U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

CMET

Integrated Master in Bioengineering (Biological Engineering Branch)

# Single-cell Protein production on methanol by co-cultivation of a bacterium and a yeast

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Master's Dissertation in partnership with Centre of Microbial Ecology and Technology (CMET, UGent), through the Erasmus+ Programme

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September of 2021

# Acknowledgments

This thesis is not merely a work of research but also the result of a collaboration of people that made me grow tremendously and helped me feel at home in the beautiful city that is Ghent. I would like to start by thanking the one inspiring person that gave me twice the opportunity to come to CMET and do my research there: Prof. Korneel Rabaey, who never says no to a motivated person. To Prof. Nuno Azevedo* back at home, thank you for the great advice, patience, and guidance.

I would also like to express my deepest gratitude to the force of nature that is my mentor Myrsini. Thank you for spotting me last year and giving me the opportunity to come back, which turned into this fantastic experience. I greatly admire you not only for your incredible work and passion for research but also for how helpful you are to everyone. Despite working during the most chaotic time of your research, you were always there for me, both professionally and personally. Learning from you made all the occasional frustration that comes with scientific work worth it. *Ευχαριστώ!*

I was also lucky enough to be able to count on one of the smartest people I have ever met: Dr. Frederiek-Maarten Kerckhof, which I thank for analyzing some of my results during his very little free time. I would like to extend my thanks to Elisa who helped me learn a new programming language in the process. Then, I would like to thank the entire team of CMET, that became a big inspiration to me during this period. A special thanks to Adri, Guillaume, and Kevin, for always lifting my spirits and being supportive. To Albérte, Barbara, Brecht, Celia, Camille, Lotte, Quinten, and Xavier, for helping me and inspiring further my love of science (and existential crisis at lunch times).

I wish also to show my deepest gratitude to my roommates at Home Göttingen. Thanks for all the *tiramisu* after long lab days and the bullying you endured from me (remember I secretly like you all). I have no idea how I will survive adulthood without you living with me.

Lastly, I would like to thank all my friends and family. Many thanks to my curiously minded grandma, asking me questions such as “If the planets are made of rocks, why don’t they fall down from the sky?” that made me never take education for granted. To my parents for all the unconditional love and support (and to my dogs for the same reason). I cannot finish without thanking all my friends who put up with my *telenovelas* for 5 years and always reminded me of “*O que é o pior que pode acontecer*?”.

*O Prof. Nuno Azevedo, orientador desta dissertação, é membro integrado do LEPABE – Laboratório de Engenharia de Processos, Ambiente Biotecnologia e Energia, financiado por: Financiamento Base - UIDB/00511/2020 da Unidade de Investigação - Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia – LEPABE - financiada por fundos nacionais através da FCT/MCTES (PIDDAC).

# Resumo

O crescimento exponencial da população mundial e do interesse por dietas ricas em proteína tem conduzido à produção insustentável de fontes proteicas tradicionais (e.g. animal). A produção de *Single-Cell Protein* (SCP), recorrendo à utilização de metanol como substrato, reemerge como uma alternativa potencialmente sustentável e caracterizada por um conteúdo proteico superior. De modo a tornar o processo financeiramente viável, este estudo focou-se no cultivo individual e conjunto de *M. extorquens* e *K. phaffii* em *batch*, para melhoria dos rendimentos em biomassa e proteína, e na exploração de uma técnica de precipitação acídica da biomassa resultante.

Testes iniciais foram realizados para obter as condições ótimas de operação, demonstrando significativamente a importância do meio de cultura. Um novo meio, denominado *Methylotrophic Mineral Medium with Vitamins* (MMMV), levou à obtenção de até 8 vezes mais biomassa final que três outros meios indicados para estas estirpes em pequena-escala. O mesmo não foi observado no biorreator, em que o cultivo em *Ammonium Mineral Salts* (AMS) resultou no triplo de biomassa final de *M. extorquens*, indicando sinais de stress celular pela presença de agregados (quase 50% da população) e a potencial acumulação de *polihidroxibutirato* (PHB). Por outro lado, bactérias cultivadas em MMMV demonstraram pouco crescimento a nível de células viáveis. Entre culturas, *M. extorquens* apresentou um baixo conteúdo proteico (até 25%$_{CDW}$) e um crescimento aproximadamente 3 vezes inferior quando comparado com *K. phaffii* e com a co-cultura. *K. phaffi* obteve um conteúdo proteico (44%$_{CDW}$) semelhante à co-cultura (39%$_{CDW}$) e comparável com valores da literatura, mas a co-cultura obteve um rendimento em biomassa 1.4 vezes superior. Adicionalmente, utilizando citometria de fluxo, foi possível observar que a presença de *K. phaffii* melhorou o crescimento de *M. extorquens*. Por último, um teste de precipitação acídica, usando ácido fórmico e ácido hidroclórico com um pH ótimo entre 2.5 e 4, resultaram na precipitação máxima de 92%$_{CDW}$ da biomassa de *K. phafii*, quase o dobro do obtido por precipitação natural (54%$_{CDW}$).

Deste modo, o trabalho realizado demonstrou a importância da escolha de um meio de cultura adequado e do uso de co-culturas para o aumento da rentabilidade do processo, apresentando ainda um novo método de extração de biomassa (precipitação acídica).

