulation of PHB is likely limited to Type II methanotrophs that use the serine cycle for carbon assimilation[24].

1.4.1. Dissimilatory pathway: Methane oxidation

Methanotrophs oxidize methane terminally to carbon dioxide through a series of linked reactions shown in Figure 3[14].

The oxidation of methane to methanol is initiated by MMO, the principal defining enzyme of methanotrophs. MMO incorporates one atom of oxygen (O_2) into methane and requires reducing power to reduce the second oxygen atom to H_2O[19],[21]. This step is considered energy consuming while the remaining reactions are energy producing. There are two distinct forms of MMO, the cytoplasmic soluble form (sMMO) and the copper containing particulate form (pMMO) located in the intracytoplasmic membrane. The reducing equivalent required for the reduction of oxygen to H_2O is nicotinamide adenine dinucleotide NAD(P)H generated from

formaldehyde and formate oxidation in sMMO, while the electron donor for pMMO has not been identified yet. Also, pMMO has a higher methane affinity than sMMO, while sMMO has a broader substrate range than pMMO. The expression of both enzymes is controlled by the copper concentration. pMMO is expressed in high copper-to-biomass ratios, while sMMO is expressed when the copper-to-biomass ratio is low[14],[19],[20],[21].

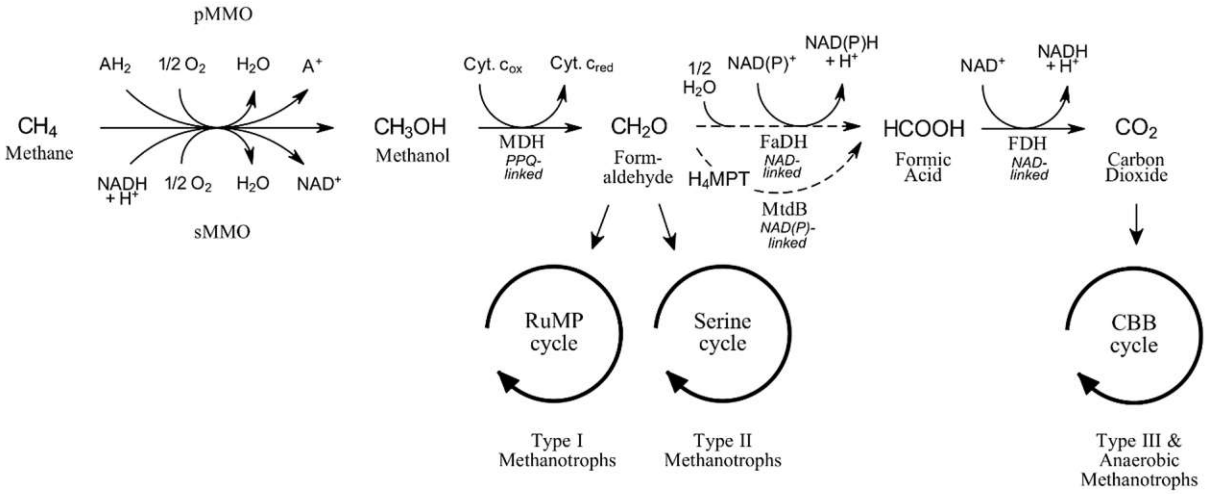


Figure 3: Methane oxidation in methanotrophs; particulate methane monooxygenase (pMMO), soluble methane monooxygeanse (sMMO), A (reducing equivalent providing 2 e^- and 2 H^+), methanol dehydrogenase (MDH), formaldehyde dehydrogenase (FaDH), tetrahydromethanopterin (H_4MPT), NAD(P)-dependent methylenetetrahydromethanopterin dehydrogenase (MtdB), formate dehydrogenase (FDH), ribulose monophosphate (RuMP) cycle, Calvin-Benson-Bassaham (CBB) cycle

In a second step the produced methanol is further oxidized to formaldehyde by methanol dehydrogenase (MDH). MDH is a quinoprotein and contains the cofactor pyrroloquinoline quinone (PQQ). Methanol oxidation to formaldehyde is accompanied by the reduction of PQQ to $PQQH_2$. $PPQH_2$ is then oxidized in turn and two electrons are transferred either to the terminal oxidase with cytochromes-c and other carriers as intermediates or to regenerate the reducing equivalents required for methane hydroxylation[14],[20],[21].

Formaldehyde is the central intermediate in methane oxidation as it can be oxidized to CO_2 for energy generation or incorporated in the carbon assimilation pathways for biosynthesis of cell material. Either way, formaldehyde is promptly metabolised due to its toxic effect on methanotrophs[14]. Different formaldehyde oxidation routes are known in methylotrophs and might be present in methanotrophs as well as a means of formaldehyde detoxification. The simplest of these systems is the oxidation of formate to formaldehyde catalysed by formaldehyde dehydrogenase (FaDH). FaDH can either be NAD-linked or PQQ-containing and

cytochrome-linked. In the tetrahydromethanopterin (H_4MPT)-linked formaldehyde oxidation pathway NAD(P)$^+$ is reduced to NAD(P)H by methylenetetrahydromethanopterin dehydrogenase (MtdB)[14],[21]. Finally, formate is oxidized to carbon dioxide catalysed by the NAD-dependent enzyme formate dehydrogenase (FDH)[14],[20].

Each of the last three oxidation steps produces two electrons, so six electrons in total. Two of these electrons supply methane oxidation in the form of reducing equivalents. The remaining four electrons traverse the electron transport chain and generate energy by producing adenosine triphosphate (ATP) through the proton motive force[14].

1.4.2. Assimilatory pathways and PHB synthesis

For the methane assimilation pathways, as previously mentioned, Type I methanotrophs undergo the RuMP pathway and Type II use the serine pathway. Whereas Type III and the anaerobic methanotrophs assimilate carbon via the CBB cycle. An overview of the major reactions and intermediates in the serin cycle and PHB cycle in Type II methanotrophs is shown in Figure 4.

The serine cycle is initiated through the condensation of formaldehyde with tetrahydrofolate (H_4F) and the formation of methylene-H_4F. Methylene-H_4F then reacts with glycine to yield serine, catalysed by serine hydroxymethyltransferase (SHMT). This C_3 compound undergoes several reactions to form phosphoenolpyruvate, which is carboxylated by phosphoenolpyruvate carboxylase (PEPC) and reduced to malate by malate dehydrogenase (MaDH). Malate is converted to malyl-CoA by malate thiokinase (MTK). Malyl-CoA lyase (ML) cleaves Malyl-CoA into two C_2 compounds, namely glyoxylate and the primary product of the serine cycle acetyl-CoA. In the second half of the cycle, glyoxylate is converted back to glycine by serine glyoxylate aminotransferase (SGAT) while acetyl-CoA undergoes a series of transformations to regenerate the second molecule of glyoxylate[20],[21].

Under nutrient-sufficient conditions methanotrophs proceed to the tricarboxylic acid (TCA) cycle while under unbalanced conditions, they switch to the PHB cycle to provide the energy required for cell maintenance[22]. A limitation of the nitrogen source is one of the most researched conditions for PHB accumulation as well as limitations in phosphorus, copper, iron, sulphur, magnesium, potassium and calcium[14].

The first entry to the PHB cycle is acetyl-CoA, which is converted to acetoacetyl-CoA by β-ketothiolase (ßK) and then reduced to β-hydroxybutryl-CoA via acetoacetyl-CoA reductase (AR). Next, PHB synthetase (PS) forms PHB from β-hydroxybutryl-CoA. In the second half of

the cycle, PHB depolymerase (PD) depolymerizes PHB to β-hydroxybutrate monomers. Acetoacetate is then formed via β-hydroxybutyrate dehydrogenase (ßD) which is converted to acetoacetyl-CoA by acetoacetate succinyl-CoA transferase (AST) to complete the cycle[14],[22].

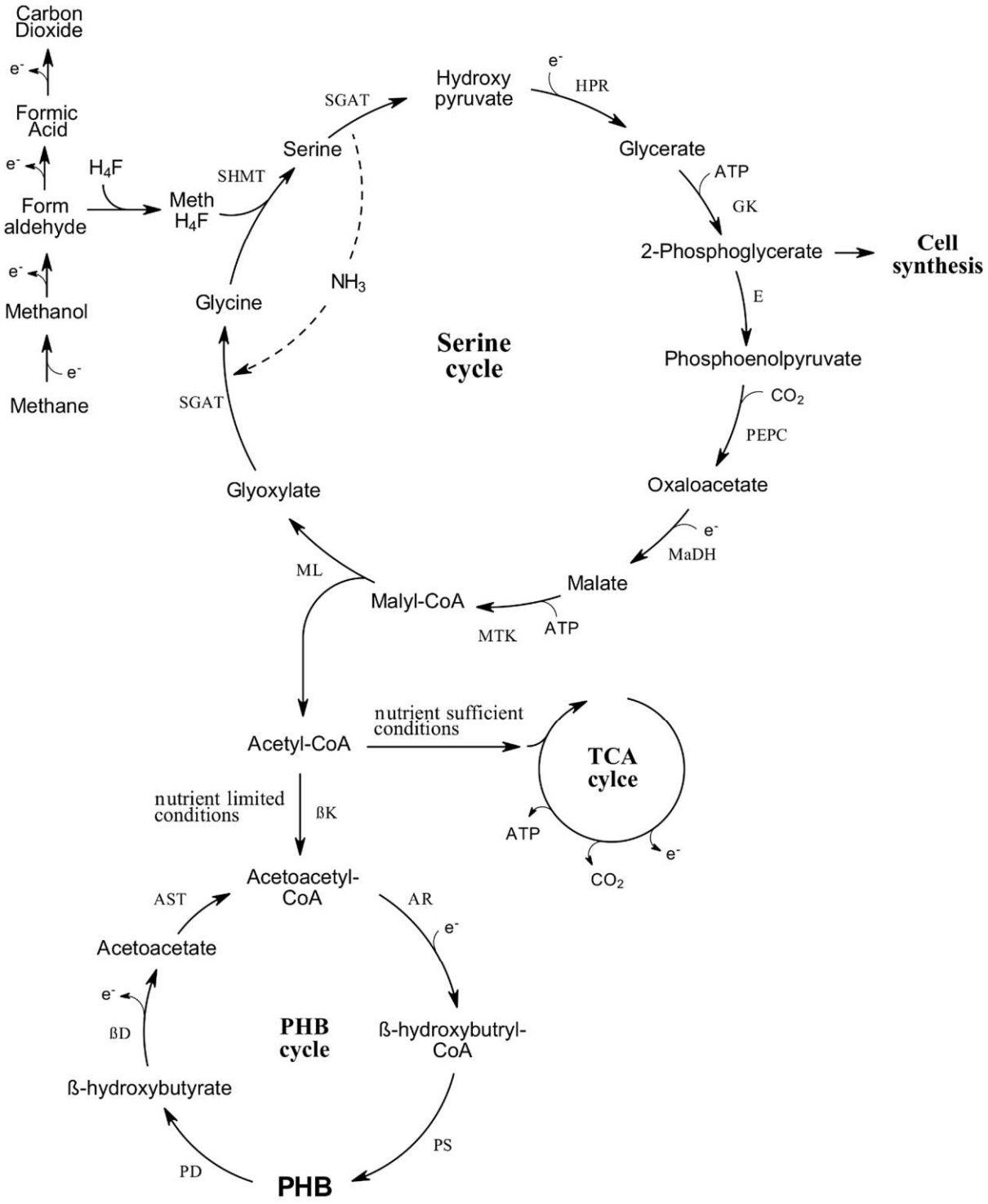


Figure 4: Serine cycle and PHB cycle in type II methanotrophs; tetrahydrofolate (H_4F), serine hydroxymethyltransferase (SHMT), serine glyoxylate aminotransferase (SGAT), hydroxypyruvate dehydrogenase (HPR), glycerate kinase (GK), enolase (E), phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MaDH), malate thiokinase (MTK), malyl-CoA lyase (ML), ß-ketothiolase (ßK), acetoacetyl-CoA teductase (AR), PHB synthetase (PS), PHB depolymerase (PD), ß-hydroxybutyrate dehydrogenase (ßD), acetoacetate succinyl-CoA transferase (AST), tricarboxylic acid (TCA) cycle

1.4.3. PHB accumulation potential of methanotrophs

An overview of several methanotrophic strains and reported PHB yields is given Table 2[22].

Table 2: PHB accumulation potential of methanotrophs

Strain	PHB [% of total biomass]
Methylocystis parvus OBBP	70
	60
	36 ± 8
Methylosinus trichosporium OB3P	10
	30
	25 ± 4.2
	38 ± 3.4
	29
Methylosinus trichosporium IMV 3011	32
	35
Methlocystis sp. GB25 DSMZ 7674	28.3−51.3
Methylocystis SC2	30 ± 13
Methylocystis 42/22	25 ± 7

1.4.4. Methane solubility and challenges for bioreactor setup

One of the major obstacles that the cultivation of methanotrophs is facing, is the low water solubility of methane. Methane shows a water solubility of 24.4 $mg \cdot L^{-1}$ (at 20 °C and 1 atm) compared to a water solubility of 45.5 $mg \cdot L^{-1}$ (at 20 °C and 1 atm) for oxygen[25]. This low solubility hampers the transport of CH_4 to the cells and leads to lower biomass concentrations and overall process performance[14].

Bioreactor configuration and operation have the most significant impact on the gas-to-liquid mass transfer[15]. Stirred tank bioreactors (STR) were most commonly used for the production of PHB from methane in recent research[26],[27],[28]. In a previous study, a PHB content of 51 % PHB of biomass could be reached in a non-sterile production process in a pressurized bioreactor equipped with a stirrer system utilizing a methanotrophic mixed culture (*Methylocystis* sp. GB 25 DSMZ 7674 ≥ 90 % of total dry weight)[29]. However, the required power input, heat removal and usually large shear stress exerted on the cells render this reactor configuration less attractive for industrial applications[15].

Another group of reactors that have been utilized for the conversion of methane are airlift reactors. In the bubble column reactor (BCR), substrate gas is introduced at the bottom of the reactor vessel and the rising bubbles induce agitation[15]. Because of its simple construction, the BCR offers advantages such as low operating cost, reduced effort for maintenance as no moving parts are present and reduced shear stress for the cells[30]. A PHB content of 30.5 % of cell dry weight could be reached in a fermentation of *Methylocystis hirsuta* from natural gas in a 1-L BCR setup[31].

Besides bioreactor setup, adding components with higher affinity to methane and oxygen like vectors, polymers, nanoparticles, electrolytes, and non-ionic surfactants can improve methane mass transfer[15].

1.5. Methylotrophs

Methylotrophic microorganisms utilize reduced one carbon compounds such as methanol as their sole carbon and energy source.

1.5.1. Dissimilatory and assimilatory pathway

Analogous to methanotrophy, methylotrophy can be divided into a dissimilatory and an assimilatory pathway. In the dissimilatory pathway, the primary C_1 substrate is oxidized to formaldehyde which is in turn oxidized to CO_2.

Also, the serine cycle is utilized for C_1 assimilation in the methylotroph *M. extorquens* AM1. Methylene-H_4F is the entry point of reduced C_1 compounds into the serine cycle and can be generate via two routes. The direct route comprises the spontaneous condensation of formaldehyde and H_4F to form methylene-H_4F. The indirect route involves the oxidation of formaldehyde to formate via the H_4MPT-dependent pathway followed by ATP-dependent condensation with H_4F to form formyl-H_4F. The latter reaction is catalysed by formate tetrahydrofolate ligase (FtfL). The produced formyl-H_4F is then converted to methenyl-H_4F and subsequently to methylene-H_4F[32],[33]. Contradicting data is available whether the direct or indirect pathway is of higher importance for the carbon assimilation of methylotrophs[32].

1.5.2. PHB production from methanol

One of the most extensively studied methylotrophs is the *alpha-proteobacterium M. extorquens*. Strain *M. extorquens* AM1 in particular has served as important model organism for enzyme and pathway discovery[32],[34].

M. extorquens has also served as microbial host for the production of various value-added products such as PHAs, amino acids, single cell protein and dicarboxylic acids. Although the strain is able to utilize a wide range of different carbon sources (one to five carbon atoms including alcohols, amines, mono-, and dicarboxylic acids) most biotechnological processes thus far are based on the substrate methanol[32]. A concentration of 136 $g \cdot L^{-1}$ PHB (66 % of cell dry weight (CDW)) at a yield of 0.18 $g \cdot g^{-1}$ methanol was reached in 1986 by Suzuki et al. in a fed-batch cultivation and a limitation in ammonium[32],[35]. Later in 2009, Mokhtari-Hosseini et al. reached a concentration of 46.2 g L^{-1} of PHB (up to 35% of CDW) and an overall yield of biomass and PHB of 0.44 $g \cdot g^{-1}$ and 0.15 $g \cdot g^{-1}$ respectively, employing strain *M. extorquens* DSMZ 1340[36].

2. Motivation and Objective

The motivation of this work is the sustainable production of the biopolymer PHB by utilizing one carbon-compounds. C_1 compounds are either cheap and naturally abundant or can be sustainably produced from CO_2 and renewable energy[13].

In a first approach, the feasibility of PHB production with the gaseous one-carbon compound methane is evaluated in a 16-L BCR setup. The utilization of methane as substrate for PHB production is already studied in lab-scale experiments but is not yet implemented on an industrial scale[22]. Most studies are carried out in smaller scale bioreactors with working volumes between 1 to 4 L[14]. Also, to overcome the low water solubility of methane, energy-intensive measures like high pressure and agitation are often applied[29].

To not compromise the sustainable aspect of the process by high energy demands, this work aims to overcome the low water solubility of methane through an effective bioreactor configuration. The BCR offers an energy-efficient setup and low operating cost for the cultivation of methanotrophic strains. With a working volume of 16 L, the BCR also constitutes a bigger-scale setup as a step towards industrial applications.

To implement a sustainable process for the production of PHB from methane in the BCR setup, this work aims to answer the following questions

- Which methanotrophic strains are suited for the production of PHB?
- Which process parameters are critical for the cultivation of methanotrophs (temperature, ammonium concentration)?
- Which antifoam agents are suited for the cultivation of methanotrophs?
- Which agents can improve methane mass transfer?
- Which k_La values are reached in the BCR setup compared to STRs?

In a second approach, the production of PHB via utilization of the C_1 compounds methanol and formate is investigated. As mentioned, methanol has been explored as substrate for PHB production both scientifically and commercially and high product concentrations and yields have already been reached. On the other hand, formate has not yet been explored as substrate for PHB production.

In this work, the addition of formate as an auxiliary carbon source to methanol is investigated in a first step, to potentially implement a process with formate as substrate in the future. As discussed before, methylene-H_4F is the entry-point into the serine cycle for carbon assimilation

and can be synthesized via a direct and an indirect route. The indirect pathway involves the condensation of formate and H_4F. Thus, the addition of formate might have a positive impact. To investigate the effect of formate as auxiliary carbon source, this work aims to answer the following questions

- How does the substrate influence the growth of different strains of *M. extorquens* (DSMZ 1337, DSMZ 1338, DSMZ 1339 and DSMZ 1340)?
- How does the substrate influence PHB accumulation of different strains of *M. extorquens*?
- Evaluation of formate as auxiliary carbon source in a STR system

3. Materials and Methods

3.1. Cultivations utilizing gaseous C_1 compounds

3.1.1. Strains

The methanotrophic strains used within this work were *Methylocystis parvus* OBBP, *Methylocystis rockwell* and *Methylocystis* sp. GB25 DSMZ 7674. *M. parvus* OBBP and *M. rockwell* were obtained from Valladolid and *M.* sp. GB25 DSMZ 7674 was obtained from 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (DSMZ). All strains were kept in the freezer at -80 °C in 75% (w/w) glycerol for long-term storage.

3.1.2. Media

For the cultivation of methanotrophic strains, three main types of media were used. The minimal ammonium mineral salts (AMS) medium 1 is derived from Wendlandt et al. [29] and the components are listed in Table 3. No MES Monohydrate is added for the cultivations in the BCR, as the pH is adjusted with acid and base in the reactor. As the ammonium concentration varied throughout the experiments, it is stated in the respective sections.

Table 3: Components and respective concentrations of minimal AMS medium 1

Component	Concentration [L^{-1}]
$MgSO_4 \cdot 7\ H_2O$	25.0 mg
$CuSO_4 \cdot 5\ H_2O$	0.785 mg
$MnSO_4 \cdot H_2O$	1.389 mg
$FeSO_4 \cdot 7\ H_2O$	1.678 mg
$ZnCl_2$	0.322 mg
$Al_2(SO_4)_3 \cdot 16\ H_2O$	0.176 mg
$CaCl_2 \cdot 2\ H_2O$	0.415 mg
$Na_2MoO_4 \cdot 2\ H_2O$	0.041 mg
H_3BO_3	1.286 mg
$CoCl \cdot 6\ H_2O$	0.030 mg
NH_4Cl	Stated in the respective section
KH_2PO_4	1.36 g
MES Monohydrate	10.66 g

AMS medium 2 is derived from Pieja et al.[37] and the components are listed in Table 4 to 6. As the ammonium concentration varied throughout the experiments, it is stated in the respective sections.

Table 4: Components and respective concentrations of AMS medium 2

Component	Concentration [L^{-1}]
$MgSO_4 \cdot 7\ H_2O$	0.20 g
$CaCl_2 \cdot 2\ H_2O$	0.016 g
$NaHCO_3$	0.13 g
KH_2PO_4	0.32 g
K_2HPO_4	0.59 g
$Na_2MoO_4 \cdot 2\ H_2O$	5.01 mg
$CuSO_4 \cdot 5\ H_2O$	0.16 mg
Fe-EDTA	3.44 mg
NH_4Cl	Stated in the respective section
TE solution	1 $mL \cdot L^{-1}$
Vitamin solution	10 $mL \cdot L^{-1}$

Table 5: Components and respective concentrations of trace element solution for AMS medium 2

Component	Concentration [L^{-1}]
$FeSO_4 \cdot 7\ H_2O$	500 mg
$ZnSO4 \cdot 7\ H_2O$	400 mg
$MnCl_2 \cdot 7\ H_2O$	20 mg
$CoCl_2 \cdot 6\ H_2O$	50 mg
$NiCl_2 \cdot 6\ H2O$	10 mg
H_3BO_3	15 mg
EDTA	250 mg

Table 6: Components and respective concentrations of vitamin solution for AMS medium 2

Component	Concentration [L^{-1}]
Biotin	2 mg
Folic acid	2 mg
Thiamine·HCl	5 mg
Calcium pantothenate	5 mg
Vitamin B12	0.1 mg
Riboflavin	5 mg
Nicotinamide	5 mg

Components of previously described AMS medium 3 are listed in Table 7 to 10[38].

Table 7: Components and respective concentrations of AMS medium 3

Component	Concentration [L^{-1}]
$MgSO_4 \cdot 7\ H_2O$	1.0 g
NH_4Cl	0.5 g
$CaCl_2 \cdot H_2O$	0.2 g
3.8% (w/v) solution Fe-EDTA	0.1 ml
0.1% (w/v) $NaMo \cdot 4\ H_2O$	0.5 ml
TE solution	1 $mL \cdot L^{-1}$
Phosphate stock solution	10 $mL \cdot L^{-1}$
Vitamin solution	10 $mL \cdot L^{-1}$

Table 8: Components and respective concentrations of trace element solution for AMS medium 3

Component	Concentration [L^{-1}]
$FeSO_4 \cdot 7\ H_2O$	500 mg
$ZnSO_4 \cdot 7\ H_2O$	400 mg
$MnCl_2 \cdot 7\ H_2O$	20 mg
$CoCl_2 \cdot 6\ H_2O$	50 mg
$NiCl_2 \cdot 6\ H_2O$	10 mg
$CuCl_2 \cdot 2\ H_2O$	1.4 mg
H_3BO_3	15 mg
EDTA	250 mg

Table 9: Components and respective concentrations of phosphate stock solution for AMS medium 3

Component	Concentration [L^{-1}]
KH_2PO_4	26 g
$Na_2HPO_4 \cdot 7\ H_2O$	62 g

Table 10: Components and respective concentrations of vitamin solution for AMS medium 3

Component	Concentration [L^{-1}]
Biotin	2.0 mg
Folic acid	2.0 mg
Thiamine HCl	5.0 mg
Calcium pantothenate	5.0 mg
Vitamin B12	0.1 mg
Riboflavin	5.0 mg
Nicotinamide	5.0 mg

3.1.3. Cultivations in serum bottles

3.1.3.1. Preparation of serum bottles

To prepare the serum bottles for cultivation with minimal AMS medium 1, all components listed in Table 3 were first dissolved in deionized water and the pH was adjusted to 6.8 with 1 M KOH. Then, a volume of 39 mL of the solution was dispensed in serum bottles with 80 mL headspace. The serum bottles were sealed airtight and autoclaved at 121 °C for 20 minutes.

To prepare the serum bottles for cultivation with AMS medium 2, all base components listed in Table 4 were first dissolved in deionized water and the pH was adjusted to 6.8 with 1 M HCl. Then, a volume of 39 mL of base solution was dispensed in serum bottles with 80 mL headspace. The serum bottles were sealed airtight and autoclaved at 121 °C for 20 minutes. The components of the trace element (TE) solution (Table 5) and vitamin solution (Table 6) were dissolved in deionized water separately. 1 $mL \cdot L^{-1}$ of TE solution and 10 $mL \cdot L^{-1}$ vitamin solution were added to the autoclaved serum bottles through a 0.2 µm filter.

To prepare the serum bottles for cultivation with AMS medium 3, the base components listed in Table 7 were dissolved in deionized water. The components of the TE solution listed in Table 8 were dissolved in deionized water separately and 1 mL L^{-1} was added to the base components.

Then, a volume of 39 mL of the solution was dispensed in serum bottles with 80 mL headspace. The serum bottles were sealed airtight and autoclaved at 121 °C for 20 minutes. The components of the phosphate stock solution listed in Table 9 were dissolved in deionized water and autoclaved at 121°C for 20 minutes. 10 mL L^{-1} of the autoclaved phosphate stock was then added to the serum bottles. Lastly, 10 mL L^{-1} of the vitamin stock solution (all vitamins listed in

Table 10 dissolved in deionized water) was added to the serum bottles through a 0.2 µm filter. Methanol was added to the serum bottles through a 0.2 µm filter. Methane was provided by flushing the serum bottles with methane:air 40:60 (v/v) with 0.25 standard litre per minute (slpm) for 1 minute.

3.1.3.2. Cultivation of methanotrophic strains in serum bottles

Growth of *M. parvus* OBBP, *M. rockwell* and *M.* sp GB25 DSMZ 7674 was investigated in serum bottles with 40 mL medium and 80 mL headspace. An initial methanol concentration of 7 mM and an ammonium concentration of 10 mM were supplied throughout all culturing steps. Precultures were inoculated with 1 mL of the respective liquid cryo-cultures in serum bottles containing 40 mL AMS medium 2. After 29 hours of cultivation, the main cultures were inoculated with 0.5 mL of the respective preculture in serum bottles containing 40 mL AMS medium 2. To further increase the biomass concentration of the main cultures, a methanol pulse (14 mM) was added to the serum bottles after 24 hours of cultivation. All cultures were incubated at 30°C and 150 rpm. An overview of the cultivation conditions is given in Table 11. The experiment was executed in triplicates.

Table 11: Conditions during cultivation of methanotrophic strains in serum bottles

	Cultivation conditions
Strains	*M. parvus* OBBP, *M. rockwell*, *M.* sp GB25 DSMZ 7674
Medium	AMS medium 2
pH	6.8
Temperature	30°C
rpm	150 rpm
Initial ammonium concentration	10 mM
Initial methanol concentration	7 mM

3.1.3.3. Effect of temperature, ammonium concentration, antifoam agents and gas-transfer-promoting agents on methanotrophic growth

The effect of temperature, ammonium concentration, antifoam agents and gas-transfer-promoting agents on the growth of *M.* sp GB 25 DSMZ 7674 was investigated in serum bottles with 40 mL medium and 80 mL headspace. An initial supply of methane was provided by flushing the serum bottles with methane:air (40:60 (v/v), 0.25 slpm) for 1 minute throughout all culturing steps.

A preculture was inoculated with 1.6 mL of liquid cryo-culture of *M.* sp GB 25 DSMZ 7674 in a serum bottle containing 40 mL minimal AMS medium 1. After 41 hours of cultivation the main cultures were inoculated with 2 mL of the preculture in serum bottles containing 40 mL of minimal AMS medium 1. To resupply oxygen and methane, the main cultures were flushed with methane:air (40:60 (v/v), 0.25 slpm) for 1 minute two more times at 28 and 48 hours of cultivation.

To investigate the effect of temperature and ammonium concentration, cultures were cultivated at three different temperatures (30, 37 and 38 °C) and three different ammonium concentrations (2.0, 8.5 and 15.0 mM) respectively. To investigate the effect of different antifoam agents, PEG 400 (0.1 $g \cdot L^{-1}$), Struktol J673-A (0.1 $g \cdot L^{-1}$) and Struktol 2020 (0.1 $g \cdot L^{-1}$) were added to the serum bottles. A control culture without added agents was cultivated under the same conditions. An overview of the cultivation conditions is shown in Table 12. The experiment was executed in duplicates.

Table 12: Cultivation conditions during experiments to investigate the effect of different temperatures, ammonium concentrations, antifoams agents and gas-transfer-promoting agents on methanotrophic growth

	Effect of temperature	**Effect of ammonium concentrations**	**Effect of agents, Experiment I**	**Effect of agents, Experiment II**
Strain	*M.* sp GB25 DSMZ 7674	*M.* sp GB25 DSMZ 7674	*M.* sp GB25 DSMZ 7674	*M.* sp GB25 DSMZ 7674
Medium	Minimal AMS medium 1	Minimal AMS medium 1	Minimal AMS medium 1	Minimal AMS medium 1
pH	6.8	6.8	6.8	6.8
Temperature	30, 37, 38 °C	38 °C	38 °C	37 °C
rpm	200	200	200	200
Initial ammonium concentration	15 mM	2.0, 8.5, 15.0 mM	15.0 mM	15.0 mM
Substrate	Methane	Methane	Methane	Methane

To investigate the effect of additional antifoam agents and gas-transfer-promoting agents, a second experiment was conducted. Therefore, a preculture was inoculated with 1.6 mL of liquid cryo-cultures of *M.* sp GB 25 DSMZ 7674 in serum bottles with 40 mL minimal AMS medium 1. After 41 hours of cultivation, the main cultures were inoculated with 2 mL of the preculture in serum bottles containing 40 mL of minimal AMS medium 1. To resupply oxygen and methane, the cultures were flushed with methane:air (40:60 (v/v), 0.25 slpm) for 1 minute two more times at 29 hours and 53 hours of cultivation.

The agents were added in the following concentrations: Hydraffin CC12x40 (1 $g \cdot L^{-1}$), Paraffin (5%), PPG 2000 (0.1 $g \cdot L^{-1}$) and PEG 400 (0.1 $g \cdot L^{-1}$). The experiment was conducted in duplicates. A control culture without added agents was cultivated under the same conditions.

3.1.3.4. Cultivation of *M. parvus* OBBP in serum bottles

Strain *M. parvus* was cultivated in minimal AMS medium 1 and AMS medium 3. The serum bottles contained 40 mL of the respective medium and 80 mL headspace. To provide an initial supply of methane, the serum bottles were flushed with methane:air (40:60 (v/v), 0.25 slpm) for 1 minute. A preculture was inoculated with 1.6 mL of liquid cryo-cultures of *M. parvus* OBBP in a serum bottle containing 40 mL of AMS medium 3. After 24 hours of cultivation, the main cultures were inoculated with the preculture in serum bottles containing 40 mL of the respective medium, yielding a start OD_{600} of 0.1. To resupply oxygen and methane, the cultures were flushed with methane:air (40:60 (v/v), 0.25 slpm) for 1 minute two times at 16 hours and 24 hours of cultivation. All cultures were incubated at 37 °C and 200 rpm.

3.1.3.5. Sampling

To monitor cell growth and substrate consumption, samples were taken in regular intervals.

3.1.4. Cultivations in the Bubble Column Reactor

3.1.4.1. Setup

The reactor vessel is made of Hastelloy C-22 (2.4602), which is a nickel-based alloy. It has a total volume of 21.1 L and a working volume of 15 L. The vessel has a diameter of 0.134 m and a height of 1.1 m, resulting in a height-to-width ratio of about 8:1. It has a pressure rating of -1/+5 barg and a temperature rating of 0/+90 °C. The fermenter is equipped with an electric heating jacket. [30]

The BCR setup is shown in Figure 5 and 6 and a process flow diagram is shown in Figure 9. The depicted membrane is not used in this process. Two gassing systems were applied to introduce substrate gas, namely a vortex provided by our project partner shown in Figure 7 and a sparger shown in Figure 8.

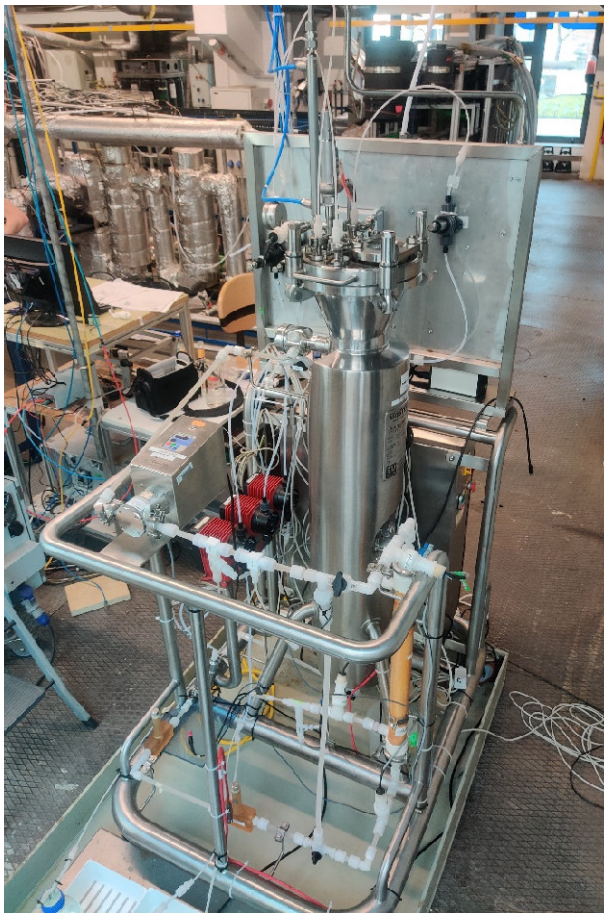


Figure 5 (left) and Figure 6 (right): BCR setup with view from the back on the left and view from the front on the right, the combustion hood can be seen in the top left corner

Figure 7 (left) and Figure 8 (right): Gassing devices, vortex on the left and sparger on the right

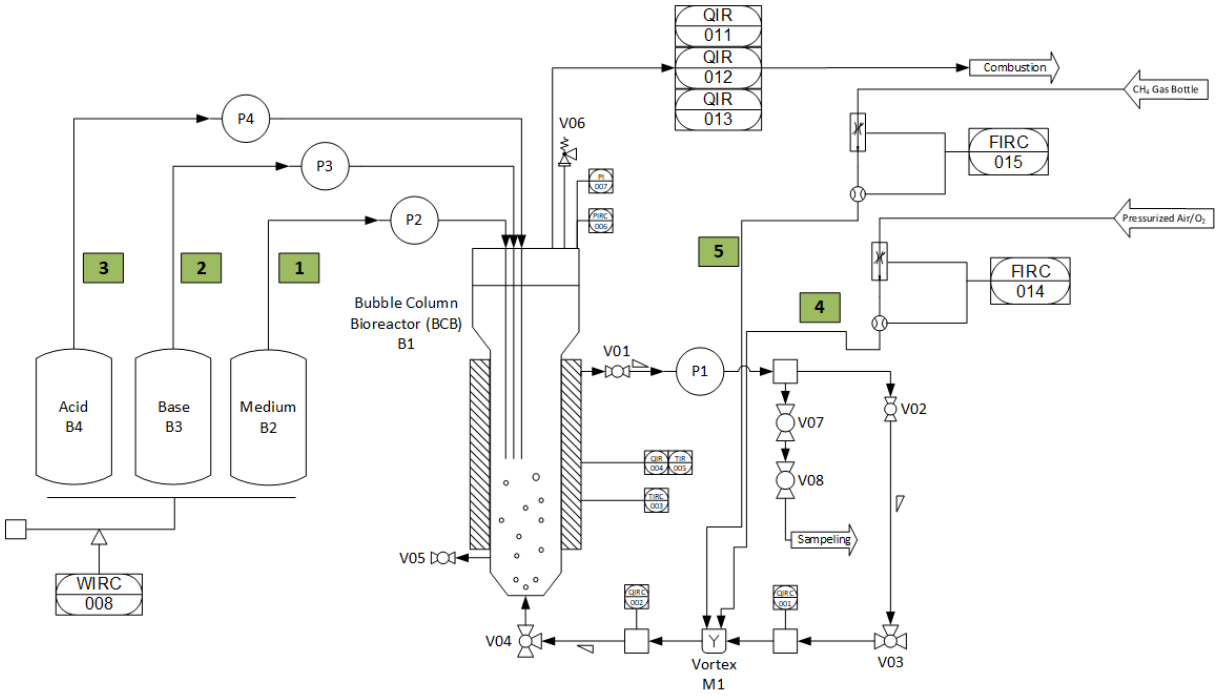


Figure 9: Process flow diagram of the BCR setup with vortex as gassing device

The BCR (B1) is connected to base (B3) and acid (B4) for pH control in the reactor. To facilitate additional feeding during the process, medium (B2) can be attached to the BCR. The dosing of base and acid is achieved by two identical magnetically actuated membrane pumps (P3 and P4) of the type Magdos LB1 supplied by Lutz-Jesco. The pumps can achieve a flow rate of up to 0.76 $L \cdot h^{-1}$ at 16 barg counterpressure. The dosing of medium is achieved by a magnetically actuated membrane pump (P2) of the type Magdos LA4 supplied by Lutz-Jesco with a maximum flow rate of 3.4 $L \cdot h^{-1}$ at a pressure of 16 barg. Base, acid and medium are placed on a scale (WIRC 008) to monitor mass differences. The cell broth is removed from the top of the fermenter and introduced to the loop via a 4-piston diaphragm pump (P1) supplied by ALMATEC Maschinenbau. The pumphead is made of polypropylene and offers low shear rate. Pressurized air (4) and methane (5) are introduced through one of two gassing devices located in the loop line, before the cell broth enters the fermenter on the bottom. The vortex depicted in Figure 7 is a gas mixing chamber that is placed in the loop line. It has two separate gas inlets, and the gases are mixed in the central chamber of the vortex within the liquid phase. In contrast to the vortex, the sparger shown in Figure 8, is placed inside the vessel above the loop inlet. The gases are mixed before entering the liquid phase. The flow rates of air and methane are controlled by mass flow controllers (FIRC 014, 015) obtained from Brooks and of the type GF40.

A safety valve is located on the lid (V06) which opens at an overpressure of 5 barg. Two sampling valves are available. Two probes to measure dissolved oxygen (DO) were placed in the loop line and the reactor vessel, respectively. The pH was measured in the loop line, while the temperature was measured in the reactor vessel around half height. A pressure sensor is located on top of the vessel. As a safety measure, the offgas is conducted to combustion to prevent accumulation of unutilized methane. Concentrations of CH_4 in the offgas are measured by a BCP-CH_4 gas sensor obtained from BlueSens. Concentrations of O_2 and CO_2 are measured in the offgas by a BlueVary sensor obtained from BlueSens as well. An overview of all appliances can be found in the appendix. The process is controlled by Lucullus Process Information Management System®.

3.1.4.2. Determination of the k_La-value in the BCR

The k_La-value is a parameter that describes the mass transfer from gas bubbles to the liquid phase according to Equation 1. It is the product of the mass transfer coefficient (k_L) and the interface area between gas and liquid phase (a).

$$\frac{d[O_2]}{dt} = k_La \cdot ([O_2]^* - [O_2])$$

Equation 1

$[O_2]^*$ is the saturated oxygen concentration in the liquid phase and $[O_2]$ is the concentration of dissolved oxygen in the liquid phase. t represents the time. ($[O_2]^*$- $[O_2]$) represents the driving force for oxygen mass transfer[30].

The k_La of methane can be estimated through a linear relationship to the k_La of oxygen according to Equation 2[15].

$$k_La_{CH_4} = 0.855 \cdot k_La_{O_2}$$

Equation 2

To investigate the influence of the aeration rate and the loop pump speed on the volumetric mass transfer coefficient (k_La), a Design of Experiment (DoE) was performed with both gassing devices, the vortex and the sparger. Additionally, the effect of the antifoam agent Struktol J-673A on the k_La-value was examined. According to the DoE (Figure 10), the k_La-values were determined at loop pump speeds of 1500 rpm, 2100 rpm and 2700 rpm and aeration rates of 0.2 vvm, 0.6 vvm and 1.0 vvm.