**Palavras-chave:** *Single-Cell Protein*, comunidades sintéticas, comida sustentável, fonte proteica

# Abstract

Due to the increasing global population and the switch to protein-rich diets, conventional protein sources are unable to meet the demand without further compromising the environment. Single-cell Protein (SCP) production on methanol re-emerges as a potentially more sustainable protein source with high yields. To improve its cost-effectiveness, this thesis focused on batch mono and co-cultivation of the methylotrophs *M. extorquens* and *K. phaffii* with attainment of process yields and biomass characterization, and biomass recovery through a novel acid precipitation assay.

Initial tests to optimize the operational conditions showed the importance of medium composition on microbial growth. A new medium, named Methylotrophic Mineral Medium with Vitamins (MMMV), resulted in up to 8 times more final cell dry weight (CDW) than 3 other established media for these strains at small scale. The same was not seen in the bioreactor, where Ammonium Mineral Salts (AMS) medium provided 3 times higher final CDW of *M. extorquens.* but showed signs of stress through the presence of aggregates (up to 50%) and the potential accumulation of polyhydroxybutyrate (PHB). On the other hand, MMMV-grown bacteria demonstrated little increase of intact cells throughout time. Both AMS-grown and MMMV-grown *M. extorquens* performed poorly in terms of protein content (up to 25%$_{CDW}$) compared to the yeast monoculture (44%$_{CDW}$), with the latter comparing favorably with literature. Although the co-culture exhibited similar protein content (39%$_{CDW}$) and final CDW to the yeast monoculture, it had significantly higher (1.4 times) biomass yield. Through flow cytometry, bacterial and yeast population were distinguished, and it was observed that the presence of yeast aided in bacterial growth. Finally, a biomass acid precipitation test, using formic and hydrochloric acid at an optimal pH range between 2.5 and 4, resulted in biomass recovery values up to 92%$_{CDW}$, almost twice of the amount achieved by natural precipitation (54%$_{CDW}$).

These experiments demonstrated the importance of medium composition for increased cell growth, the potential of using co-cultures to increase process yields and the use of acid precipitation as a promising method for biomass harvesting.

**Keywords:** Single-Cell Protein, synthetic community, nutritional quality, sustainable food

# Declaration

I declare, on my word of honour, that the information provided on this work is original and that all non-original contributions were duly referenced with identification of the source.

Maria Alexandra Pinto Martins Pereira, September, 2021

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# Abbreviations

| | |
|---|---|
| **AMS** | Ammonium Mineral Salts |
| **CDW** | Cell Dry Weight |
| **COD** | Chemical Oxygen Demand |
| **DM** | Dry Matter |
| **MeOH** | Methanol |
| **MMM** | Methylotrophic Mineral Medium |
| **MMMV** | Methylotrophic Mineral Medium with Vitamins |
| **MQ** | Milli-Q |
| **PBS** | Phosphate Buffer Solution |
| **PHA** | Polyhydroxyalkanoate |
| **PHB** | Polyhydroxybutyrate |
| **SCP** | Single Cell Protein |

# Chapter 1

# Introduction

## 1.1 Context

The world's population is increasing at an accelerated rate and is expected to reach more than 9 billion people by 2050 (Kc & Lutz, 2017). At the same time, rising incomes and urban population is taking place, with an expected increase by more than 3 billion people between 2010 and 2050 (Buhaug & Urdal, 2013). These events contribute to high protein demand for human consumption and protein-rich diets (Godfray et al., 2010; Henchion et al., 2017) with production being already insufficient in many parts of the world. The resulting malnutrition, starvation, food insecurity and related disease currently lead up to 12,000,000 deaths every year (Akanni et al., 2014).

Conventional processes of protein production are focused on animal or plant-based proteins. Although vegetal sources currently dominate protein supply worldwide (57%), demand for animal sources is rising (Henchion et al., 2017). For example, global consumption of meat has increased by 60% between 1990 and 2009 and it is expected to continue to rise (Henchion et al., 2014). Production of meat is strongly associated with climate change due to high greenhouse gas (GHG) emissions, chemical inputs, land, and water use. Another important animal protein source is fish, whose sustainability is disputable considering that conventional fishing practices lead to the depletion of wild capture fisheries. The main current alternative is aquaculture, that is growing globally and is expected to be the main source of fish by 2030 (Henchion et al., 2017). However, it depends on fish itself as feed, in the form of the increasingly expensive fishmeal, and alternatives are typically plant-based, such as soy (Tlusty et al., 2017b).

Although plant-based protein is often described as a more sustainable alternative due to lower land use and GHG emissions (Henchion et al., 2017), to meet the high demand for animal feed as well as food, it needs to be intensively produced in industrialized farming systems, leading to deforestation, habitat loss, soil degradation, and pollution as well as socio-economic concerns (Vermeulen et al., 2012). Currently, 19-29% of all anthropogenic GHG emissions come from agriculture practices, which are also responsible for consumption of 70% of freshwater and 30% of ice-free land (Sakarika et al., 2020). Furthermore, the places that have the richest-protein diet often need to import their demand from less developed and geographically distant countries, which further contributes to the environmental and social impact. For example, Europe imports up to 70% of high-quality protein

feed from outside its borders (Buckwell & Nadeu, 2018). If protein demand continues to rise, an increase of 30-60% in global crop production until 2050 is expected (Pikaar et al., 2018), which will have a damaging environmental impact.

Unconventional protein sources currently being considered include insects, that have a lower environmental impact than livestock but raise concerns regarding safe consumption, and *in vitro* meat, which has high energy requirements and still faces many technical challenges (Henchion et al., 2017). Thus, there is clearly a need for alternative protein sources that could be used as food and feed and are sustainably and ethically produced.