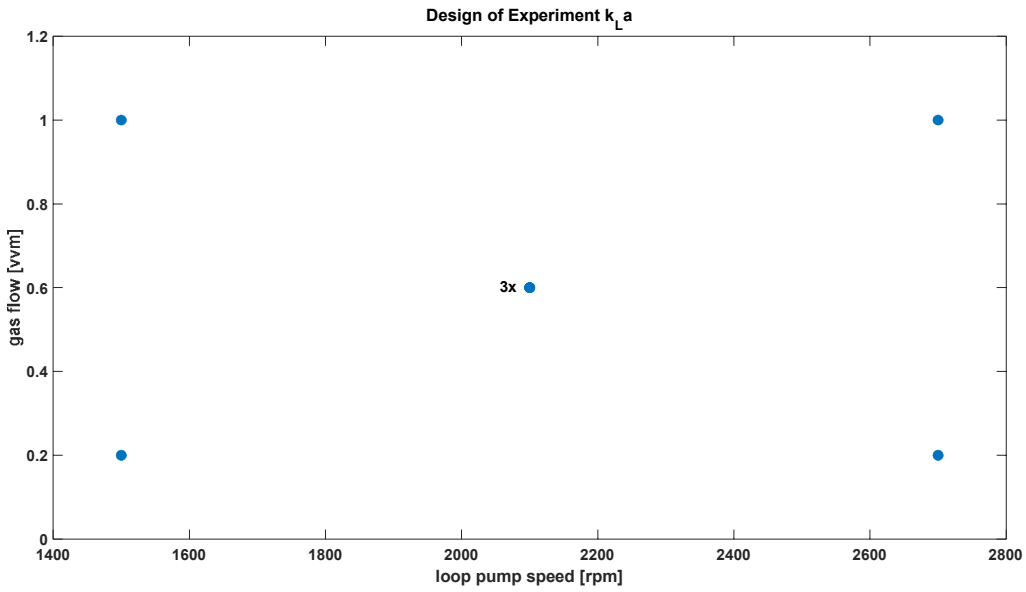


Figure 10: DoE for determination of k_La values in the BCR

The k_La-values were determined by the dynamic gassing-out method in deionized water and AMS medium 1 (20-fold concentration of nutrients) at 38 °C. The fermenter was filled with the respective liquid to a working volume of 16 L and heated to 38 °C. In a first step, the reactor was flushed with nitrogen, until the DO is stable at 0 %. In a second step, the fermenter was flushed with air at until the water/medium is saturated and the DO is stable at maximum value. This procedure was repeated for the respective loop pump speeds and aerations rates and with the sparger and vortex as gassing devices.

The k_La-values were determined by fitting the DO measurements according to Equation 3.

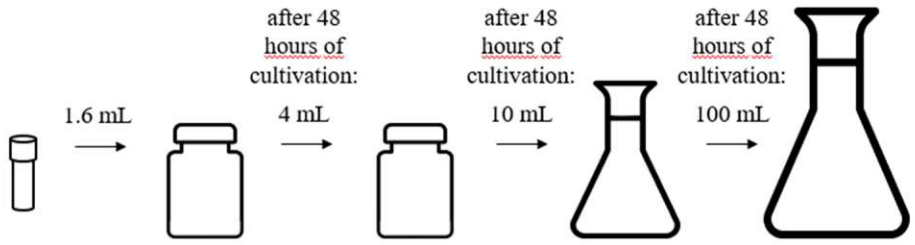
$$DO = DO_{max} \cdot \left(1 - e^{-k_L a \cdot (t - t_0)}\right)$$

Equation 3

DO is the measured DO signal from the DO probe at a time t, whereas t_0 is the start time of the DO measurement. DO_{max} is the maximum DO signal during the respective experiment.

3.1.4.3. Precultures

The precultures for cultivation in the BCR utilizing methanol as substrate were prepared in four steps as shown in Figure 11. High methanol concentrations can fully or partially inhibit growth of the methanotrophic cells, therefore methanol is added incrementally[14],[38].



	Serum Bottle	Serum Bottle	1L Shake Flask	5L High Yield Shake Flask
Media Volume	40 mL	40 mL	100 mL	1 L
Initial methanol concentration	1 mL·L^{-1}	1 mL·L^{-1}	1 mL·L^{-1}	1 mL·L^{-1}
Initial ammonium concentration	15.0 mM	15.0 mM	15.0 mM	15.0 mM
Additional methanol pulses	-	-	1 mL·L^{-1} after 24 hours of cultivation	1 mL·L^{-1} after 24 hours of cultivation

Figure 11: Preparation of precultures for fermentation in the BCR with methanol as substrate

An initial methanol concentration of 1 mL·L^{-1} and an ammonium concentration of 15.0 mM was supplied throughout all four preculture steps. The first culture was inoculated with 1.6 mL of liquid cryo-culture of *M.* sp. GB25 DSMZ 7674 in a serum bottle containing 40 mL minimal AMS medium 1 and 80 mL headspace. After around 48 hours, the second culture was inoculated with 4 mL of the previous culture in a serum bottle containing 40 mL of minimal AMS medium 1 and 80 mL headspace. In the next step, another culture was inoculated with 10 mL of the previous culture in 100 mL of minimal AMS medium 1 in a 1-L shake flask after around 48 hours of cultivation. To further increase the biomass concentration another pulse of 1 mL·L^{-1} methanol was added after 24 hours of cultivation. After around 48 hours of cultivation, the fourth culture was inoculated with the whole volume (100 mL) of the previous culture in 1 L of minimal AMS medium 1 in a 5-L high-yield-shake-flask. To further increase the biomass concentration another pulse of 1 mL·L^{-1} methanol was added after 24 hours of cultivation. The final preculture was cultivated for another 48 hours before the whole volume (1 L) was used to inoculate the BCR.

Table 13: Overview of OD_{600} values of precultures for fermentations in the BCR

	Fermentation 1	Fermentation 2	Fermentation 3	Fermentation 4
Strain	*M.* sp. GB25 DSMZ 7674	*M.* sp. GB25 DSMZ 7674	*M.* sp. GB25 DSMZ 7674	*M. parvus* OBBP
Substrate	Methanol	Methanol	Methanol	Methane
Medium	Minimal AMS medium 1	Minimal AMS medium 1	Minimal AMS medium 1	AMS medium 3
pH	6.8	6.8	6.8	6.8
Temperature	38 °C	38 °C	37 °C	37 °C
rpm	200	200	200	200
OD_{600}	1.07	2.16	1.55	0.81

The precultures cultivated with the gaseous substrate methane were conducted in airtightly sealed serum bottles. To supply an initial carbon and oxygen source, all serum bottles were flushed with methane:air (40:60 (v/v), 0.25 slpm) for 1 minute. An inital ammonium concentration of 15 mM was supplied in all cultures. The first culture was inoculated with 1.6 mL of a liquid cryo-culture of *M. parvus* OBBP in a serum bottle containing 40 mL of AMS medium 3 and 80 mL headspace. After 22 hours of cultivation, 3 serum bottles containing 150 mL of medium each, were inoculated with 10 mL of the previous culture. The precultures were cultivated for another 45 hours before the combined volume (450 mL) was used to inoculate the BCR. An overview over the culturing conditions and reached OD_{600} values of the precultures used for inoculation of the BCR is given in Table 13.

3.1.4.4. Procedure for a fermentation in the BCR

For sanitation, the reactor was first treated with 0.5 M NaOH and rinsed with distilled water afterwards. The reactor vessel was filled with autoclaved distilled water to a working volume of 16 L and the components of the respective medium were added. The vessel content was heated to the according temperature and the pH was adjusted with acid and base. The pH probes were calibrated by a two-point calibration at pH 7.0 and pH 4.0. The DO probes were also calibrated by a two-point calibration at 0 % oxygen and 100 % oxygen, whereby 100 % oxygen amounts to the percentage of oxygen in the air at atmospheric pressure. Then the vessel was inoculated with the respective preculture.

Table 14: Process parameters during fermentation 1 to 4 in the BCR

	Process parameters			
	Fermentation 1	**Fermentation 2**	**Fermentation 3**	**Fermentation 4**
Strain	*M.* sp. GB25 DSMZ 7674	*M.* sp. GB25 DSMZ 7674	*M.* sp. GB25 DSMZ 7674	*M. parvus* OBBP
Medium	AMS medium 1 (60-fold concentration of nutrients)	AMS medium 1 (20-fold concentration of nutrients)	AMS medium 1 (20-fold concentration of nutrients)	AMS medium 3
Temperature	38°C	38°C	37°C	37°C
pH	6.8	6.8	6.8	6.8
Gassing system	Vortex	Vortex	Sparger	Sparger
Loop pump speed [rpm]	2400	1500-1800	1500	1500
Aeration rate [vvm]	0.3, 0.5	0.3	0.2	0.1
Air:CH_4	50:50, 70:30	70:30	70:30	70:30
Acid	1 M H_2SO_4	1 M H_2SO_4	1 M H_2SO_4	1 M H_2SO_4
Base	1 M NaOH	1 M NaOH	1 M NaOH	1 M NaOH
Antifoam	-	Struktol J673-A	PEG400	

As methane is a highly flammable gas, certain safety measures are required. The combustible range of methane in a methane-air mixture is around 5–15 v%, which was avoided at all times. Also, a methane detector was placed next to the setup. An overview of the process parameters during fermentation 1 to 4 in the BCR is shown in Table 14.

3.1.5. Cultivation in a DASbox® Mini Bioreactor System

3.1.5.1. Setup

Cultivation of methanotrophic strains was also performed in a DASbox® Mini Bioreactor System for microbiology with a fourfold setup provided by Eppendorf. The DASbox® system consists of four parallel operable glass reactors with a total volume of 350 mL. It is equipped with a multi-port stainless steel head plate and a direct overhead drive. The impeller is a Rushton-type. The temperature is controlled through thermoelectric heating and cooling. Each unit is equipped with a DO, pH, temperature and level sensor located on the head plate. Gas mixing is mass flow-controlled and the gas is introduced via a submerged sparger. The process is monitored and controlled via DASware® control.

3.1.5.2. Preculture

A preculture was inoculated with a liquid cryo-culture of *M.* sp. GB25 DSMZ 7674 in a serum bottle containing 40 mL medium AMS 3 and 80 mL headspace. Substrate is provided by flushing the serum bottle with methane:air (40:60 (v/v), 0.25 slpm) for 1 minute. The preculture was incubated at 37°C at 200 rpm for 24 hours.

3.1.5.3. Procedure for a fermentation in the DASbox® system

Each vessel was filled with the base solution (Table 7 and Table 8) of AMS medium 3. Then the vessels were sealed airtight and autoclaved at 121 °C for 20 minutes. After autoclavation, the phosphate stock solution (Table 9) and vitamin solution (Table 10) were added to the reactors through a 0.2 µm filter yielding a working volume of 200 mL.

The vessel content was heated to a temperature of 37 °C and the pH was adjusted to 6.8 with acid and base. The pH probes were calibrated by a two-point calibration at pH 7.0 and pH 4.0. The DO probes were also calibrated by a two-point calibration at 0 % oxygen and 100% oxygen, whereby 100 % oxygen amounts to the percentage of oxygen in the air at atmospheric pressure. Then the vessels were inoculated with the preculture. The process parameters of fermentation 5 in the DASbox® system are summarized in Table 15.

Table 15: Process parameters of fermentation 5 in the DASbox® system

Parameter	Setpoint
Strain	*M.* sp. GB25 DSMZ 7674
Medium	AMS medium 3
Temperature	37°C
pH	6.8
Aeration rate	2 $L \cdot h^{-1}$, 0.16 vvm
Gas composition	11-14.5 % O_2, 30 % CH_4
Acid	-
Base	1 M NaOH
Antifoam	-

The DO setpoint was set to a minimum of 20 % and was controlled via the gas flow rate (1-9 $l \cdot h^{-1}$) and stirrer speed (200-1000 rpm).

An initial ammonium concentration of 10 mM was supplied in both reactors. To further increase biomass, ammonium was resupplied in concentrations of 12 mM and 30 mM at 40 and 46 hours of cultivation in reactor C respectively. A total ammonium concentration of 52 mM was supplied in reactor C. In reactor D ammonium was resupplied in concentrations of 5 mM, 12 mM and 28 mM at 30, 46, and 52 hours of cultivation respectively, yielding a total of 55 mM ammonium.

3.2. Cultivations utilizing liquid C_1 compounds

3.2.1. Strains

The methylotrophic strains used within this work were *M. extorquens* DSMZ 1337, *M. extorquens* DSMZ 1338, *M. extorquens* DSMZ 1339 and *M. extorquens* DSMZ 1340. The strains were obtained from 'DSMZ' and stored in the freezer at -80 °C in 75% (w/w) glycerol for long-term storage.

3.2.2. Media

Two types of media were used for the cultivation of the methylotrophic strains. The first medium is an undefined medium recommended by DSMZ[39], while the second medium (AMS medium 4) is derived from Bourque et al.[40]

To prepare undefined medium 1, all nutrients listed in Table 16 were dissolved in deionized water and autoclaved afterwards at 121°C for 20 minutes.

Table 16: Components and respective concentrations of undefined medium 1

Component	Concentration [L^{-1}]
Peptone	5.0 g
Meat extract	3.0 g

To prepare AMS medium 4, the base components listed in Table 17 were dissolved in deionized water, except for $FeSO_4 \cdot 7\ H_2O$. The resulting solution was autoclaved at 121°C for 20 minutes. The components of the phosphate stock solution listed in Table 18 were dissolved in deionized water and autoclaved at 121°C for 20 minutes separately and added to the base solution.

$FeSO_4 \cdot 7\ H_2O$ (dissolved in deionized water) was added to the final medium through a 0.2 µm filter. The ammonium sulphate concentration is stated in the respective sections, as it varied throughout the experiments.

Table 17: Components and respective concentrations of AMS medium 4

Component	Concentration [L^{-1}]
$(NH_4)_2SO_4$	stated in the respective section
$MgSO_4 \cdot 7\ H_2O$	0.45 g
$CaCl_2 \cdot 2\ H_2O$	20 mg
$FeSO_4 \cdot 7\ H_2O$	20 mg
$MnSO_4 \cdot H_2O$	4.9 mg
$ZnSO_4 \cdot 7\ H_2O$	2.6 mg
$CuSO_4 \cdot 5\ H_2O$	800 µg
$Na_2MoO_4 \cdot 2\ H_2O$	800 µg
$CoCl_2 \cdot 6\ H_2O$	800 µg
H_3BO_3	600 µg
Phosphate stock solution	100 $mL \cdot L^{-1}$

Table 18: Components and respective concentrations of phosphate stock solution for AMS medium 4

Component	Concentration [L^{-1}]
KH_2PO_4	13.05 g
$Na_2HPO_4 \cdot 7\ H_2O$	40.2 g

3.2.3. Cultivations in shake flasks

3.2.3.1. Growth on methanol

Cultivations of *M. extorquens* DSMZ 1337, *M. extorquens* DSMZ 1338, *M. extorquens* DSMZ 1339 and *M. extorquens* DSMZ 1340 with methanol as substrate were carried out in 500-mL shake flasks. Initial concentrations of 1 v% (≙ 0.25 $mol \cdot L^{-1}$) methanol as carbon source and 1.5 $g \cdot L^{-1}$ ammonium sulphate as nitrogen source were supplied throughout all culturing steps. Precultures were inoculated with 1.6 mL of the respective liquid cryo-culture in 25 mL of undefined medium 1. After 6.6 hours of cultivation, cultures were inoculated with 0.5 mL of the respective precultures in 50 mL of AMS medium 4 as an intermediate step. The main cultures were inoculated after 41.5 hours of cultivation with the respective intermediate cultures

in 50 mL of AMS medium 4 in duplicates. The inoculation volume for the main cultures was chosen to yield a start OD_{600} value of 0.1. All cultures were incubated at 30°C and 200 rpm.

3.2.3.2. Growth on formate

Cultivations of *M. extorquens* DSMZ 1337, *M. extorquens* DSMZ 1338, *M. extorquens* DSMZ 1339 and *M. extorquens* DSMZ 1340 with formate as substrate were carried out in 500-mL shake flasks. Initial concentrations of 16.8 $g \cdot L^{-1}$ (≙ 0.25 $mol \cdot L^{-1}$) sodium formate as carbon source and 1.5 $g \cdot L^{-1}$ ammonium sulphate as nitrogen source were supplied throughout all culturing steps. Precultures were inoculated with 1.6 mL of the respective liquid cryo-culture in 25 mL of undefined medium 1. After 21.6 h of cultivation, the intermediate cultures were inoculated in 50 mL of AMS medium 4 to yield a start OD_{600} value of 0.1. As the intermediate cultures were stagnating in growth, the main cultures could not be inoculated. All cultures were incubated at 30°C and 200 rpm.

3.2.3.3. PHB accumulation

The PHB accumulation capability of *M. extorquens* DSMZ 1337 and *M. extorquens* DSMZ 1338 was examined in 500-mL shake flasks with three different substrate variations (methanol and formate individually and in combination).

Precultures were inoculated with 1.6 mL of the respective liquid cryo-culture in 25 mL undefined medium 1 with 1 v% methanol as carbon source. The main cultures of *M. extorquens* DSMZ 1337 were inoculated with 3.0 mL of the preculture in 50 mL AMS medium 4 after 8 hours of cultivation, while the main cultures of *M. extorquens* DSMZ 1338 were inoculated with 1.5 mL of the respective preculture in 50 mL AMS medium 4 after 25 hours of cultivation. Initial substrate concentrations of 1 v% (≙ 0.25 $mol \cdot L^{-1}$) methanol, 16.8 $g \cdot L^{-1}$ (≙ 0.25 $mol \cdot L^{-1}$) sodium formate and a combination of 0.5 v% (≙ 0.12 $mol \cdot L^{-1}$) methanol and 8.4 $g \cdot L^{-1}$ (≙ 0.12 $mol \cdot L^{-1}$) sodium formate were supplied in the main cultures respectively. An initial concentration of 1.5 $g \cdot L^{-1}$ ammonium sulphate was provided in all cultures.

To switch from the growth phase to the PHB production phase, the cells were transferred from the growth medium to the production medium (AMS medium 4 with no nitrogen source present). Therefore, each culture was centrifuged at 14.000 rpm and 4°C for 10 minutes. The supernatant was discarded and the cells were gently resuspended in the production medium. The same initial substrate concentrations were supplied after the transfer.

All cultures of *M. extorquens* DSMZ 1337 were transferred after 24 hours of cultivation. The cultures of *M. extorquens* DSMZ 1338 grown on methanol were transferred at 49 hours of cultivation while the cultures grown on formate and methanol and formate in combination were transferred after 76 hours of cultivation.
The experiment was conducted in duplicates. All cultures were incubated at 30°C and 200 rpm.

3.2.3.4. Sampling

To monitor cell growth and substrate depletion, samples were taken in regular intervals.

3.2.4. Cultivations in a DASGIP® Parallel Bioreactor System

3.2.4.1. Setup

Methylotrophic strains were cultivated in a benchtop DASGIP® Parallel Bioreactor System for microbiology with a fourfold setup provided by Eppendorf. The fermentation setup is depicted in Figure 12. A more detailed view on the individual vessel and the multi-port head plate is shown in Figure 13 and Figure 14.
The DASGIP® Parallel Bioreactor System consists of four parallel operable glass vessels with a maximum working volume of 2.7 L. Each vessel is equipped with a multi-port head plate made of stainless steel, a direct overhead drive and Rushton-type impellers. The temperature is controlled by heat blankets and cooling fingers. The exhaust gas is condensed by a water-cooled condenser before it is conducted to a DASGIP® GA4 Exhaust Analyzing Module provided by Eppendorf. To protect the exhaust analysing module from foam and fermentation broth, foam traps are installed between the reactors and the analysing module. Gas mixing is achieved by a DASGIP® MX4/4 gas mixing module provided by Eppendorf and the gas is introduced via a submerged sparger. The vessels are connected to a DASGIP® MP8 peristaltic Multi Pump Module through tubing, where acid and base are attached for pH control and additional feed can be supplied. Each unit is equipped with a DO, pH, temperature and level sensor located on the head plate. Also, a port for sampling is available on the lid. The process is monitored and controlled via DASware® control.

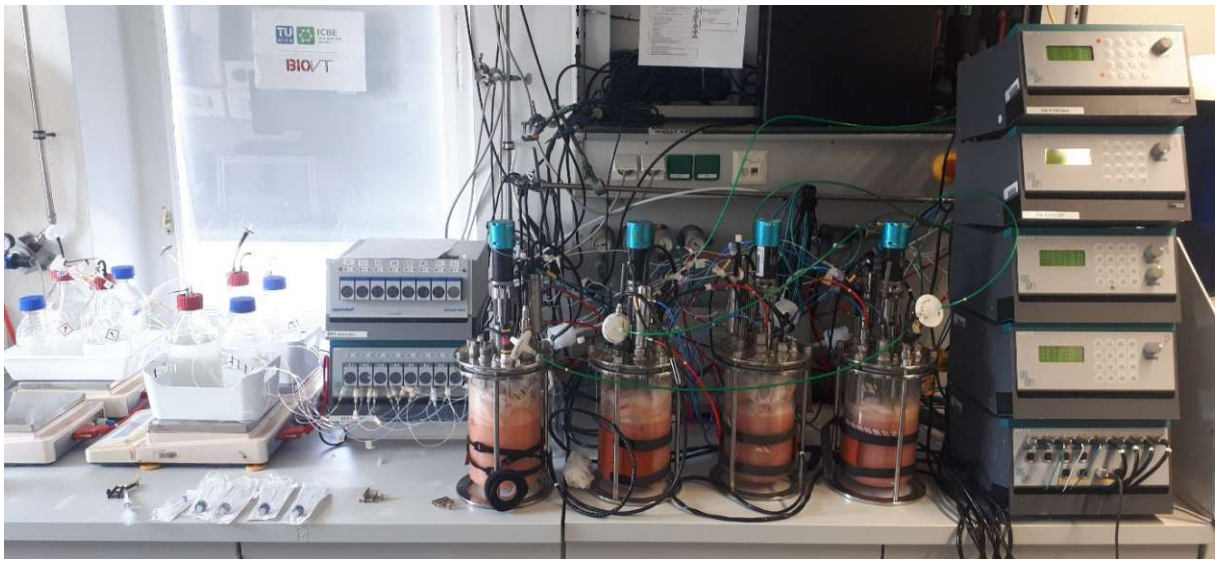


Figure 12: Overview of the DASGIP® Parallel Bioreactor System with a fourfold setup

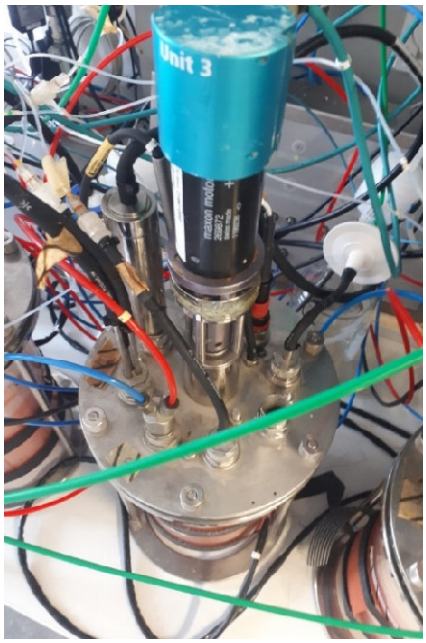


Figure 13 (left) and Figure 14 (right): Individual reactor vessel on the left and view on the multi-port head plate on the right

3.2.4.2. Preculture

In a first step, a culture was inoculated with 1.6 mL of a liquid cryo-culture in 50 mL undefined medium 1 in a 500-mL shake flask. Initial substrate concentrations of 1 v% methanol and 1.5 $g \cdot L^{-1}$ ammonium sulphate were supplied in both culturing steps.

In a second step, the actual preculture was inoculated with the whole volume of the previous culture (50 mL) in 500 mL AMS medium 4 in a 5-L ultra-yield shake flask after 24 hours of

cultivation. The preculture was cultivated for another 24 hours before it was used to inoculate the reactor vessels. Both cultures were incubated at 30°C and 200 rpm.

3.2.4.3. Procedure for a fermentation in the DASGIP® System

Each reactor vessel was filled with the base solution of AMS medium 4 (Table 17). The vessels containing the base medium were sealed airtight and autoclaved at 121°C for 20 minutes. Then the respective amount of phosphate stock solution (Table 18) and $FeSO_4 \cdot 7\ H_2O$ dissolved in deionized water was added to the base medium via a 0.2 µm filter, yielding a working volume of 1.5 L.

The reactor content was heated to a temperature of 30 °C. The pH probes were calibrated by a two-point calibration at pH 7.0 and pH 4.0. The DO probes were calibrated by a two-point calibration as well at 0% oxygen and 100% oxygen, whereby 100% oxygen amounts to the percentage of oxygen in the air at atmospheric pressure. The pumps were calibrated gravimetrically. A pH of 7.0 was adjusted with acid and base. The DO setpoint was set to a minimum of 20 % and was controlled via the gas flow rate and agitation speed.

The process parameters during the cultivation of *M. extorquens* DSMZ 1337 in the DASGIP® system are summarized in Table 19.

Table 19: Process parameters during cultivation of M. extorquens DSMZ 1337 in the DASGIP® system

Parameter	Setpoint
Temperature	30°C
pH	7.0
Agitation speed	Cascade, according DO
Aeration	45 $L \cdot h^{-1}$, air
Initial reactor volume	1.5 L
Medium	AMS medium 4
Inoculation volume	à 50 mL
Acid	H_2SO_4, 1 M
Base	KOH, 1 M
Antifoam	PEG 400

Two substrate variations were investigated: methanol as sole carbon source and formate as additional carbon source to methanol.

The fermentation consisted of an initial batch phase where carbon and nitrogen were supplied and biomass was propagated. In the fed-batch phase the biomass concentration is increased by feeding the respective carbon source. The PHB production phase is entered when ammonium is depleted.

For the first substrate variation, an initial methanol concentration of 10 $mL \cdot L^{-1}$ (≙ 0.25 $mol \cdot L^{-1}$) was supplied in the batch phase. After 29 hours of cultivation the supplied methanol was depleted, and the fed-batch with a feedrate of 10 $mL \cdot h^{-1}$ was started. First, a feed with a concentration of 100 $mL \cdot L^{-1}$ (≙ 2.5 $mol \cdot L^{-1}$) methanol was applied. After 72 hours of cultivation the feed concentration was increased to 250 $mL \cdot L^{-1}$ (≙ 6.2 $mol \cdot L^{-1}$) methanol. An initial ammonium concentration of 10 mM was supplied. After 26 hours of cultivation ammonium was depleted and another pulse of 6 mM ammonium was added, to further increase the biomass concentration. The production phase was entered at 46 hours of cultivation, when ammonium was consumed.

For the second substrate variation, an initial methanol concentration of 5 $mL \cdot L^{-1}$ (≙ 0.12 $mol \cdot L^{-1}$) and a sodium formate concentration of 8.4 $g \cdot L^{-1}$ (≙ 0.12 $mol \cdot L^{-1}$) was supplied in the batch phase. At 26 hours of cultivation the fed-batch was started and first a feedrate of 1 $mL \cdot h^{-1}$ was applied, and later increased to 10 $mL \cdot h^{-1}$. First the feed had a concentration of 50 $mL \cdot L^{-1}$ (≙ 1.2 $mol \cdot L^{-1}$) methanol and 84 $g \cdot L^{-1}$ (≙ 1.2 $mol \cdot L^{-1}$) sodium formate. The second feed had a concentration of 125 $mL \cdot L^{-1}$ (≙ 3.1 $mol \cdot L^{-1}$) methanol and 210 $g \cdot L^{-1}$ (≙ 3.1 $mol \cdot L^{-1}$) sodium formate and was applied for 72 hours of cultivation. An initial ammonium concentration of 10 mM was supplied. After 46 hours of cultivation the ammonium was used up and another shot of 9 mM ammonium was added, to reach more biomass before entering the production phase. The production phase was entered at 70 hours of cultivation, when no more ammonium was available.

3.2.4.4. Sampling

To monitor cell growth, PHB accumulation and substrate depletion, samples were taken in regular intervals.

3.3. Analytical methods

3.3.1. Determination of optical density

To measure the optical density of the samples, a ONDA V-10 PLUS Visible Spectrophotometer at a wavelength of 600 nm was used. The measurements are performed in the linear range of 0.2 to 0.8. Samples exceeding this linear range were diluted accordingly with deionized water. Plastic cuvettes with a sample volume of 1 mL and a light path of 1 cm were used.

3.3.2. Determination of cell dry weight

The cell dry weight (CDW) was determined in triplicates. 2 mL micro test tubes were dried for at least 24 hours at 80°C for constant weight. The weighed micro test tubes were filled with 2 mL of culture broth. To separate the cells from the broth, the tubes were centrifuged for 10 minutes at 14.000 rpm and 4°C and the supernatant was discarded. As a washing step, 2 mL of a 0.9 % saline solution was added and the cell pellet was dissolved before it was centrifuged for 10 minutes at 14.000 rpm and 4 °C again. The supernatant was discarded. Lastly, the tubes containing the cell pellet were dried for at least 24 hours at 80°C and weighed.

3.3.3. Determination of ammonium and phosphate concentrations

The determination of the ammonia and phosphate concentration was performed on a Roche Cedex Bio HT Analyzer. For the quantification of ammonium, the NH3 Bio HT test kit was used. It is based on the principle, that solved ammonia in presence of glutamate dehydrogenase reacts with 2-oxoglutarate and NADPH in a reductive amination to form L-glutamate and $NADP^+$. The decrease of NADPH is directly proportional to the ammonia concentration and is measured photometrically. For the quantification of phosphate, the Phosphate Bio HT test kit was used. The underlying principle is, that inorganic phosphate and ammonium molybdate in the presence of sulfuric acid form a coloured ammonium phosphomolybdate complex. The formation of this complex can be measured by increase of absorbance at 340 nm and is directly proportional to the phosphate concentration of the sample. Automatic dilution was used.
To abstract solid particles, samples were either filtered through disposable syringe filters with a pore size of 0.2 µm or centrifuged for 10 minutes at 14.000 rpm and 4°C before measurements.

3.3.4. Determination of methanol, formate and PHB concentration

Quantification of methanol, formate and PHB was performed by high performance liquid chromatography (HPLC) on a Vanquish UHPLC systems provided by Thermo-Fisher, with an Aminex HPX-87H column (Bio-Rad) at 60 °C, an isocratic eluent of 4 mM sulfuric acid in Milli-Q water with a flow of 0.6 mL min^{-1} followed by UV detection at 210 nm and RI detection (RefracoMax520, ERC, Germany).

To prepare the samples for measurements of methanol and formate, samples were either filtered by disposable syringe filters with a pore size of 0.2 µm or centrifuged for 10 minutes at 14.000 rpm and 4°C to abstract solid particles. The supernatant was diluted (1:10) with 40 mM sulfuric acid, vortexed and centrifuged again. Calibration standards were treated accordingly.

PHB samples were prepared according to Mittermair et al.[41] The cell pellet of 2 mL culture broth was dried at 80°C for 24 hours. Then, 1 mL of conc. H_2SO_4 was added and the samples were incubated on a heating block at 95°C for 60 minutes to convert PHB to crotonic acid, vortexing regularly every 10 minutes. Lastly, the samples were centrifuged at 4°C and 20.000 g for 10 min. 100 µL of the supernatant was added to 1.900 µL H_2O, mixed, and filtered through a 0.22 µm PTFE filter. For the preparation of calibration standards, PHB from natural origin was treated accordingly.

4. Results and Discussion

4.1. Gaseous C_1 compounds

4.1.1. Determination of a suitable methanotrophic strain for PHB production

Several methanotrophic strains are reported to accumulate PHB under nutrient-limited conditions (Table 2). To determine a suitable methanotrophic strain for the following production of PHB in the BCR setup and to assess preculture preparation, strains *M. parvus* OBBP, *M. rockwell* and *M.* sp. GB25 DSMZ 7674 were first cultivated in serum bottles and their growth was assessed on methanol as carbon source.

The biomass formation and methanol consumption of strain *M.* sp. GB25 DSMZ 7674 are depicted in Figure 15.

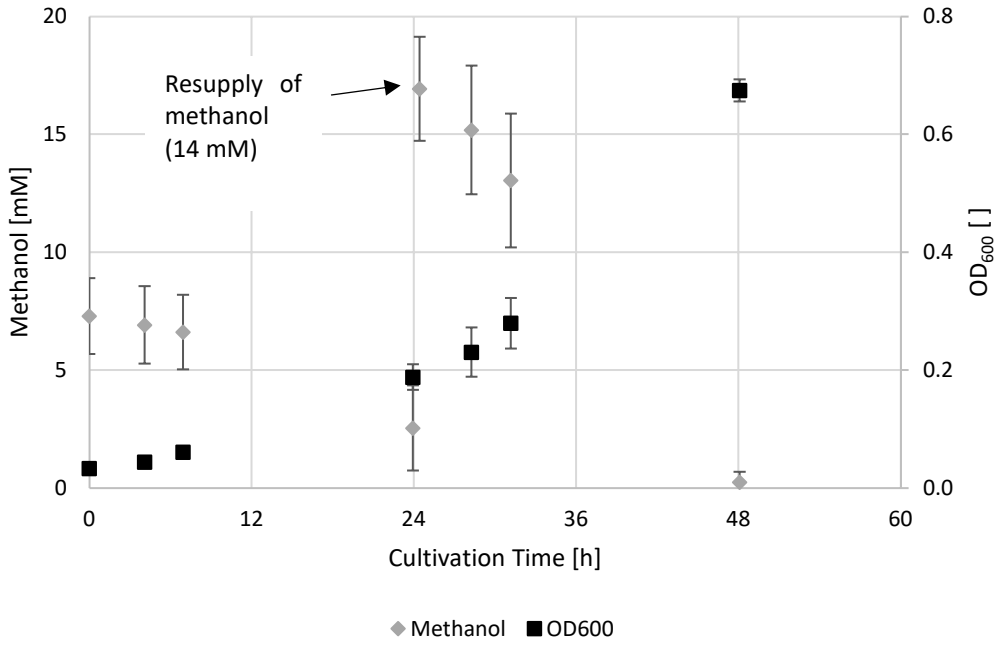


Figure 15: Biomass formation and methanol consumption of strain M. sp. GB25 DSMZ 7674 in AMS medium 2 at pH 6.8 and 30 °C, with an initial methanol concentration of 7 mM as carbon source and an initial ammonium concentration of 10 mM as nitrogen source

Strain *M.* sp. GB25 DSMZ 7674 reached a OD_{600} value of 0.675 ± 0.019 after 48 hours of cultivation and a growth rate of 0.0631 ± 0.0008 h^{-1}. Strain *M. rockwell* showed only limited growth and reached a OD_{600} value of 0.092 ± 0.006 after 48 hours of cultivation. Strain *M. parvus* OBBP showed no growth under the chosen experimental conditions. Thus, *M.* sp. GB25 DSMZ 7674 was chosen for the following cultivations in the BCR setup, as this strain showed the highest growth under the given experimental conditions.

Growth rates for strains of the genus *Methylocystis* are between 0.121 - 0.154 h^{-1} with methane as substrate[14]. The reached growth rate of 0.0631 ± 0.0008 h^{-1} of *M.* sp. GB25 DSMZ 7674 is lower than those reported, as methanol is not the preferred carbon source of methanotrophs. Even though growth rates are lower on methanol as carbon source, the liquid substrate is easier in handling than the gaseous substrate methane and was therefore chosen for the cultivation of the precultures.

4.1.2. Initial assessment of methanotrophic growth in the BCR setup

M. sp. GB25 DSMZ 7674 was first cultivated in the BCR to assess the growth of the strain in this setup and to evaluate whether the setup is suited for cultivations utilizing methane. The

cultivation conditions were based on the experiments by Wendlandt et al.[29] Mixing of gaseous and liquid phase was enabled through the vortex as gassing system.

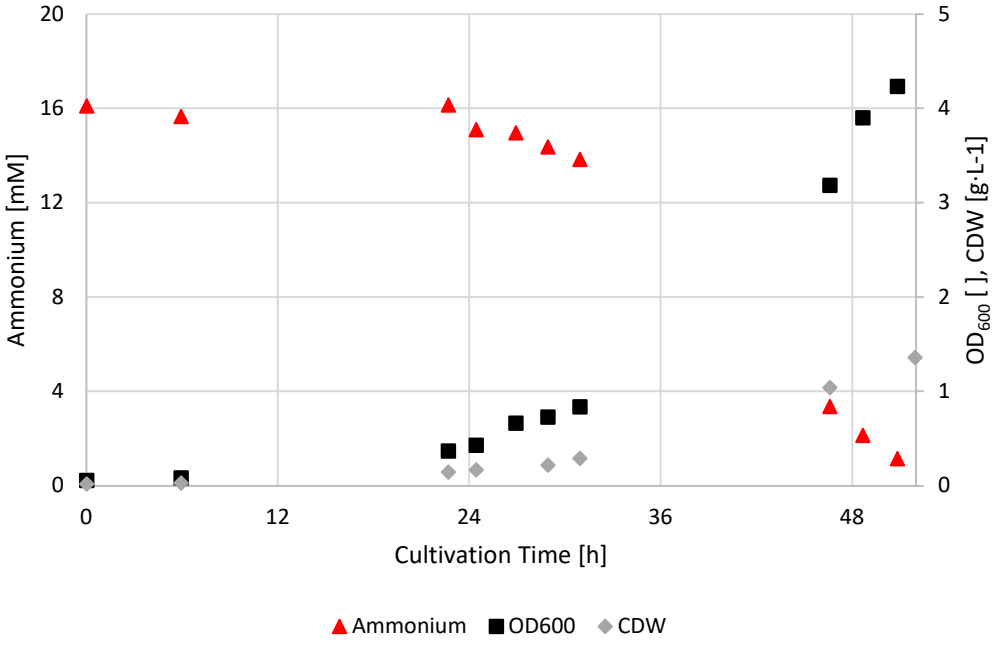


Figure 16: Biomass formation and ammonium consumption during fermentation 1 in the BCR with strain M. sp GB25 DSMZ 7674 in minimal AMS medium 1 (60-fold concentration of nutrients) at pH 6.8 and 38°C; employing the vortex as gassing device

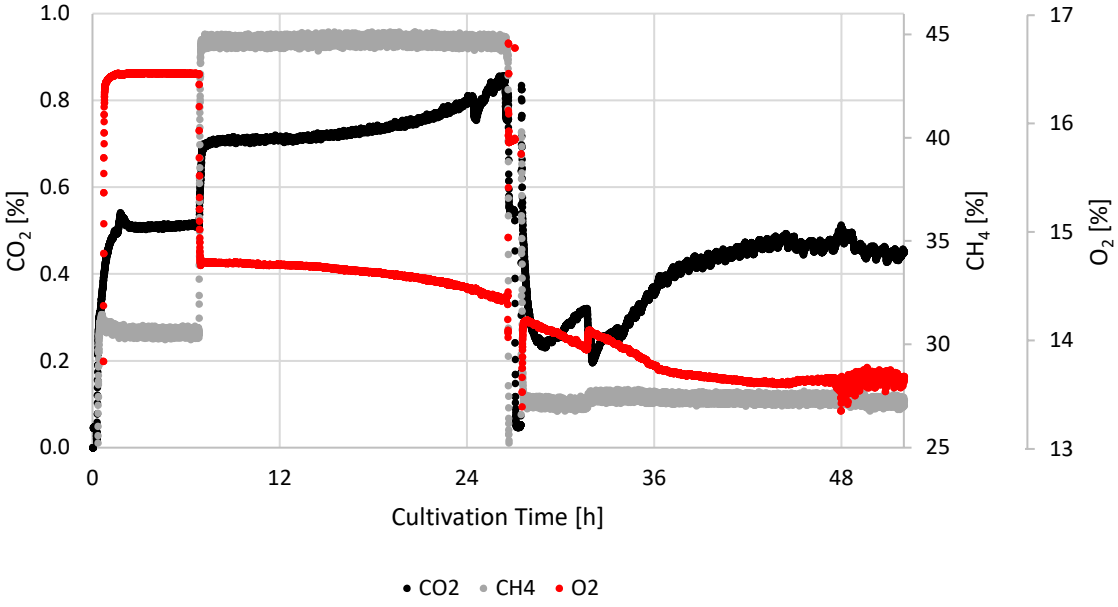


Figure 17: Course of CO_2 [%], CH_4 [%] and O_2 [%] in offgas during fermentation 1 in the BCR with strain M. sp GB25 DSMZ 7674 in minimal AMS medium 1 (60-fold concentration of nutrients) at pH 6.8 and 38°C; employing the vortex as gassing device

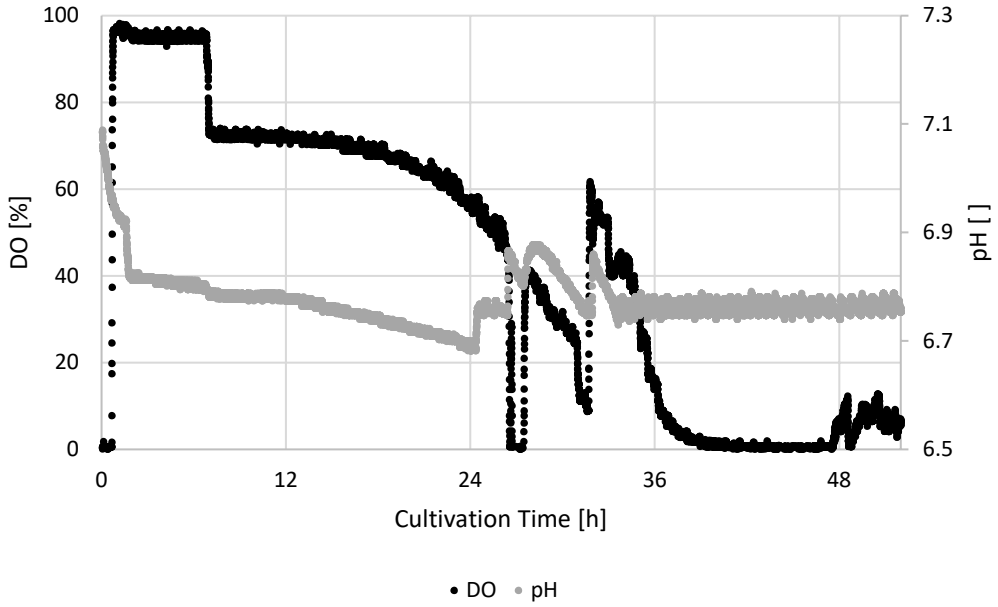


Figure 18: Course of DO [%] and pH [ ] during fermentation 1 in the BCR with strain M. sp GB25 DSMZ 7674 in minimal AMS medium 1 (60-fold concentration of nutrients) at pH 6.8 and 38°C; employing the vortex as gassing device

Biomass formation and ammonium consumption are depicted in Figure 16. The course of CO_2, O_2, CH_4 in the offgas is shown in Figure 17 and the course of DO and pH is shown in Figure 18.