## 1.2 Motivation

Single-cell protein (SCP), the biomass of yeasts, bacteria, and microalgae grown on various carbon sources, has become of increased interest as alternative protein sources (Ware, 1977). SCP has a high protein content of between 43-85 % in dry mass, can be grown on waste products, and does not depend on climate conditions, requiring low land use (Upadhyaya et al., 2016) whilst guaranteeing higher productivity than reached by larger biota (Pikaar et al., 2018). Furthermore, it could also be easily genetically modified to meet a specific composition of amino acids (Upadhyaya et al., 2016). However, most commonly used carbon sources for SCP production are sugars or non-renewable methane (i.e. natural gas) (Calysta, 2019) that compromise the sustainability of the product. A promising feed-stock alternative could be methanol, a one carbon ($C_1$) compound that is widely abundant, cheap and can be produced sustainably from industrial by-products such as $CO_2$.

Although SCP production on methanol has been present since the beginning of its history, the production was abandoned due to uncompetitive prices with conventional sources of protein in the oil crisis of the 70s (Bernauer et al., 2021). Recently, interest in this substrate has re-emerged but the research remains scarce, and improvements are still needed when concerning the process itself. To maintain low environmental impact and investment, optimization of the media supplementation and process design are necessary, minimizing waste, and keeping only the essential nutrients while guaranteeing high productivities. The choice of microorganism also influences greatly the success of the process.

Methanol is used by few microorganisms called methylotrophs, such as the bacterium *Methylorubrum extorquens* and the yeast *Komagataella phaffii*. Due to its bigger size, biomass harvesting is easier for yeast strains, making its selection advantageous. On the other hand, bacteria benefit from higher protein content (Ritala et al., 2017). *M. extorquens* specifically can produce added-value products

such as carotenoids (Tlusty et al., 2017b) and polyhydroxyalkanoates (PHA) (Mokhtari-Hosseini et al., 2009).

### 1.3 Objectives

The present thesis focused on the lab-scale production of SCP using methanol as the single substrate for the batch cultivation of *M. extorquens*, *K. phaffii* and the combination of the two species. The aim was to understand the effect of the methanol concentration, temperature, media composition and selected strains on growth yields, protein content and biomass composition. To further analyze the SCP production process, the potential of acid precipitation for harvesting of the biomass was evaluated by performing a small assay on *K. phaffii* culture.

## 1.4 Thesis Organization

This thesis contains 5 more chapters. Chapter 2 is a literature review of the work performed in the project's area of expertise, from the general history and production aspects of SCP to the use of methanol as a promising long-term viable substrate by methylotrophs. Chapter 3 presents the materials and methods. Firstly, cultures and medium used are explained followed by the explanation of the preliminary tests, main reactor experiments and the assay for biomass harvesting from acid precipitation. Finally, analytical techniques for biomass characterization and formulas for yields calculations are explained, as well as the statistical analysis used. Chapter 4 presents the results of the methods and experiments described in the previous chapter. Chapter 5 discusses the experimental data through critical thinking and comparison with previous literature on the subject. Finally, Chapter 6 gives the main conclusions and future perspectives of the work performed.

# Chapter 2

# Literature review

## 2.1 Single-cell protein as alternative feed and food

### 2.1.1 Historical context

Since most microorganisms grow as single or filamentous individual cells (Upadhyaya et al., 2016) microbial protein can be more correctly and preferably referred as "single cell protein" (SCP), a term that was coined in 1966 at Massachusetts Institute of Technology (MIT) to ease public's reaction on related products (Ware, 1977). The use of microorganisms in food production is not a new concept: for thousands of years, biomass has been used to produce consumables such as alcoholic beverages, cheese, or yogurt (Anupama & Ravindra, 2000). However, the use and development of SCP as a direct source of protein was only more recently explored, with the surge of the First (1914-1915) and Second World War (1939-1945), leading Germany to use yeasts as food and feed supplement (Tlusty et al., 2017b).

Although commercialization of SCP was already available in the 60s, limitations regarding the competitive low prices of conventional animal feed, under-developed process technology and efficiency combined with low environmental awareness led to its cease in the 80s. Since these constrains are being tackled by increased awareness and knowledge in bioengineering, SCP production has seen recent renewed interest with products obtained on methane and sugar-based substrates already being commercially available (Pikaar et al., 2018). One of the companies that has maintained consistent production of SCP is Marlow Foods Ltd with its mycoprotein Quorn$^{TM}$, which is approved by certified agencies across the world such as the FDA (Ritala et al., 2017).

### 2.1.2 General production process and considerations

Multiple microorganisms have been grown on natural or waste products through fermentation on an industrial scale, and thereafter harvested and subjected to downstream processing steps that can include washing, cell disruption, protein extraction and purification (Anupama & Ravindra, 2000). In terms of fermentation process design, reactors can be operated in batch, fed-batch or continuous mode. The continuous process offers many advantages due to its steady-state nature, leading to higher productivities, and greater control and monitoring ability (Ugalde & Castrillo, 2002).

No matter the process, the final product needs to be accepted for final consumption as food and feed by meeting five conditions (Tlusty et al., 2017b):

1. It must be accepted by the species
2. It should result in equivalent survival and growth
3. It should not cause illness or other maladies
4. It should result in an organoleptically suitable product
5. It should be economically viable to produce

These conditions widely depend on the application of SCP for either feed or food. SCP has been more commonly used as feed since regulation is tighter for human consumption, which means diversification of substrates and microorganisms used for final product is also higher for animal consumption (Ritala et al., 2017).

Although some experts view SCP use for human consumption as more economically favorable (Ware, 1977), it is important to consider that this product is relatively new and unknown to the general public which greatly impacts its market potential. For example, using waste streams as a substrate for SCP production is recommended for reducing cost and decreasing environmental impact, but the consumer might distrust it further and safe consumption needs to be ensured. Contaminants such as carcinogenic factors may be derived from the substrates used (Anupama & Ravindra, 2000). Since it is also new to the human body, SCP can lead to immunological responses such as nausea, vomiting and allergic reactions (Bratosin et al., 2021). Mitigating these symptoms through purification, decontamination, safety tests and research is essential for approval.

Furthermore, depending on growth conditions and species of microorganism, SCP can contain between 6 to 25% of nucleic acids. Although some animals such as ruminants are able to metabolize nucleic acids, humans do not have uricase in the human intestine to do so (Álvarez-Lario & Macarrón-Vicente, 2010), potentially leading to health problems (e.g. kidney stones). Further treatment is then required to remove or reduce the percentage of nucleic acids in SCP to make it consumable. The reduction should respect the World Health Organization (WHO) limit of 2 g nucleic acid per day for human ingestion of RNA from SCP (Jonas et al., 2001). This can be done through control of growth rate, base-catalyzed hydrolysis, chemical extraction, cell disruption and treatment with exogenous or endogenous enzymes (Ware, 1977). However, choosing the treatment should consider its cost and the effect it can have in product quality.

A common problem for feed and food usage is the presence of toxins in SCP, particularly in fungal and bacterial SCP, that should be below the allowed toxicity level in each case (Litchfield, 1980). The removal of toxins depends on its type: mycotoxins (produced by filamentous fungi) can be removed through ammoniation (Norred et al., 1991); bacterial toxins, which can be either exotoxins,

easily removed through heat or chemical denaturation, or endotoxins, harder to remove, generally requiring genetic engineering to inactivate the toxin-producing gene (Anupama & Ravindra, 2000).

Genetic engineering could also be performed to improve the nutritional value of SCP, broaden the substrates that a strain can metabolize, or facilitate the downstream processing. For example, in cases where the cell wall needs to be degraded, enzymes could be produced. However, approval of GMOs is a long process that has yet to meet public acceptance in places like Europe (Ritala et al., 2017).

### 2.1.3 Environmental impact of SCP production

In general, SCP production can have a protein yield up to 10 times higher than that of meat production (Anupama & Ravindra, 2000) with less resource usage and waste. Similarly, SCP marketed as FeedKind (Unibio, 2016) has recently demonstrated a production process from natural gas resulting in high volumetric productivities (3–4 kg SCP dry matter (DM) per $m^3$ reactor volume per hour) by continuous cultures of *Methylococcus capsulatus*, with a smaller physical footprint by a factor of 1000 than conventional vegetable protein production systems (Strong et al., 2015).

In terms of water use, SCP needs 77% to 98% less water than conventional agricultural feed ingredients, including soybean (Cumberlege et al., 2016). Regarding land use, the powdered SCP requires no arable land and the pelletized form requires an estimated 0.052 $m^2$/kg protein due to the vegetable oil used (Matassa et al., 2016). This is 128 times less than soybean land requirements (Cumberlege et al., 2016) and up to 44 thousand times less than beef (Wiedemann et al., 2015).

Microorganisms can also use nutrients from waste sources (nitrogen, organic carbon, phosphorus) which can enable a circular economy (Matassa et al., 2016) and decrease the high disposal costs (Ritala et al., 2017). Estimations show that by 2050, SCP could replace 10-19% of conventional crop-based animal feed protein, resulting in a decrease of global cropland area (6%), global nitrogen losses from croplands (8%) and greenhouse gas emissions (7%) (Pikaar et al., 2018).

Nitrogen pollution is an especially big concern since fertilizers are an expensive part of the farming process and plant-soil systems have low nitrogen uptake efficiency (Dimkpa et al., 2020). For example, the nitrogen uptake efficiency of SCP in feed is 33% higher than observed in conventional agriculture. Concerning SCP in food, the efficiency is higher by 39% (Pikaar et al., 2017).

### 2.1.4 Economic impact of SCP production

SCP production is gaining attention not only due to its low environmental impact but also from an economic point of view. Conventional protein sources are becoming more expensive which means there is space for a cheaper alternative. For example, aquaculture currently depends on fishmeal whose price rose from about $500 per ton in the 1990s to $2500 in recent years (Matassa et al., 2016).

However, when looking into the total product cost, capital investment and profitability, SCP production still struggles to be economically competitive. Generally, raw material costs range from 35-55% of the manufacturing costs and operation costs (e.g. labor, energy and consumables) represent 45-55% (Aggelopoulos et al., 2014). One solution to this problem lays in utilizing waste and side-streams as substrates, taking into consideration possible regulatory issues regarding human consumption. These streams are often used to produce biomass for use as a fertilizer in order to reduce the costs of waste disposal. However, SCP is a generally more preferred end-product (Ritala et al., 2017). The operation variables should also be optimized to increase the substrate to biomass conversion efficiency and decrease nutrient waste, using strategies such as recycling (Goldberg, 1985) or considering pH strategies to reduce harmful products usage. Finally, operation mode has also been demonstrated to play a role in profitability, with continuous design being considered the most profitable (Bratosin et al., 2021).