A OD_{600} value of 4.23 was reached after 50 hours of cultivation which corresponds to a biomass concentration of 1.36 $g \cdot L^{-1}$. A growth rate of 0.090 h^{-1} could be determined during the exponential growth phase.

After 27 hours of cultivation, oxygen limitation occurred in the reactor. Therefore, the aeration rate was increased from 0.3 vvm to 0.5 vvm and the air:methane ratio was adjusted from 50:50 to 70:30. After 40 hours of cultivation oxygen limitation occurred again as well as foaming problems. Also, a precipitate formed over the span of the cultivation.

Because of these problems during the growth phase, ammonium was not fully depleted before the cells were limited in oxygen. Therefore, the PHB production phase could not be reached.

To ensure better conditions during the growth phase, the cultivation of *M.* sp. GB25 DSMZ 7674 in the BCR was repeated with two adaptions. As a precipitate formed in the previous run, a less concentrated medium (20-fold concentration of nutrients) was used. Also, the antifoam agent Struktol J673-A (0.5 $ml \cdot L^{-1}$) was added due to the foaming problems in the previous run.

As no growth of the cells could be observed in the OD_{600} values, the online parameters are depicted. The course of CO_2, O_2, CH_4 in the offgas is shown in Figure 19 and the course of DO and pH is shown in and Figure 20.

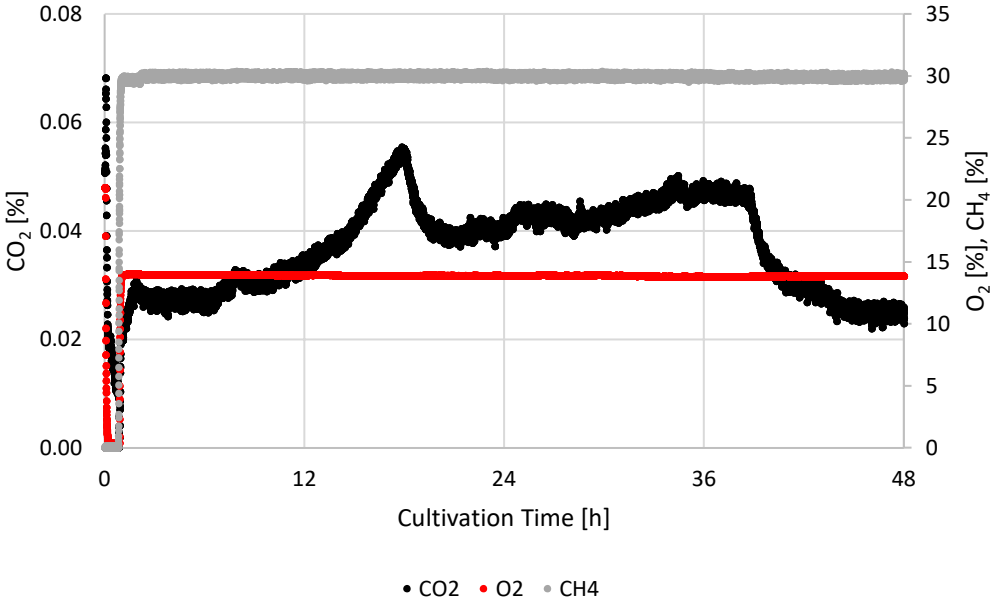


Figure 19: Course of CO_2 [%], CH_4 [%] and O_2 [%] in offgas during fermentation 2 in the BCR with strain M. sp GB25 DSMZ 7674 in minimal AMS medium 1 (20-fold concentration of nutrients) at pH 6.8 and 38°C; employing the vortex as gassing device

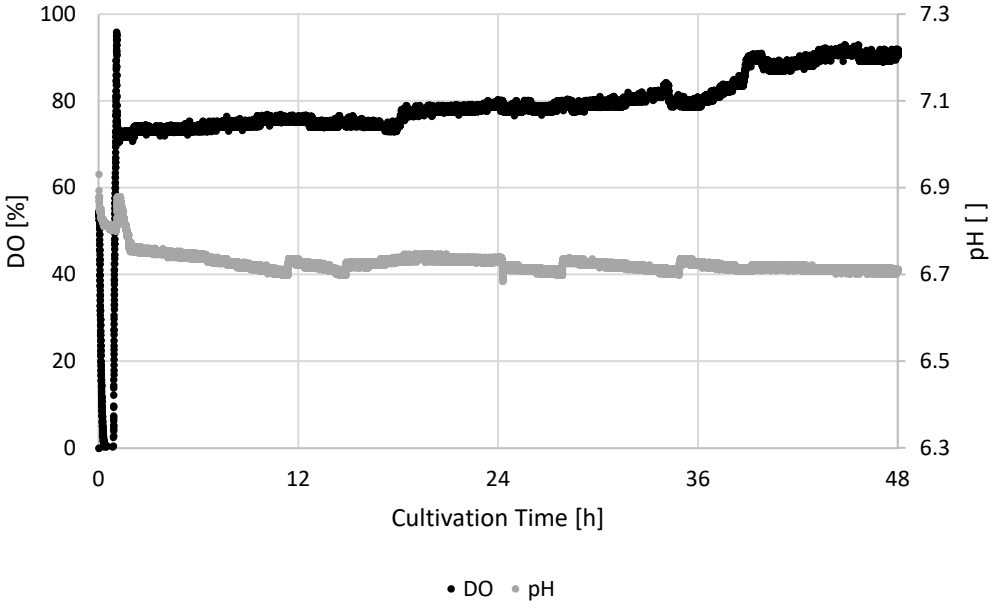


Figure 20: Course of DO [%] and pH [ ] during fermentation 2 in the BCR with strain M. sp GB25 DSMZ 7674 in minimal AMS medium 1 (20-fold concentration of nutrients) at pH 6.8 and 38°C; employing the vortex as gassing device

The initial increase in the CO_2 signal in the offgas indicates growth of the cells. However, after the initial increase in CO_2, no microbial activity is observable in the online signals. The lack of growth might be caused by the added antifoam agent Struktol J673-A.

As several problems occurred during the first two fermentation runs in the BCR, further experiments in serum bottles were conducted, to determine ideal growth conditions. Temperature and ammonium concentration are important factors that affect methanotrophic growth and are assessed[14]. Also, as foaming problems occurred during the first fermentation and the added antifoam agent Struktol J673-A might has inhibited growth of the cells, suitable antifoam agents are determined. Furthermore, the k_La values for the BCR setup were determined at this point, as oxygen limitation occurred during the first fermentation and gas-to-liquid mass transfer is critical.

4.1.3. Effect of temperature and ammonium concentration on methanotrophic growth

The effect of ammonium concentration (2.0 mM, 8.5 mM 15.0 mM) on the growth of *M.* sp. GB25 DSMZ 7674 on methane as carbon source was investigated in serum bottles. The time-resolved growth curves of the experiments can be seen in Figure 21A. Corresponding growth rates are shown in Figure 21B.

Also, the growth of *M.* sp. GB25 DSMZ 7674 on methane as carbon source at temperatures of 30 °C, 37 °C and 38 °C was examined and is shown in Figure 22AFigure 21. The according growth rates are depicted in Figure 22B.

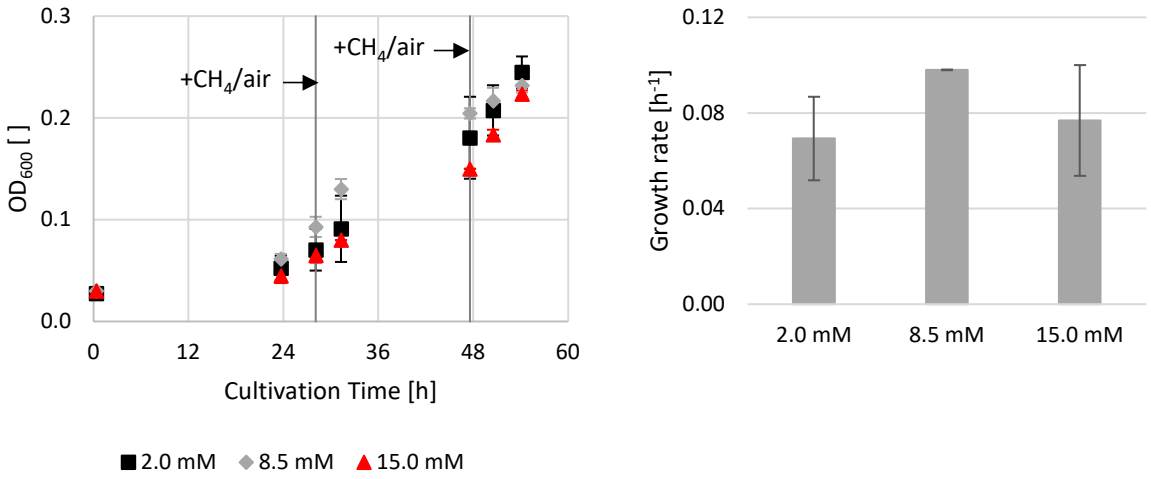


Figure 21A (left) and B (right): Growth of M. sp GB25 DSMZ 7674 in minimal AMS medium 1 at pH 6.8 and 38° with methane as carbon source and ammonium concentrations of 2.0 mM, 8.5 mM 15.0 mM

The cultures grown on an initial ammonium concentration of 8.5 mM showed limitation in methane or oxygen before the second pulse of methane/air, which is visible in the flattening of the exponential growth curve at around 30 hours of cultivation.

The culture grown on 8.5 mM of ammonium reached a growth rate of 0.098 ± 0.000 h^{-1} in the exponential phase, while the culture grown on 15.0 mM of ammonium reached a growth rate of 0.077 ± 0.023 h^{-1}. The culture grown on 2.0 mM of ammonium achieved a growth rate of 0.069 ± 0.018 h^{-1}. The optimal ammonium concentration for the growth of strain *M.* sp GB25 DSMZ 7674 could be determined with 8.5 mM on methane as carbon source within the tested range. To reach higher biomass concentrations, more frequent resupply of methane/oxygen is necessary.

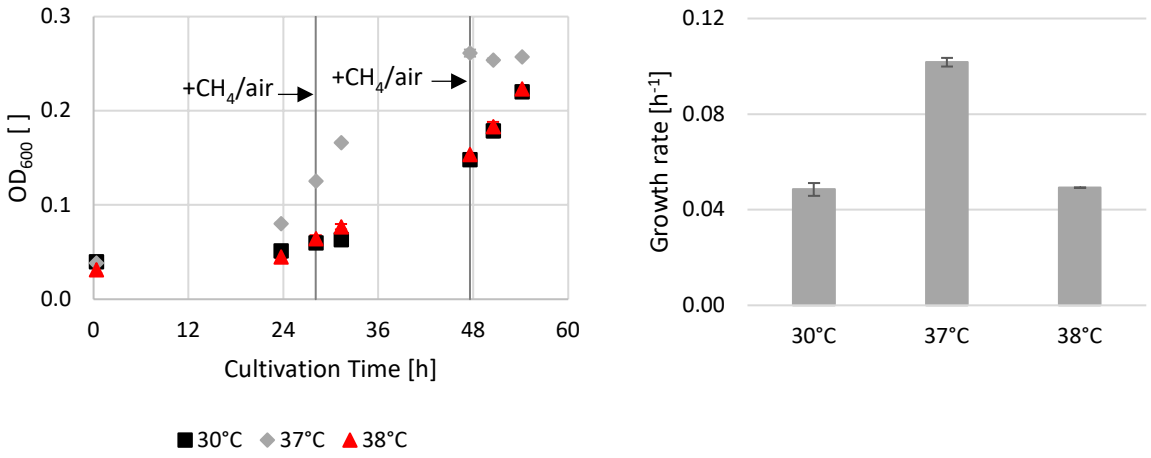


Figure 22A (left) and B (right): Growth of M. sp GB25 DSMZ 7674 in medium minimal AMS medium 1 at pH 6.8 and temperatures of 30°C, 37°C and 38°C with methane as carbon source and 15.0 mM ammonium as nitrogen source

The cultures grown at 37 °C showed distinct limitation in methane or oxygen before the second flush of methane/air, which is visible in the flattening of the exponential growth curve after around 30 hours of cultivation.

The culture grown at 37 °C showed the highest growth rate in the exponential growth phase with 0.102 ± 0.002 h^{-1}. The culture cultivated at 30 °C showed a growth rate of 0.049 ± 0.003 h^{-1} and the culture cultivated at 38 °C showed the same growth rate of 0.049 ± 0.000 h^{-1}. The optimal temperature for the growth of *M.* sp GB 25 DSMZ 7674 on methane was determined at 37 °C in this experiment.

4.1.4. Effect of antifoam agents and gas-transfer-promoting agents on methanotrophic growth

Foaming is a significant problem linked with gas fermentations[17]. To determine suitable antifoam agents for the cultivation of *M.* sp GB 25 DSMZ 7674 and to ensure they are not inhibiting cell growth, several agents were tested in serum bottles. The investigated antifoam agents were PEG 400 (0.1 $g \cdot L^{-1}$), Struktol J673-A (0.1 $g \cdot L^{-1}$) and Struktol 2020 (0.1 $g \cdot L^{-1}$). Control cultures without antifoam were cultivated under the same conditions. The OD_{600} values are shown in Figure 23 and the resulting growth rates are shown in Figure 24.

In a second experiment, further antifoam agents and gas-transfer-promoting agents were investigated. Han et al. reported on the positive effect of paraffin oil on the cell growth of *Methylosinus trichosporium* OB3b. Cell concentration could be increased from 2 $g \cdot L^{-1}$ in the control culture without oil, to 6 $g \cdot L^{-1}$ in the culture with 5 v% paraffin[42].

Therefore, strain *M.* sp GB25 DSMZ 7674 was cultivated in serum bottles in the presence of agents Hydraffin CC12x40 (1.0 $g \cdot L^{-1}$), Paraffin (5 v%), PPG2000 (0.1 $g \cdot L^{-1}$) and PEG 400 (0.1 $g \cdot L^{-1}$). A control culture without added agents was cultivated under the same conditions. The OD_{600} values are shown in Figure 25 and the resulting growth rates are shown in Figure 26.

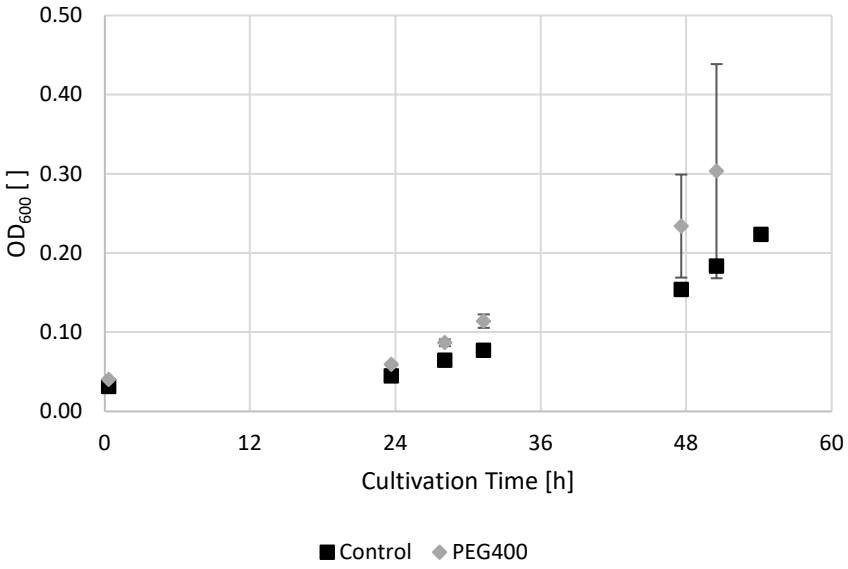


Figure 23:Growth of M. sp GB 25 DSMZ 7674 in AMS medium 1 at pH 6.8 and 38°C with methane as carbon source, 15 mM ammonium as nitrogen source and antifoam agents PEG400 (0.1 $g \cdot L^{-1}$) as well as a control culture without added antifoam agent

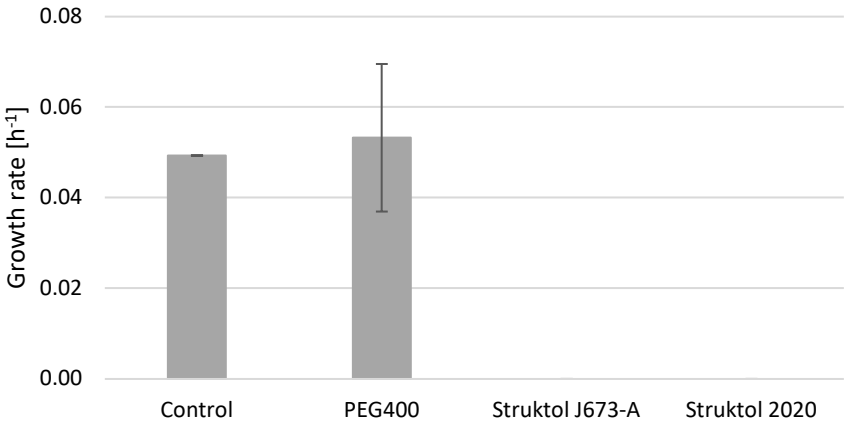


Figure 24: Growth rates of M. sp GB 25 DSMZ 7674 in AMS medium 1 at pH 6.8 and 38°C with methane as carbon source, 15 mM ammonium as nitrogen source and antifoam agents PEG400 (0.1 g·L^{-1}), Struktol J673-A (0.1 g·L^{-1}) and Struktol 2020 (0.1 g·L^{-1}) added, as well as a control culture without added antifoam agent

The culture grown with added PEG400 reached a OD_{600} value of 0.304 ± 0.135 and showed a slightly higher growth rate of 0.053 ± 0.016 h^{-1} then the control culture without added antifoam, which reached a OD_{600} value of 0.224 ± 0.004 and a growth rate of 0.049 ± 0.000 h^{-1}. The high standard deviation between the duplicates in the cultures with added PEG400 is caused by an untight septum in one of the two bottles. The antifoam agents Struktol J673-A and Struktol 2020 completely inhibited the growth of *M.* sp GB25 DSMZ 7674 and are not suitable for the cultivation of this strain.