### 2.1.5 Parameters for evaluation of SCP quality

One of the many parameters to evaluate the quality of SCP is the amino acid profile. Although dependent on the conditions and sources used for production, it has shown similar amino acid profiles compared to conventional sources of feed as well as the FAO/WHO standard for human nutrition (Matassa et al., 2016). Figure 1 shows the amino acid profile of various conventional protein sources as well as microorganisms grown on methanol.

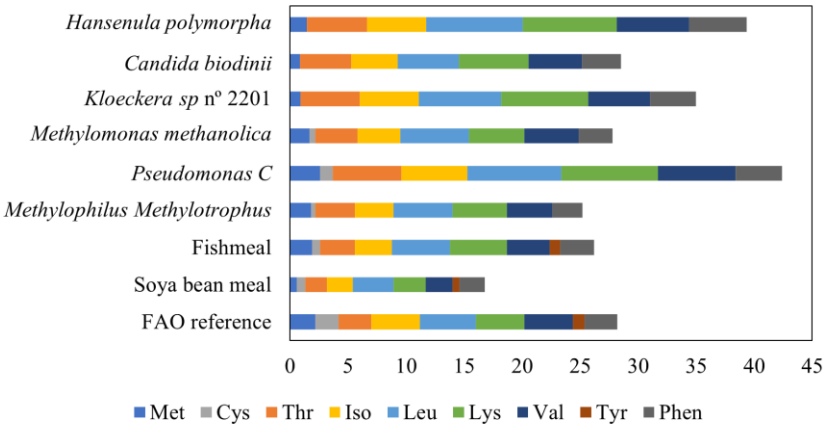


**Figure 1:** Essential amino acid profiles recommended by the Food and Agriculture Organization of the United Nations (FAO), and for conventional protein sources and SCP (g/100 g protein or g/16gN) (Abou-Zeid & Baghlaf, 1983; Goldberg, 1985).

Apart from the amino acid profile, another two parameters for feed quality evaluation stand out: digestibility and protein efficiency ratio (PER). Digestibility is expressed as a percentage and reported values range from 65-96% (Anupama & Ravindra, 2000). When compared to conventional sources such as fishmeal and soybean meal, one feeding trial tested on Atlantic Salmons has shown that bacterial SCP has similar values of digestibility (Storebakken et al., 1998). On the other hand, PER

expresses the weight gain per unit of protein consumed and typically ranges between 0.6 to 2.6 (Anupama & Ravindra, 2000), which compares favorably to reported values for plant proteins (e.g., soy protein, beans) ranging between 1.2–2.4 (Mariotti, 2017).

For human consumption, other parameters can be used to evaluate its nutritional value, such as Protein Digestibility-Corrected Amino Scores (PDCAAS) or the digestible indispensable amino acid scores (DIAAS). FAO and WHO recognized the latter as being more accurate to assess protein quality since it uses ileal digestibility, based on the digestibility of each AA individually, instead of fecal digestibility, based on the digestibility of nitrogen (Mathai et al., 2017). However, only studies on PDCAAS of mycoprotein have been reported, indicating a value of 91%, fractionally behind beef at 92% (Gilani & Lee, 2003).

### 2.1.6 Choice of microorganism

The choice of microorganism used for SCP production is one of the most important aspects and it should be made according to the following characteristics: high specific growth rate and biomass yield, high affinity for the substrate, low nutritional requirements, ability to utilize the substrate of interest, ability to develop high cell density, capacity for genetic modification, broad range of temperature and pH conditions for growth, required nutritional value with balanced protein and lipid composition, low nucleic acid content, good digestibility and no toxic compound generation (Upadhyaya et al., 2016). A summary of the characteristics of different microorganisms used for SCP production is shown in Table 1.

**Table 1:** Main characteristics and composition of microorganisms used for SCP production.

| **Microorganism** | **Bacteria** | **Yeast** | **Filamentous Fungi** | **Microalgae** | **Reference** |
|---|---|---|---|---|---|
| **Growth rate** | Highest | High | Lower than bacteria and yeast | Low | (Anupama & Ravindra, 2000) |
| **Substrate** | Wide range and low-cost | Wide Range and low-cost except carbon dioxide | Mostly lignocellulosic | Light, carbon dioxide or inorganic samples | (Anupama & Ravindra, 2000) |
| **Protein content** | 50-80% | 45-55% | 30-50% | 60-80% | (Ritala et al., 2017; Underkofler et al., 1976) |
| **Nucleic Acid Content** | 8-12% | 6-12% | 7-10% | 3-8% | (Underkofler et al., 1976) |
| **Fat Content** | 1-3% | 2-6% | 2-8% | 7-20% | (Underkofler et al., 1976) |
| **Ash Content** | 3-7% | 5-10% | 9-14% | 8-10% | (Underkofler et al., 1976) |
| **Toxin** | Endotoxins from gram negative bacteria | - | Mycotoxins | - | (Anupama & Ravindra, 2000) |

Bacteria possess many advantages due to their generally high specific growth rate in multiple substrates. The amino acid content is expected to be comparable or higher than the FAO recommendations and methionine content is higher than obtained in algal or fungal SCP (Ritala et al., 2017). Bacterial SCP could potentially contain lipids and vitamins from the B group. However, utilization for human consumption requires sterility and intensive screening due to many of these species being pathogenic. Other disadvantages include the high nucleic acid content, meaning further treatment is necessary, and difficult harvesting process due to their small size (Upadhyaya et al., 2016). Large quantities of oxygen are also required, which can account for a large part of the production cost and is also a common problem in yeasts (Ware, 1977).

On the other hand, yeast is the best studied and more accepted by consumers SCP due to its common use as baker's yeast. For this reason, it has a global production volume of 3 ,000,000-ton dry matter (DM)/year with 9.2 billion euro market value, growing yearly at 7.9% (Matassa et al., 2016). Due to their larger size, the separation process is easier, but their amino acid profile often is low on sulphated amino acids, such as methionine. The ability of yeasts to grow in acidic conditions can inhibit bacterial contamination since bacteria grow generally between a pH of 5-7 (Upadhyaya et al., 2016).

Other well studied microorganisms are microalgae that are currently used in form of supplements and in processed products, such as snacks and pastas, but primarily used for animal nutrition in aquaculture due to their fatty acids and carotenoid content (Ritala et al., 2017). Currently, global production is at 9,000-ton DM/year with a market value of 2.4 billion euro with a projected yearly growth of 10% (Matassa et al., 2016). Unlike other microorganisms, they do not need to be grown with organic substrates and need to be supplied with $CO_2$ in either outdoor ponds or indoor photobioreactors, being either prone to contamination or in need of expensive artificial light (Blanken et al., 2013). Lastly, the cell wall of microalgae is non-digestible for humans, which means treatment is necessary to disrupt it. However, they offer a big advantage in their low nucleic acid content and their nutritional value, providing vitamins (A, B, C, D and E), mineral salts, and chlorophyll (Ritala et al., 2017).

Lastly, fungi can also be used for SCP but have a low growth rate compared to yeasts and bacteria, are easily contaminated, and can produce toxins (Ware, 1977). Although they have a generally favorable amino acid profile, methionine content and total protein content are often low (Ritala et al., 2017).

### 2.1.7 Choice of substrate

Initially, by-products from the petrochemical industry, such as higher paraffins, were used as substrates to decrease the disposal cost of waste (Ware, 1977). However, due to the rising costs of these non-renewable substrates and the low price of conventional protein sources (Øverland et al., 2010), the interest in SCP production decreased significantly. Currently, as the protein scarcity is starting to be a concern, there is increased interest in using renewable substrates such as cellulosic and non-cellulosic carbohydrate wastes (e.g. starch, molasses, fruit and vegetable wastes) (Ware, 1977). Many of these wastes need expensive pre-treatments, can be seasonal and often come from the cultivation of crops, which pose difficulties on its sustainable and reliable use.

The ideal substrate should be available at high quantities in pure form, be low in cost, oxygen requirements toxicity and versatility (growth of a limited number of microorganisms to decrease probability of contamination), be miscible in water, easy to store and handle, and lead to high biomass yields (Ware, 1977). Finally, consumer acceptance of the substrate also plays an important role.

## 2.2 Methanol as a promising substrate for SCP production

Methanol is gaining interest in the biochemical industry, not only for SCP production but also for other compounds such as polyhydroxyalkanoates (Yang & Zhang, 2018). It is abundant, has a low price and has higher purity than other sustainable low-cost feedstocks like molasses, which makes it an attractive alternative. It can be supplied with inexpensive, defined media and simple nitrogen sources (e.g. ammonia) which further reduce the downstream processing costs (Cotton et al., 2020). Furthermore, a limited amount of microorganisms can use it as a carbon and energy source (Yang & Zhang, 2018), known as methylotrophs, which significantly lower the risk of contamination.

Since it is a liquid compound, it also presents advantages to more widely studied and used C1 gas compounds (e.g. methane, carbon monoxide and hydrogen/$CO_2$) due to its complete miscibility in water, potentially leading to increased productivity, and ability to be easily handled and store (Cotton et al., 2020). Furthermore, although the chemical composition of bacterial meal produced on methane and methanol are similar, it seems that methanol supports higher amino acid digestibility than methane (Øverland et al., 2010).

However, it is important to keep in mind the disadvantages of methanol when using it as a substrate. Firstly, methanol assimilation by microorganisms leads to the formation of intermediate products, formaldehyde, and hydrogen peroxide, that can accumulate and reach toxic concentrations (Pfeifenschneider et al., 2017), leading to the inactivation of proteins and other macromolecules (Cotton et al., 2020). There is no defined threshold for non-toxic methanol concentration since it

varies between microorganisms, but reports suggest an optimal concentration range between 0.3-2% (v/v) (Cotton et al., 2020; Kim et al., 2003). Concentrations above 4-6% can completely inhibit the growth of microorganisms (Cotton et al., 2020). Therefore, it is important to choose the appropriate feed concentration of methanol. Furthermore, methanol is more reduced than sugars which means the aerobic process has a high oxygen demand, leading to considerable heat production. Reactor cooling is then necessary which is often expensive (Pfeifenschneider et al., 2017).

Another important aspect is the production of methanol itself. As stated before, the sustainability of the substrate is an important aspect when choosing a feedstock. Currently, around 90% of methanol is industrially produced through syngas (synthetic gas) (Frazão & Walther, 2020), which originates from feedstocks such as coal, oil, and natural gas. However, syngas can also be produced from renewable sources such as biogas and agricultural wastes. Therefore, the former is expected to increase in demand (Dimian et al., 2019).