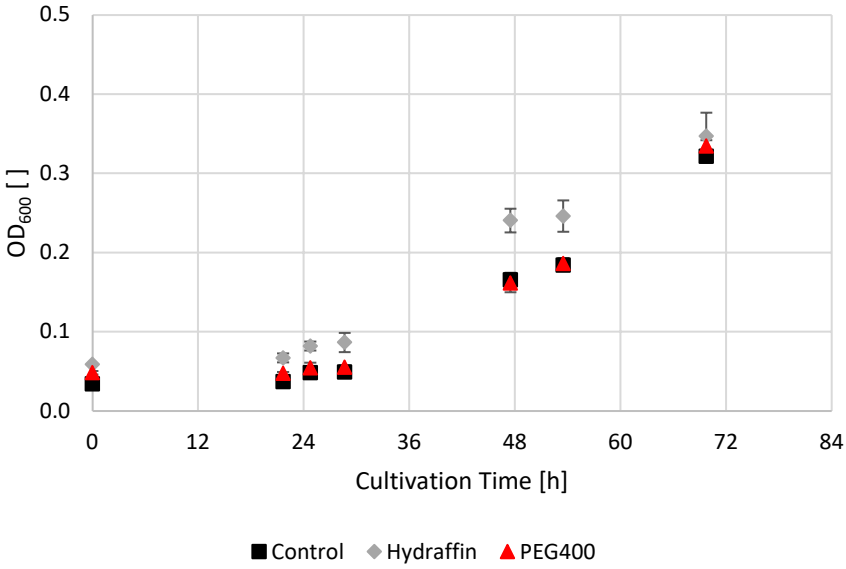


Figure 25: Growth of M. sp GB 25 DSMZ 7674 in AMS medium 1 at pH 6.8 and 37°C with methane as carbon source, ammonium as nitrogen source and agents Hydraffin (1.0 g·L^{-1}) and PEG400 (0.1 g·L^{-1}) added as well as a control culture with no additional agents

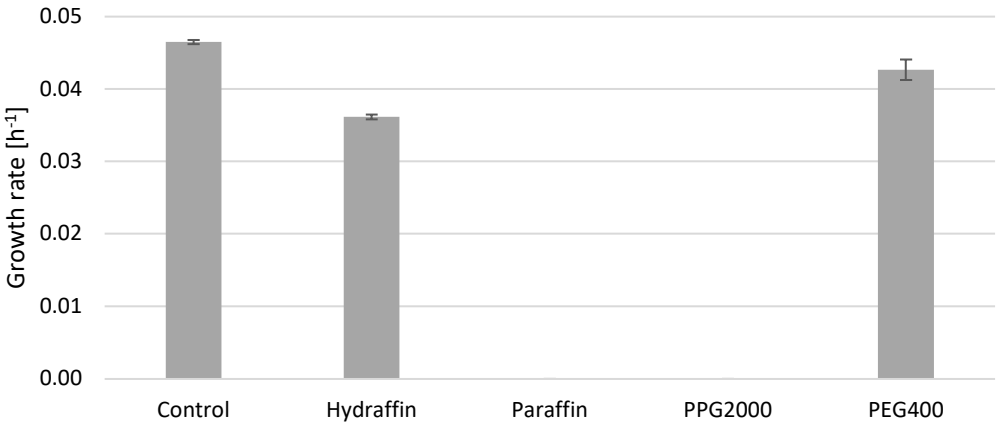


Figure 26: Growth rates of M. sp GB 25 DSMZ 7674 in AMS medium 1 at pH 6.8 and 37°C with methane as carbon source, ammonium as nitrogen source and agents Hydraffin (1.0 g·L^{-1}), Paraffin (5%), PPG 2000 (0.1 g·L^{-1}) and PEG400 (0.1 g·L^{-1}) added as well as a control culture with no additional agents

The control culture without added agents reached a OD_{600} value of 0.322 ± 0.008 and a growth rate of 0.046 ± 0.000 h^{-1}. The culture with added PEG 400 showed a OD_{600} of 0.334 ± 0.008 and a growth rate of 0.043 ± 0.001 h^{-1}, while the culture with added hydraffin reached a OD_{600} of 0.347 ± 0.030 and a growth rate of 0.036 ± 0.001 h^{-1}. Paraffin and PPG2000 completely inhibited the growth of the cells. The positive effect of paraffin oil reported by Han et al. could not be confirmed for the growth of *M.* sp GB25 DSMZ 7674 in this experiment. PEG400 could be determined as suitable antifoam agent for the cultivation of *M.* sp GB25 DSMZ 7674 under the given experimental conditions.

4.1.5. Determination of k_La-values in the BCR

As the introduction of substrate gas into the liquid culture medium is critical in gas fermentations, the k_La-values were determined for the BCR setup to characterize this mass transfer.

The effect of loop pump speed and aeration rate on the k_La-value was evaluated according to a DoE (Figure 10) for both gassing devices (vortex and sparger). The k_La-values were determined in deionized water and AMS medium 1 (20-fold concentration of nutrients) to investigate, whether the added salts in the medium hamper the k_La-values. Additionally, the effect of the antifoam agent Struktol J-673A (AF) on the k_La-value was investigated.

The results are depicted as contour plot in Figure 27 (sparger) and Figure 28 (vortex). The numeric values are summarized in Table 20.

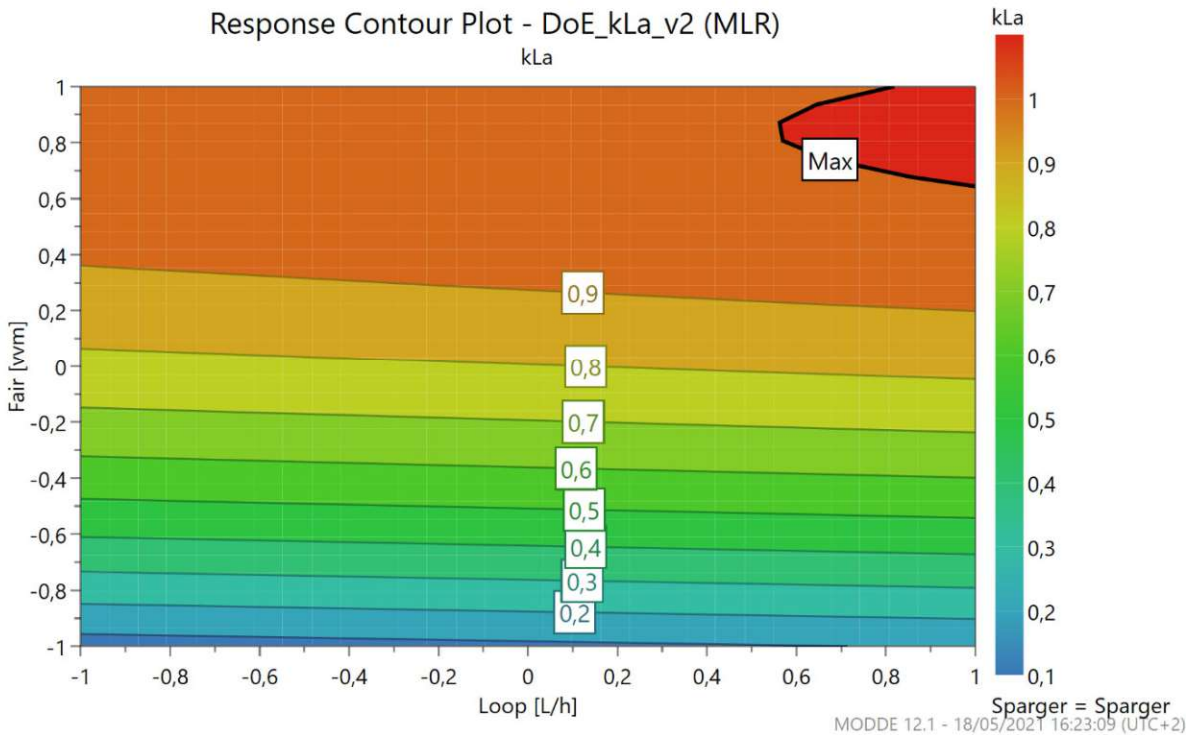


Figure 27: Contour Plot, sparger as gassing device

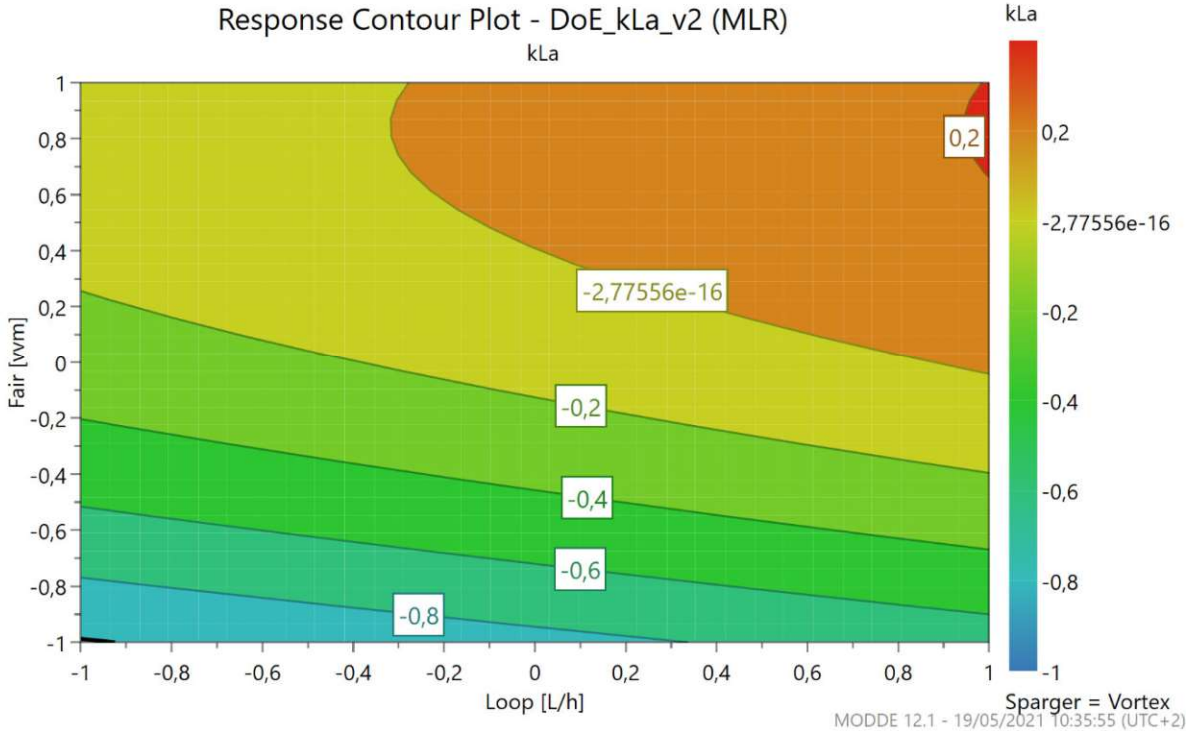


Figure 28: Contour plot, vortex as gassing device

Table 20: Results of k_La-value determination in the BCR in deionized water (H_2O) and AMS medium 1 (20-fold concentration of nutrients) at 38°C with two gassing devices (sparger and vortex)

Aeration Rate		Loop pump speed		Sparger			Vortex	
				H_2O	Medium	Medium +AF*	H_2O	Medium
	[vvm]		[rpm]	k_La [h^{-1}]	k_La [h^{-1}]	k_La [h^{-1}]	k_La [h^{-1}]	k_La [h^{-1}]
-1	0.2	-1	1500	31.01	31.14	13.86	12.38	14.07
-1	0.2	1	2700	49.92	34.84	17.36	17.99	19.08
0	0.6	0	2100	43.55	47.91	23.22	27.33	27.95
0	0.6	0	2100	45.18	48.79	25.28	25.58	28.07
0	0.6	0	2100	47.77	48.79	24.05	27.34	28.91
1	1.0	-1	1500	50.21	49.19	-	29.32	30.21
1	1.0	1	2700	49.33	46.67	-	37.15	35.75

*AF – antifoam agent Struktol J-673A

As can be seen in the contour plots, higher loop pump speed and aeration rate led to higher k_La values and the optimum range for both gassing devices could be determined at maximum loop pump speed and aeration rate of 2700 rpm and 1.0 vvm respectively.

The loop pump speed has a higher impact when the vortex is used as gassing device. The reason for this might be that the vortex is placed in the loop line (instead of inside the reactor vessel) and a higher loop pump speed might lead to better mixing and more turbulent conditions.

Comparing the numeric values (Table 20), considerably higher k_La-values of 31-49 h^{-1} (in water) were reached with the sparger used as gassing device, compared to k_La-values of 12-37 h^{-1} (in water) when the vortex was employed. Thus, the sparger could be determined as more favourable over the vortex in terms of gas-to-liquid mass transfer.

The k_La-values determined in water and medium are similar, indicating that the added salts in the medium do not significantly hamper the gas-to-liquid mass transfer. In contrast, antifoam agent Struktol J673-A drastically decreased the k_La-values to 14-24 h^{-1} compared to k_La-values of 31-47 h^{-1} without added antifoam agent. Thus, the cells in the cultivation where Struktol J673-A was used to prevent foaming might have been limited in oxygen.

A maximum k_La of 84 h^{-1} is reported for the BCR at an aeration rate of 2.0 vvm[30]. The k_La of around 50 h^{-1} determined at half the aeration rate of 1.0 vvm in this experiment is in good accordance. The BCR overall reached higher k_La-values (around 49 h^{-1}) than those reported for stirred-tank reactors of 14 and 18 h^{-1} [15].

4.1.6. Assessment of methanotrophic growth in the BCR setup with adapted parameters

Strain *M.* sp. GB25 DSMZ 7674 was cultivated in the BCR with adapted parameters according to the previous experiments performed in serum bottles and the k_La value determination. Therefore, cultivation was conducted at a temperature of 37 °C and PEG400 was added as antifoam agent. The sparger was applied as gassing device.

As no growth was evident in the OD_{600} values, the course of CO_2 [%], CH_4 [%] and O_2 [%] in the off gas is depicted in Figure 29 and the course of DO [%] and pH [ ] in Figure 30.

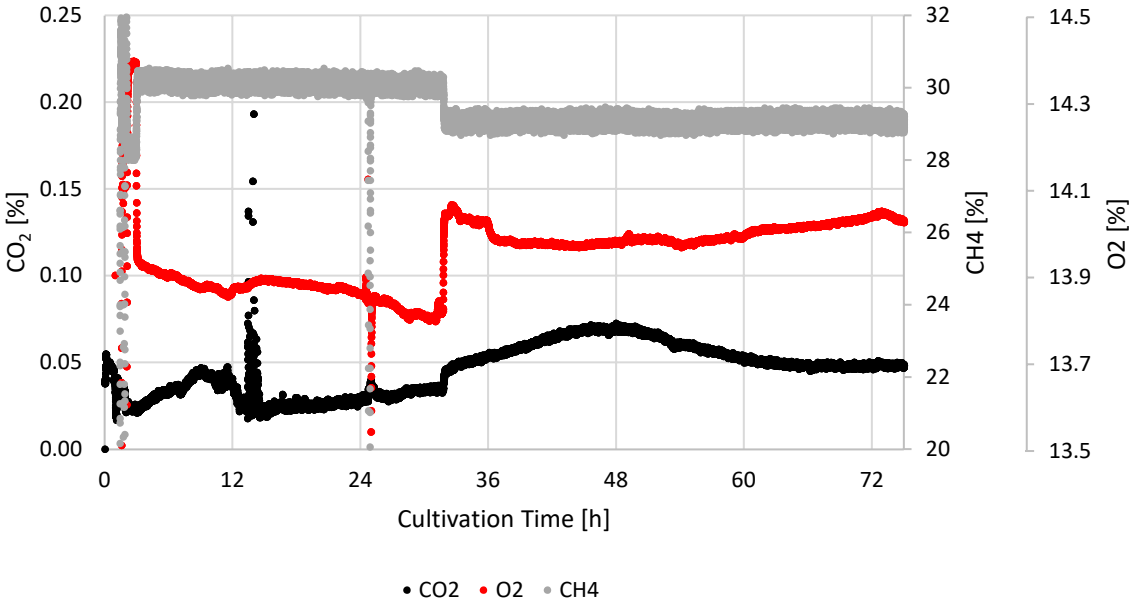


Figure 29: Course of CO_2 [%], CH_4 [%] and O_2 [%] in offgas during fermentation 3 in the BCR with strain M. sp GB25 DSMZ 7674 in minimal AMS medium 1 (20-fold concentration of nutrients) at pH 6.8 and 37°C; employing the sparger as gassing device

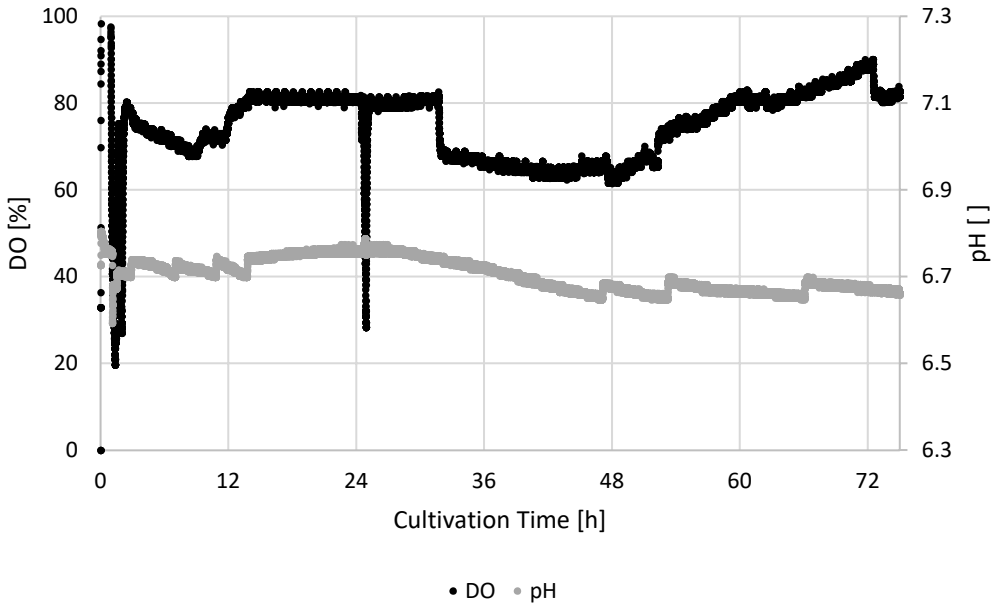


Figure 30: Course of DO [%] and pH [ ] during fermentation 3 in the BCR with strain M. sp GB25 DSMZ 7674 in minimal AMS medium 1 (20-fold concentration of nutrients) at pH 6.8 and 37°C; employing the sparger as gassing device

Initial growth of the cells is visible in the increase of CO_2 and the decrease of O_2 and DO. The pH is decreasing initially and is adjust with NaOH. After 8 hours of cultivation there is a sudden stop in microbial activity.

The successful growth of the initial first run in the BCR could not be repeated, even though the adapted parameters showed to be favourable for the growth of the strain in serum bottles. The sudden stop in microbial activity could be caused by insufficient mass transfer in the BCR, which leads to a limitation of the cells in methane. Besides an insufficient mass transfer, the cells might be lacking vital nutrients. Although, growth of strain *M.* sp GB 25 DSMZ 7674 was successful in serum bottles, AMS medium 1 adapted from Wendlandt et al.[29] contains no vitamins or trace elements. Also, the preparation of the precultures might not be favourable for this strain. The switch in carbon source from methanol in the precultures to methane in the BCR might not be beneficial. Additionally, the scale-up step from the precultures with a volume of 1 L to a reactor volume of 16 L in the BCR might be too big.

In the next step, strain *M. parvus* OBBP is cultivated in the BCR setup, to verify whether the BCR setup is suitable for cultivations of methanotrophic strains and to exclude *M.* sp GB25 DSMZ 7674 as not viable and the reason for the lack of growth.

4.1.7. Preliminary determination of a suitable medium for the cultivation of *M. parvus* OBBP in serum bottles

A suitable medium for the cultivation of strain *M. parvus* OBBP was first determined in serum bottles. Therefore, *M. parvus* OBBP was cultivated in minimal AMS medium 1 (Table 3) which contains no vitamins and trace elements and AMS medium 3 which contains vitamins and trace elements (Table 7) with methane as carbon source.

The biomass formation of *M. parvus* OBBP in both media is depicted in Figure 31.

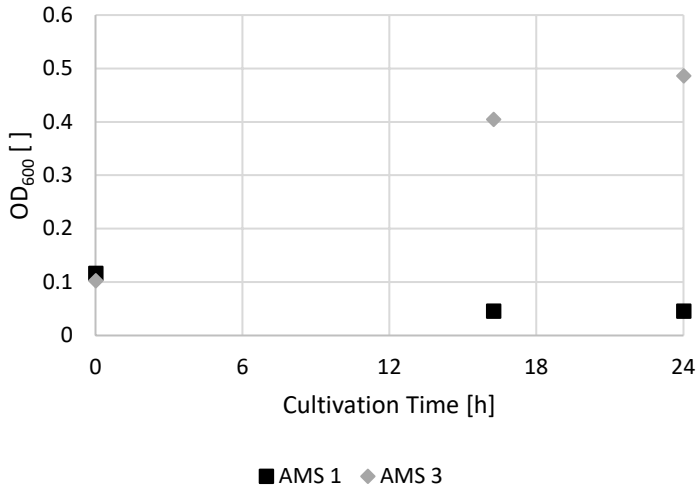


Figure 31: Biomass formation of M. parvus OBBP in minimal AMS medium 1 and AMS medium 3 at pH 6.8 and 37°C with methane as carbon source

Strain *M. parvus* OBBP reached a OD_{600} value of 0.487 after 24 hours of cultivation in AMS medium 3, while no cell growth could be observed in minimal AMS medium 1. AMS medium 1 contains no vitamins or trace elements and seems to be insufficient for the growth of *M. parvus* OBBP, therefore AMS medium 3 was chosen for the following cultivation of *M. parvus* OBBP in the BCR setup.

4.1.8. Assessment of growth of strain *M. parvus* OBBP in the BCR setup

To verify that the BCR setup is suitable for cultivations of methanotrophic strains *M. parvus* OBBP was cultivated in AMS medium 3 in the BCR.

As no growth was evident in the OD_{600} values, the course of CO_2 [%], CH_4 [%] and O_2 [%] in the off gas is depicted in Figure 32 and the course of DO [%] and pH is shown [ ] in Figure 33.

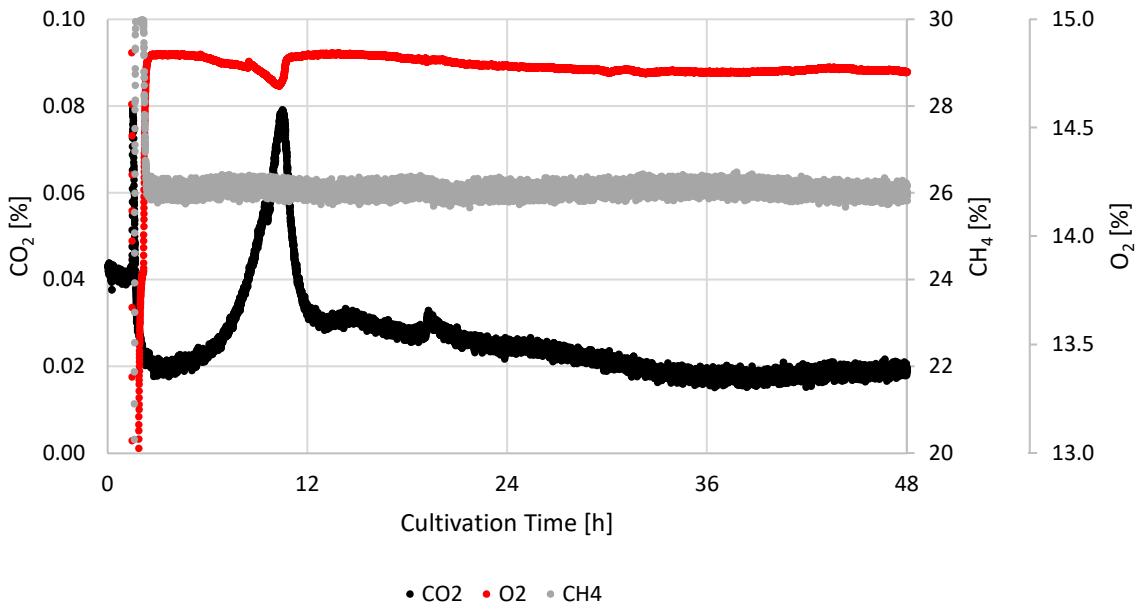


Figure 32: Course of CO_2 [%], CH_4 [%] and O_2 [%] in offgas during fermentation 4 in the BCR with strain M. parvus OBBP in AMS medium 3 at pH 6.8 and 37°C; employing the sparger as gassing device

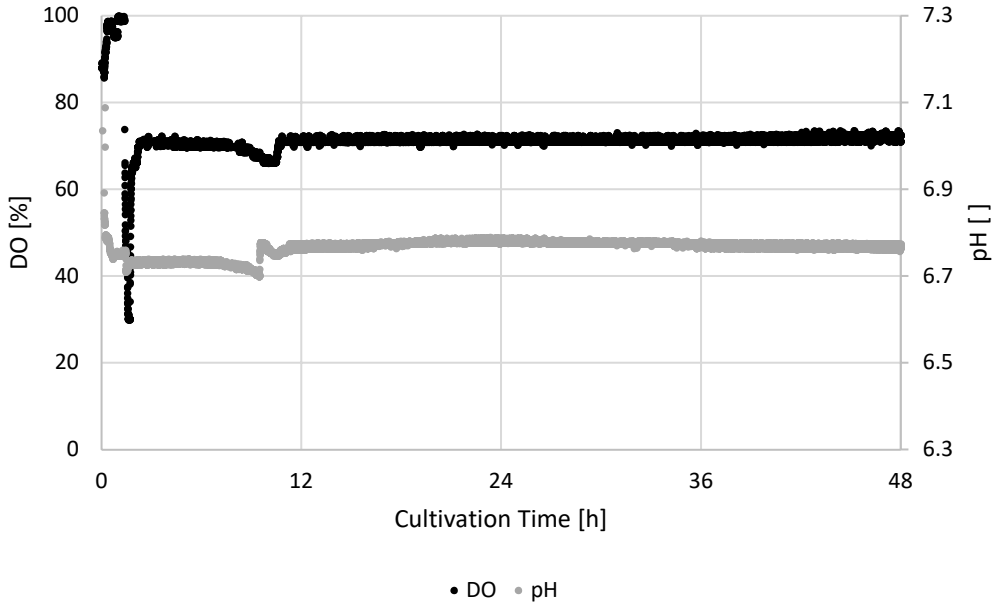


Figure 33: Course of DO [%] and pH [ ] during fermentation 4 in the BCR with strain M. parvus OBBP in AMS medium 3 at pH 6.8 and 37°C; employing the sparger as gassing device

The CO_2 signal is increasing and the O_2 as well as the DO signal are decreasing in the first 10 hours of cultivation, which indicates growth of the cells. Also, the pH is initially decreasing and is adjusted with NaOH.

After 10 hours of cultivation, strain *M. parvus* OBBP showed the same sudden stop in microbial activity as *M.* sp GB25 DSMZ 7674 in the previous cultivation in the BCR. If the methane mass transfer is not sufficient in the BCR setup, the cells might have been limited in methane again. The preculture of *M. parvus* OBBP was prepared with methane, so there was no switch in carbon source in this cultivation. But the cell concentration of the preculture might have been too low in this cultivation as well.

To verify the assumption that methane mass transfer is not sufficient in the BCR, cultivations were performed in a smaller-scale bioreactor setup equipped with stirrers.

4.1.9. Assessment of PHB production in a stirred-tank reactor system with strain *M.* sp GB25 DSMZ 7674

To verify that *M.* sp GB25 DSMZ 7674 is viable and that the chosen cultivation conditions are suitable the strain was cultivated in the DASbox® system. The course of OD_{600}, CDW and PHB concentration in reactor C is depicted in Figure 34 and the course of OD_{600}, CDW and PHB concentration in reactor D is shown in Figure 35. Reactor A and B could not be assessed, as the data was compromised by a blur during fermentation.

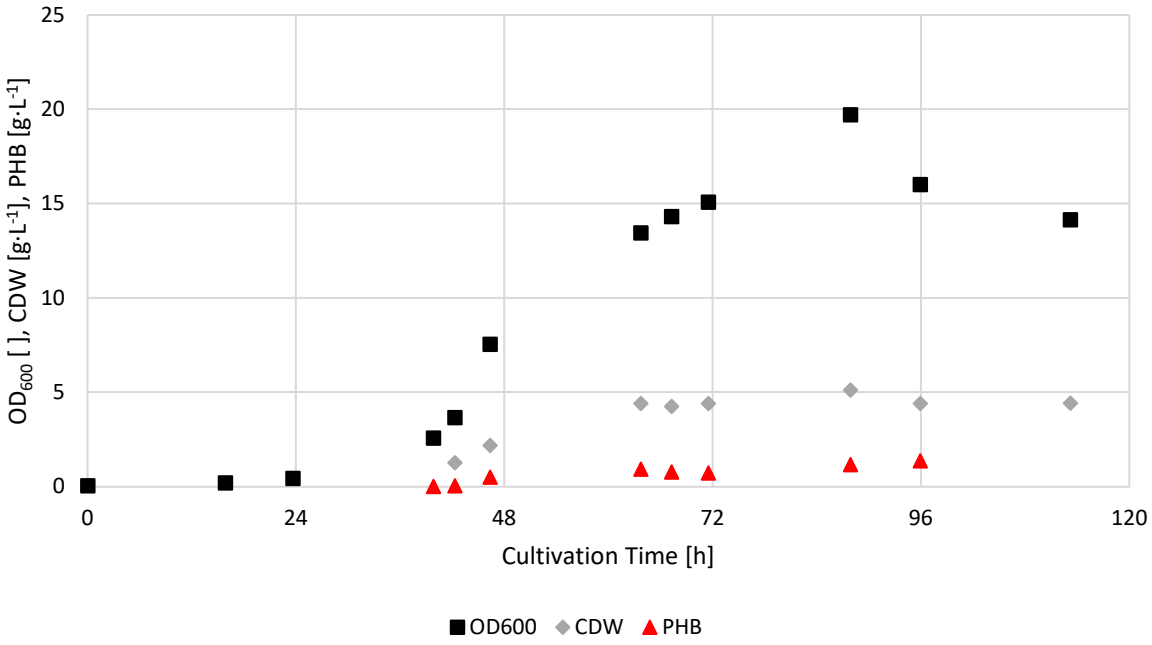


Figure 34: Course of ammonium [mM], OD_{600} [ ], CDW [g·L^{-1}] and PHB [g·L^{-1}] during fermentation 5 in the DASbox® system with M. sp. GB25 DSMZ 7674 in AMS medium 3, at pH 6.8 and and 37°C, Reactor C

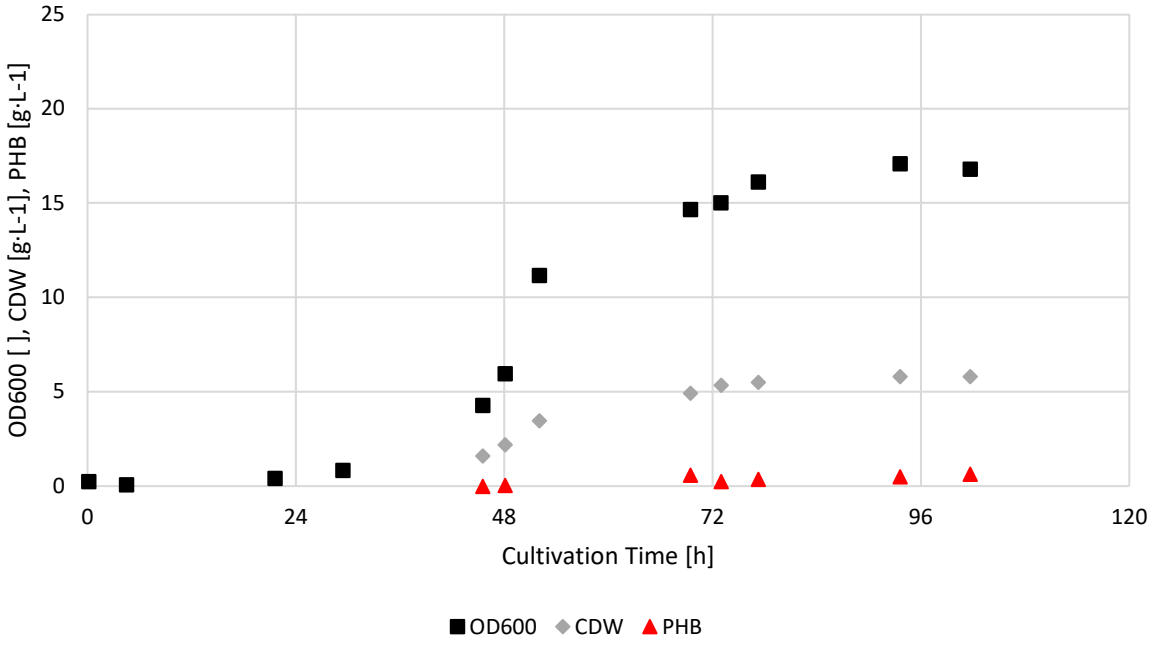


Figure 35: Course of ammonium [mM], OD_{600} [ ], CDW [g·L^{-1}] and PHB [g·L^{-1}] concentration during fermentation 5 in the DASbox® system with M. sp. GB25 DSMZ 7674 in AMS medium 3, at pH 6.8 and and 37°C, Reactor D

In reactor C a OD_{600} value of 14.13 and a CDW of 4.42 g·L^{-1} was reached after 113 hours of cultivation. A final PHB concentration of 1.35 g·L^{-1} could be determined which corresponds to a ratio of 31 % PHB per CDW. PHB was already formed at around 46 hours of cultivation, when ammonium was almost depleted. The accumulated PHB was not consumed when ammonium was resupplied. In reactor D a OD_{600} value of 16.80 and a CDW of 5.81 g·L^{-1} could be reached after 102 hours of cultivation. A final PHB concentration of 0.64 g·L^{-1} could be determined which corresponds to a share of 11 % PHB per CDW.

The successful growth and PHB accumulation of *M.* sp GB25 DSMZ 7674 in the DASbox® system, reinforces the assumption, that the mass transfer might not be sufficient in the BCR and that cells are limited in methane.

4.2. Liquid C1 compounds

4.2.1. Cultivations in shake flasks

4.2.1.1. Assessment of methylotrophic growth on methanol and formate

Strains *M. extorquens* DSMZ 1337, *M. extorquens* DSMZ 1338, *M. extorquens* DSMZ 1339 and *M. extorquens* DSMZ 1340 were cultivated on methanol and formate as carbon sources respectively to determine the influence of the substrate on the growth.

The biomass formation of the four *M. extorquens* strains on methanol is depicted in Figure 36, while the biomass formation on formate is shown in Figure 37.

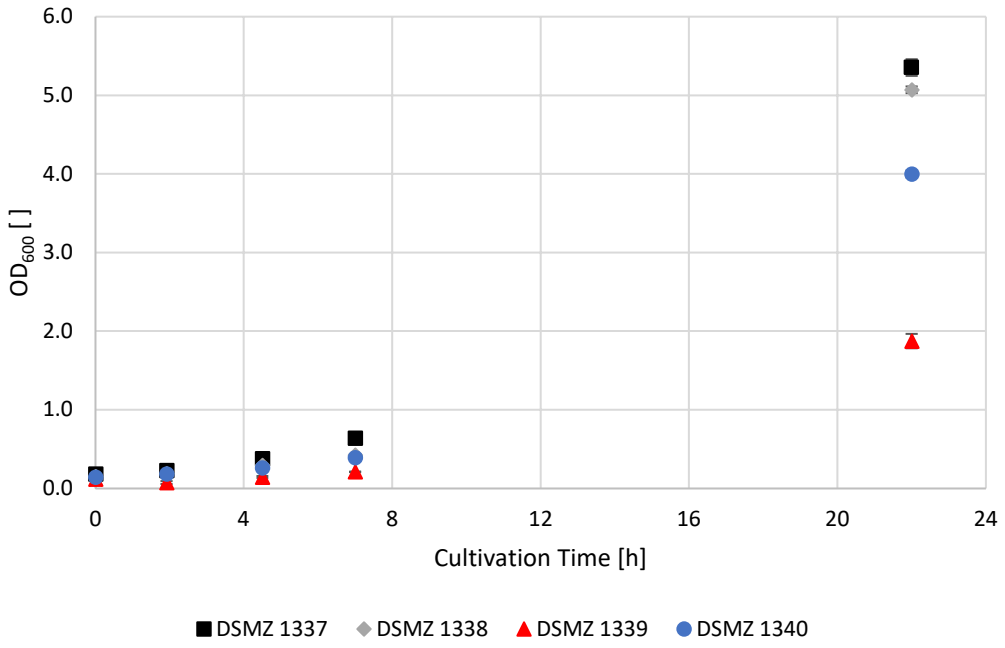


Figure 36: Biomass formation of M. extorquens DSMZ 1337, M. extorquens DSMZ 1338, M. extorquens DSMZ 1339 and M. extorquens DSMZ 1340 in AMS medium 4 at 30°C with an initial methanol concentration of 1 v% as carbon source and 1.5 g·L^{-1} ammonium sulphate as nitrogen source

Strain *M. extorquens* DSMZ 1337 showed the highest OD_{600} value of 5.36 ± 0.11 and the highest growth rate of 0.201 ± 0.009 h^{-1} with methanol as carbon source. *M. extorquens* DSMZ 1338 reached a OD_{600} value of 5.07 ± 0.04 and a growth rate of 0.163 ± 0.002 h^{-1}, while *M. extorquens* DSMZ 1340 reached a OD_{600} value of 4.00 ± 0.04 and a growth rate of 0.154 ± 0.001 h^{-1}. The lowest growth on methanol with a OD_{600} value of 1.88 ± 0.09 and a growth rate of 0.155 ± 0.003 h^{-1} showed *M. extorquens* DSMZ 1339.

The growth rate of 0.201 ± 0.009 h^{-1} determined for strain *M. extorquens* DSMZ 1337 in this experiment is even higher than the reported growth rate of 0.168 ± 0.003 h^{-1} on methanol in *M. extorquens* AM1[43].

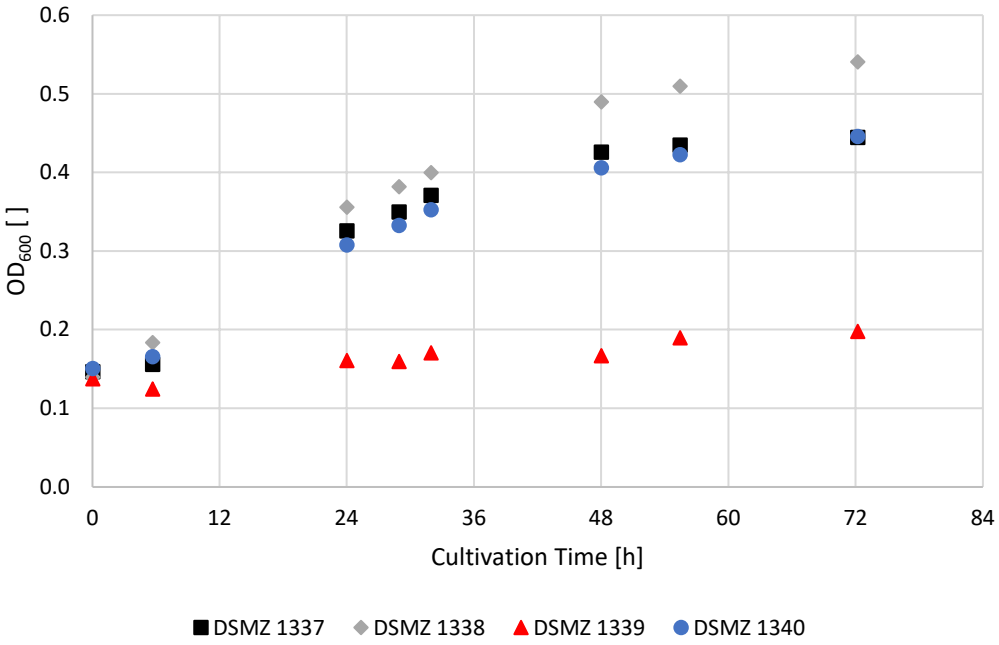


Figure 37: Biomass formation of M. extorquens DSMZ 1337, M. extorquens DSMZ 1338, M. extorquens DSMZ 1339 and M. extorquens DSMZ 1340 in AMS medium 4 at 30°C with an initial formate concentration of 16.8 g·L^{-1} as carbon source and 1.5 g·L^{-1} ammonium sulphate as nitrogen source

All four strains of *M. extorquens* (DSMZ 1337, DSMZ 1338, DSMZ 1339 and DSMZ 1340) showed stagnating growth with formate as carbon source and reached lower OD_{600} values of 0.54 to 0.44 compared to OD_{600} values of 5.36 ± 0.11 to 1.88 ± 0.09 for the growth on methanol. *M. extorquens* DSMZ 1339 showed no growth under the given experimental conditions.

Formate was supplied in the form of sodium formate, which reacts basic in aqueous solution. To better assess cell growth with sodium formate as substrate in shake flasks, the pH needs to be monitored and adjusted for optimal growth of the cells.

Overall, *M. extorquens* DSMZ 1337 and *M. extorquens* DSMZ 1338 showed the highest growth in this experiment and were chosen for the following assessment of PHB accumulation capability.

4.2.1.2. Assessment of PHB accumulation on methanol and formate as sole carbon sources and in combination

Strain *M. extorquens* DSMZ 1337 and *M. extorquens* DSMZ 1338 were cultivated in shake flasks on methanol and formate as individual carbon sources and in combination to investigate the effect of the substrate on PHB accumulation.

The PHB yield of *M. extorquens* DSMZ 1337 per consumed substrate is depicted in Figure 38 and 39 for the three different substrate variations. The PHB yield of *M. extorquens* DSMZ 1338 per consumed substrate is shown in Figure 40 and 41 also for all three substrate variations.