Interest is growing in producing methanol from $CO_2$ through hydrogenation in electrolysis processes. This would be a more sustainable route of production. $CO_2$ is naturally available in the atmosphere and as a by-product of many bio and chemical processes (e.g. bioethanol production, exhaust of factories) which make its capture and usage of great environmental and economic interest. $H_2$ is harder to produce but it can be done sustainably through the use of renewable electricity for water electrolysis (Dimian et al., 2019).

Producing methanol from $CO_2$ can be done through two different pathways but both require the catalysts based on $CuO/ZnO/Al_2O_3$. $CO_2$ can be directly hydrogenated to methanol through the following equation (Simoes Van-Dal & Bouallou, 2013):

$$CO_2 + 3H_2 \leftrightarrow CH_3OH + H_2O \ (\Delta H = -87 \frac{\text{kJ}}{mol}) \ (eq.1)$$

Another way is by converting $CO_2$ firstly to CO via reverse water gas shift (RWGS, equation 2), and only then to methanol (equation 3) (Simoes Van-Dal & Bouallou, 2013).

$$CO_2 + H_2 \leftrightarrow CO + H_2O (\Delta H = +41 \frac{kJ}{mol}) \ (\text{eq. } 2)$$

$$CO + 2H_2 \leftrightarrow CH_3O \ (\Delta H = -128 \frac{\text{kJ}}{mol}) \ (eq. \ 3)$$

The main difference between the processes is the water content, which is higher in the first pathway described. Water adsorbs on the active site of the catalysts, inhibiting adsorption of $CO_2$. By separating the conversion of $CO_2$ to methanol, it is possible to remove the water after the RWGS step, lowering the amount of purge gas needed and increasing methanol yield. This makes the process more economically competitive with the production of methanol from syngas (Joo et al., 1999).

## 2.3 Methylotrophy

Methylotrophs are microorganisms that can use reduced carbon substrates as energy and carbon source. These substrates include not only one-carbon methanol or methane but also multi-carbon compounds that contain no carbon-carbon bonds such as methylated amines (Schrader et al., 2009).

### 2.3.1 *Methylorubrum extorquens*

*Methylorubrum extorquens*, previously known as *Methylobacterium extorquens* is a methylotrophic α-proteobacterium (Lim et al., 2019). *M. extorquens* AM1 has been the most studied and understood methylotroph since its use for biotechnology purposes, including production of SCP, in the 1960s and is considered as a platform for biofuel production and development of tools for metabolic engineering (Hu & Lidstrom, 2014).

Through its metabolism, methanol is oxidized firstly into formaldehyde in the periplasm by methanol dehydrogenase. This compound can either follow a dissimilation pathway, in which successive oxidations lead to the formation of formate and final conversion to $CO_2$, or an assimilation pathway, in which it is reduced to methylene-tetrahydrofolate and assimilated in the serine cycle, where it condenses with glycine to form serine (Schrader et al., 2009). The intermediates of this cycle are partially deviated for biosynthesis. For this reason, glycine can only be partly regenerated from the serine cycle, with the remaining being regenerated from acetyl-CoA through the central pathway known as ethylmalonyl-CoA pathaway (Figure 2) (Hu & Lidstrom, 2014; Schrader et al., 2009).

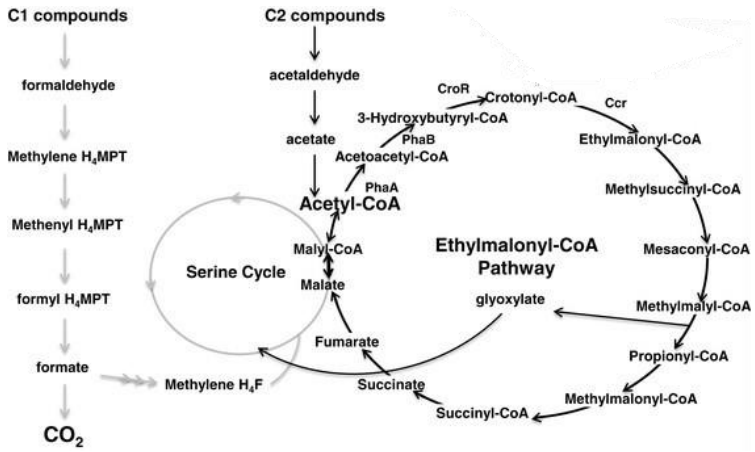


**Figure 2:** Methanol metabolism by *M. extorquens*. Adapted from: (Hu & Lidstrom, 2014).

*M. extorquens* is pink-pigmented due to its naturally produced carotenoid compounds. These compounds offer many advantages: they are precursors of vitamin A, may enhance immunity due to antioxidant properties and are able to provide color. Carotenoids are already widely used in aquaculture feed for coloring and are generally expensive. Furthermore, since most are synthetically

produced, their usage is restricted in certain jurisdictions, such as in the European Union (Tlusty et al., 2017b). Therefore, *M. extorquens* emerges as a biotechnologically interesting organism not only for nutritional purposes but also for coloring and pharmaceutical applications. Finally, it is important to mention that other valuable products can be obtained when using this bacterium, such as polyhydroxybutyrate (PHB), a component belonging to the group of PHA, intracellular storage compounds which serve as a carbon and energy reserve. Due to its biodegradability and similarities to polypropylene, it is often described as a sustainable alternative to petrochemical plastics (Mokhtari-Hosseini et al., 2009; Orita et al., 2014). It can also serve as prebiotics in aquaculture (De Schryver et al., 2010) .