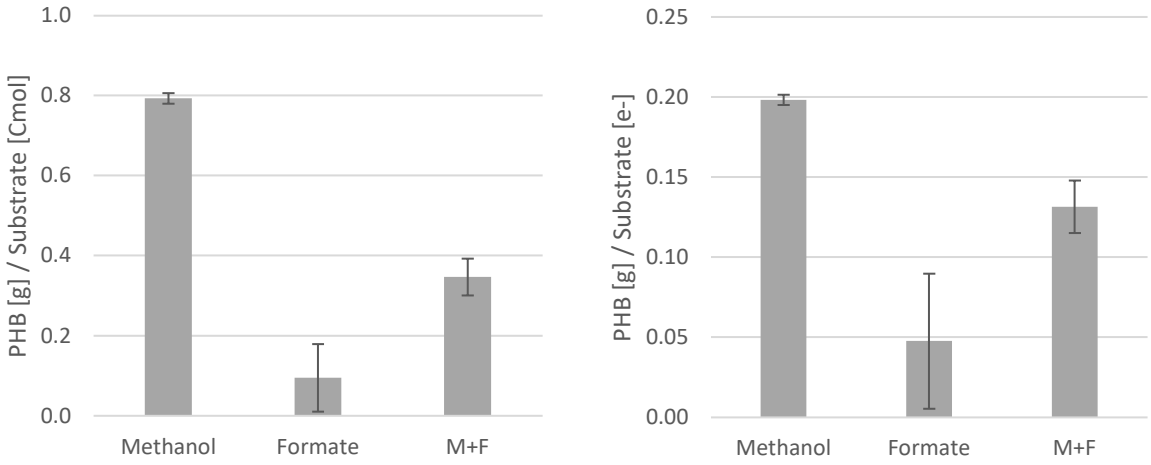


Figure 38 and Figure 39: PHB yields per consumed substrate in Cmol (left) and e- (right) of M. extorquens DSMZ 1337 in AMS medium 4 with methanol and formate as individual carbon sources and in combination (M+F)

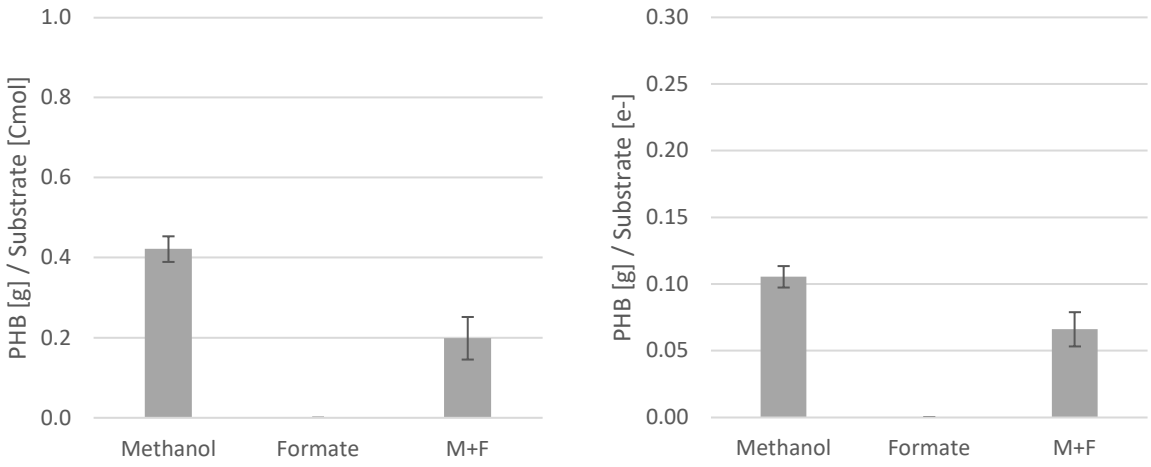


Figure 40 and Figure 41: PHB yields per consumed substrate in Cmol (left) and e- (right) of M. extorquens DSMZ 1338 in AMS medium 4 with methanol and formate as individual carbon sources and in combination (M+F)

M. extorquens DSMZ 1337 accumulated 0.793 ± 0.013 g PHB per Cmol methanol (0.198 ± 0.003 g PHB per e^- methanol), 0.095 ± 0.084 g PHB per Cmol formate (0.048 ± 0.042 g PHB per e^- formate) and 0.347 ± 0.046 g PHB per Cmol methanol and formate in combination (0.131 ± 0.016 g PHB per e^- methanol and formate). *M. extorquens* DSMZ 1338 accumulated 0.422 ± 0.032 g PHB per Cmol methanol (0.105 ± 0.008 g PHB per e^- methanol), no PHB on formate and 0.199 ± 0.053 g PHB per Cmol methanol and formate in combination (0.066 ± 0.013 g PHB per e^- methanol and formate).

Methanol showed to be the favourable carbon source for PHB accumulation in both strains and resulted in the highest PHB yields, while little to no PHB was produced on formate as carbon source under these experimental conditions. The combination of methanol and formate as substrate also led to significantly lower PHB yields than methanol as sole carbon source. Overall, *M. extorquens* DSMZ 1337 reached higher PHB yields than *M. extorquens* DSMZ 1338.

Based on these results, strain *M. extorquens* DSMZ 1337 was chosen for cultivation in the bioreactor system.

4.2.2. Cultivations in a bioreactor system

4.2.2.1. Evaluation of formate as auxiliary carbon sources for PHB production

Strain *M. extorquens* DSMZ 1337 was cultivated in a DASGIP® bioreactor system. Two substrate variations were investigated, namely methanol as sole carbon source and a combination of methanol and formate as carbon sources.

The course of OD_{600}, CDW and PHB during cultivation of *M. extorquens* DSMZ 1337 on methanol as carbon source is depicted in Figure 42 and the course of OD_{600}, CDW and PHB on methanol and formate in combination is shown in Figure 43. The most important process parameters are summarized in Table 21.Table 19

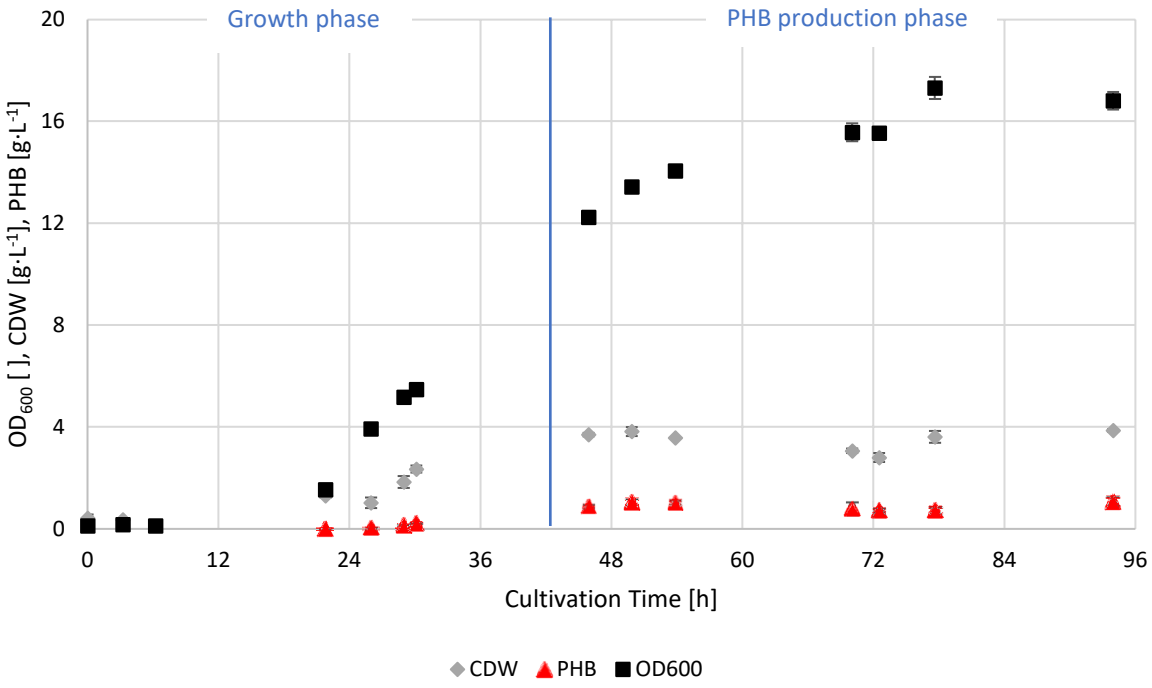


Figure 42: Course of OD_{600}, CDW and PHB during cultivation in the DASGIP® system of strain M. extorquens DSMZ 1337 in AMS medium 4 at pH 7.0 and 30°C and methanol as sole carbon source

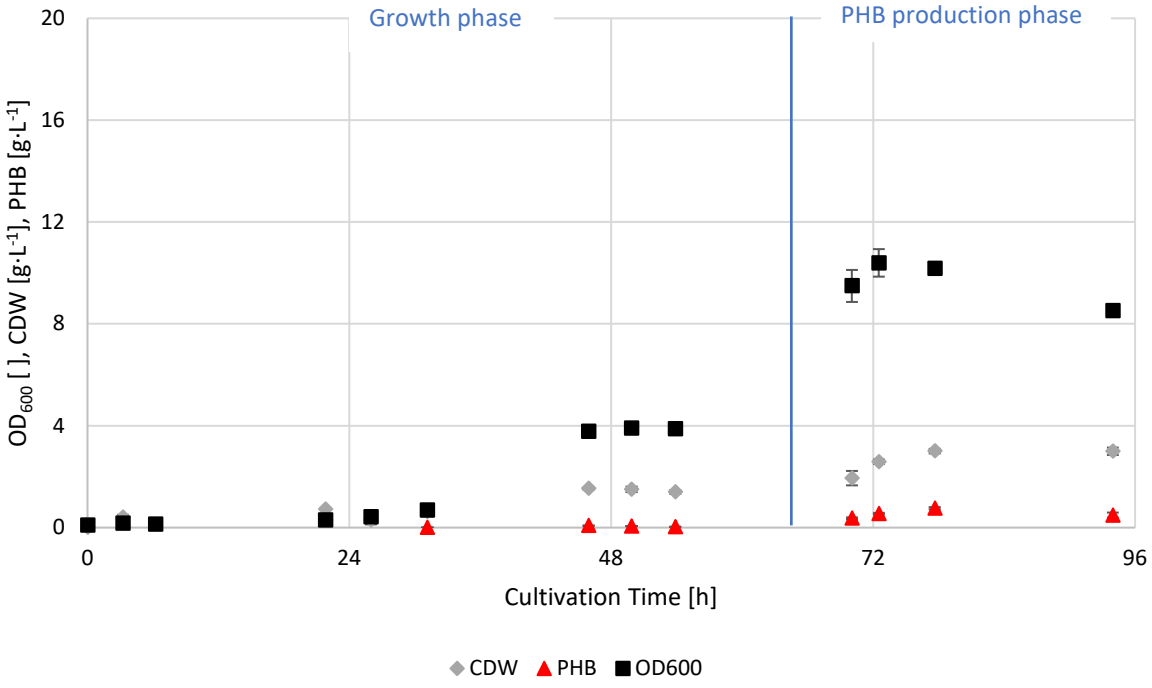


Figure 43: Course of OD_{600}, CDW and PHB during cultivation in the DASGIP® system of strain M. extorquens DSMZ 1337 in AMS medium 4 at pH 7.0 and 30°C and methanol and formate in combination as carbon sources

A biomass concentration of 3.86 ± 0.05 g·L^{-1} was reached after 94 hours of cultivation with methanol as substrate, while a biomass concentration of 3.00 ± 0.14 g·L^{-1} was reached with methanol and formate in combination after 94 hours of cultivation. A PHB concentration of 1.06 ± 0.18 g·L^{-1} was accumulated (27.4 % of CDW) with methanol as carbon source, while a PHB concentration of 0.49 ± 0.10 g·L^{-1} was produced (16.0 % per CDW) with methanol and formate as carbon sources in combination.

The reached biomass concentration of 3.86 ± 0.05 g·L^{-1} with methanol as substrate is very low compared to biomass levels between 100 g·L^{-1} and even up to 206 g·L^{-1} for different *M. extorquens* strain reported in literature[35],[36],[40]. To increase the biomass concentration, an exponential feed could be applied to reach maximum biomass levels within a shorter period of time before entering the production phase. Also, the biomass concentration in the cultivation with formate as additional carbon source to methanol (Figure 43) was initially lagging, due to problems with the pH adjustment.

Table 21: Process parameters of cultivation of M. extorquens DSMZ 1337 with methanol as sole carbon source and methanol and formate in combination, 'e'- available electrons, methanol: 4 electrons, formate: 2 electrons

		Methanol	**Methanol+Fomate**
μ (in batch phase)	**[h^{-1}]**	0.138 ± 0.005	0.049 ± 0.007
$Y_{X/S}$	**[g·g^{-1}]**	0.190 ± 0.010	0.036 ± 0.002
	[g·Cmol^{-1}]	6.083 ± 0.335	1.848 ± 0.122
	[g· e^{-1}]	1.521 ± 0.084	0.616 ± 0.041
$Y_{PHB/S}$	**[g·g^{-1}]**	0.088 ± 0.006	0.014 ± 0.002
	[g·Cmol^{-1}]	2.829 ± 0.206	0.700 ± 0.077
	[g·e^{-1}]	0.707 ± 0.051	0.233 ± 0.026
$q_{S/X}$	**[g·g^{-1}·h^{-1}]**	0.172 ± 0.007	0.440 ± 0.066
	[mmol·g^{-1}·h^{-1}]	5.365 ± 0.203	8.551 ± 1.288
C-Balance	**[ ]**	0.868 ± 0.007	0.579 ± 0.052

The maximum biomass yield of 0.190 ± 0.010 g·g^{-1} relative to the methanol consumed is lower than reported values of 0.29-0.33 g·g^{-1}. The maximum PHB yield of 0.088 ± 0.006 g·g^{-1} is in the lower range of reported PHB yields of 0.09-0.12 g·g^{-1}[40]. Reported PHB contents are between 35 % and up to 66 %[35],[36]. The PHB content of 27.4 % of CDW could be further improved by accurately adjusting the carbon to nitrogen ratio. Suzuki et al. (1986) showed in a kinetic study, that feeding a small quantity of ammonia resulted in a more rapid increase in intracellular PHB content than was the case without ammonia feeding[44]. Also, as can be seen

in the course of PHB concentration in the cultivation with methanol as carbon source (Figure 42), the PHB content could not be increased over time. So, a rapid PHB production phase is favourable.

As can be seen in Table 21, the addition of formate did not increase the PHB content and overall PHB yields. A biomass yield of 6.083 ± 0.335 g·Cmol^{-1} (1.521 ± 0.084 g·e^{-1}) was reached with methanol as substrate while a biomass yield of 1.848 ± 0.122 g·Cmol^{-1} (0.616 ± 0.041 g·e^{-1}) was reached with formate in addition. A PHB yield of 2.829 ± 0.206 g·Cmol^{-1} (0.707 ± 0.051 g·e^{-1}) was reached for methanol compared to a PHB yield of 0.700 ± 0.077 g·Cmol^{-1} (0.233 ± 0.026 g·e^{-1}) with formate as additional carbon source. The substrate uptake rate is 5.365 ± 0.203 mmol·g^{-1}·h^{-1} for methanol as substrate and 8.551 ± 1.288 mmol·g^{-1}·h^{-1} with formate as auxiliary carbon source.

Also, the C-balances with 0.868 ± 0.007 for methanol and 0.579 ± 0.052 for methanol and formate in combination are not fully closing. Potential reasons for these incomplete C-balances are, that the determination of CDW and PHB content underlies fluctuations and also the detection of CO_2 in the offgas might be erroneous.

5. Conclusion

The feasibility of PHB production with methane as substrate in the 16-L BCR setup could not be shown. Growth of *M.* sp. GB25 DSMZ 7674 was initially successful in the BCR, but problems like foaming and oxygen limitation prevented the transition into the PHB production phase.

Several critical process parameters for the cultivation of methanotrophic strains could be determined. An optimal temperature of 37°C and an ammonium concentration of 8.5 mM could be implemented for *M.* sp. GB25 DSMZ 7674. Antifoam agent PEG 400 was determined as suitable for the cultivation of *M.* sp. GB25 DSMZ 7674, whereas antifoam agent Struktol-673A inhibited growth of the cells. The reported positive effect of the gas-transfer-promoting agents hydraffin and paraffin oil could not be confirmed in our experiments. Also, the successful production of PHB in a small-scale STR system validates, that the strain *M.* sp. GB25 DSMZ 7674 is viable and the general process parameters like temperature, pH and medium are suitable for the cultivation of this strain. Even though the determined k_La-values of around 50 h^{-1} are higher than those reported for STRs (14 and 18 h^{-1} [15]), methane mass transfer might has been insufficient. Poor liquid circulation is a commonly known problem with BCRs, thus the BCR might not be the ideal reactor setup for the conversion of methane to PHB.

Another reason for the lack of growth in the BCR could be, that the scale-up step from the precultures (volume of 1 L, OD_{600} around 1-2) to the BCR (16 L working volume) might have been too big and the initial cell concentration in the reactor vessel was too low. In a next step, the preparation of precultures could be further improved and biomass concentrations increased, by performing more precultures steps or utilizing bigger vessels that can be sealed airtightly and allow the cultivation of precultures with methane as substrate.

In the second approach, PHB production with methanol and formate as auxiliary carbon source to methanol was successful. However, the process needs to be further improved by e.g., applying an exponential feed rate to exaggerate biomass followed by a rapid PHB production phase and accurately adjusting the carbon to nitrogen ratio to yield maximum PHB contents.

Also, the addition of formate did not improve the yields under the given experimental conditions. However, formate as cosubstrate is worth being further investigated. Formate has been previously tested in multiple organisms and under various conditions as an auxiliary substrate and has shown to support increased growth and product yields[45]. Also, formate can directly provide the cell with reducing power, as its reduction potential is low enough to efficiently donate its electrons to NAD(P)H[46].

6. Outlook

Methane constitutes a promising substrate to lower the cost of PHA production as it is cheap and abundant. However, its low water solubility remains as major challenge. PHB production from methane could successfully be shown mostly in lab-scale STRs. To make this process feasible for industrial applications and to achieve sufficient productivity, further process development is necessary to implement a novel reactor configuration and operation strategies on a bigger scale[22].

Forced circulation loop bioreactors (FCLBs) appear to be an attractive option for methane conversion in large scale processes. In FCLBs, fermentation broth is pumped up the riser segment by a peristaltic pump though static mixing elements and into the degassing unit from which the broth proceeds through the downcomer segment and back to the pump[15]. A PHB content of 51.6 % of cell dry weight (CDW) could be achieved in a 1.4-L forced-liquid vertical tubular loop bioreactor by *Methylocystis hirsuta* from natural gas with a limitation in the nitrogen source[31].

Out of the three investigated carbon sources, methanol is likely the most promising to meet the criteria for a cost-effective and competitive process for the production of PHB on an industrial scale. It is one of the cheapest noble substrates, is available in large volumes and can be easily stored and transported[13],[40]. To further improve the already high PHB yields on methanol, a versatile set of genetic tools (gene deletions, overexpression, random mutagenesis etc.) as well as metabolic insights might provide possibilities to further maximize the efficiency[32].

Formate has only recently been gaining interest as industrial feedstock with developments in electrochemical, photochemical, and catalytic methods for its generation[13]. The concept of a formate bioeconomy suggests that excess energy produced at off-peak hours from renewable and intermittent sources can be utilized to generate formate which in turn could serve as microbial substrate. This way energy could be efficiently stored in chemical compounds and the cost of formate production could be significantly reduced. To the authors knowledge, a process utilizing formate as sole carbon source for the production of PHB has not been developed yet. Further research is necessary to assess the most promising strategies for formate conversion[46].

List of Tables

List of Figures

Appendix

ID	Flow
1	Medium
2	Base
3	Acid
4	Air/O_2
5	CH_4

ID	Tank
B1	BCB
B2	Medium tank
B3	Base tank
B4	Acid tank

ID	Valve
V01	Valve loop entrance
V02	Valve bypass entrance
V03	Valve bypass end
V04	Valve B1 release
V05	Additional valve
V06	Security valve
V07	First sampling valve
V08	Second sampling valve

ID	Name	Sensor	Function
001	QIRC	DO	Measurement + Control
002	QIRC	pH	Measurement + Control
003	TIRC	T	Measurement + Control
004	QIR	DO	Measurement
005	TIR	T	Measurement
006	PIRC	p	Measurement + Control
007	PI	p	Indication
008	WIRC	m	Measurement
011	QIR	CH_4	Measurement
012	QIR	O_2	Measurement
013	QIR	CO_2	Measurement
014	FIRC	Flow air/O_2	Measurement + Control
015	FIRC	Flow CH_4	Measurement + Control

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