### 2.3.2 *Komagataella phafii*

*Komagatella phaffii*, previously known as *Pichia pastoris*, is a facultative methylotrophic yeast that has gained popularity over the years for biotechnological applications in food, feed and pharmaceutical industries. Compared to other widely popular yeasts, such as *Saccharomyces cerevisiae*, *K. phaffii* has great thermo- and osmo-tolerance (Bernauer et al., 2021), is able to growth to very high cell densities in a simple defined medium, has strongly inducible promoters and commercially available methods, host strains and expression vectors for genetic manipulations (Zhang et al., 2000). For these reasons, *K. phaffii* is a promising choice for SCP.

As a methylotrophic yeast, *K. phaffii* assimilates methanol through the methanol utilization (MUT) metabolic pathway (Figure 3) (Bernauer et al., 2021). In this pathway, methanol is firstly oxidized to formaldehyde and hydrogen peroxide in the peroxisomes by alcohol oxidase (AOX). AOX is encoded by two genes, AOX1 – responsible for 85% of the enzyme activity in the cell - and AOX2 (Zhang et al., 2000). This step requires oxygen as an electron acceptor which makes *K. phaffii* an aerobic methylotroph. AOX1 has low affinity for oxygen which means oxygen requirements must be ensured.

The hydrogen peroxide formed in this step is decomposed to water by a catalase, another essential enzyme to prevent toxic concentrations of this compound in the peroxisome. Both catalase and AOX enzymes are the main components of the peroxisomes, large microbodies that prevent the harmful effect of hydrogen peroxides. These structures are destroyed in presence of other substrates such as glucose and ethanol since these enzymes are not present (Zhang et al., 2000).

After this step, two pathways can be followed. In the dissimilatory pathway, some of the formaldehyde produced enters the cytosol where it is oxidized by formaldehyde dehydrogenase, producing formate. Through a series of consecutive steps, the formate is completely oxidized to carbon dioxide for energy generation. In the assimilatory pathway, the remaining formaldehyde stays

in the peroxisome and reacts with xylulose-5-phosphate, forming dihydroxyacetone and glyceraldehyde-3-phosphate. These compounds enter the cytosol and follow a cyclic metabolism, regaining xylylose-5-phosphate and forming glyceraldehyde-3-phosphate for central metabolism (Yurimoto & Sakai, 2019).

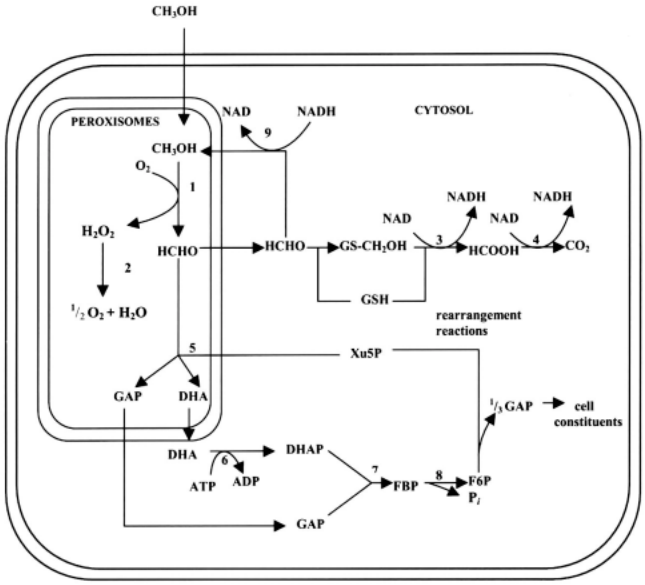


**Figure 3:** Methanol metabolism pathways in *K. phafii*. 1) alcohol oxidase, 2) catalase, 3) formaldehyde dehydrogenase, 4) formate dehydrogenase, 5) dihydroxyacetone synthase, 6) dihydroxyacetone kinase, 7) fructose 1,6-bisphosphate aldolase, 8) fructose-1,6-bisphosphotase, 9) formaldehyde reductase. Taken from: (Zhang et al., 2000).

## 2.4 Methylotrophic SCP production

SCP production using methylotrophs has been present since its early history, particularly on methanol (Table 2). The methylotrophic yeast *K. phaffii* was firstly cultivated by Philips Petroleum Company in the early 1970s until the production was no longer economically viable due to the oil crisis that led to the consequent rise in price of methanol (Bernauer et al., 2021). Similarly, Imperial Chemical Industries used the bacterium *Methylophilus methylotrophus* for pig feed production, but it was unable to compete with conventional protein sources.

Early SCP feeding trials were done on pigs and resulted in high amino acid digestibility (Braude & Rhodes, 1977; D'Mello et al., 2007), and on broiler chicks, with varied effects on feed intake, conversion, and growth rates (Øverland et al., 2010). More recently, KnipBio Meal, the SCP from *M. extorquens*, received positive reviews from feeding trials. *Tlusty et al* (2017) tested feed supplemented with 30, 55 and 100% of KnipBio Meal on shrimps and fish and the digestibility, growth and health were equivalent to standard aquaculture diet for all conditions. Another study of soybean feed containing 5% or 10% KnipBio meal supplied to fish observed no statistically significant differences in final fish weight and nutrient retention indices were similar to standard diet (Hardy et al., 2018).

**Table 2:** Industrial applications and patents related to SCP production on methanol.

| Microorganism | Organization | Type | Production Process Remarks | Reference |
|---|---|---|---|---|
| ***K. phaffii*** | Phillips Petroleum Company | Industrial Process | Biomass: 130 g (CDW)/L Productivity: 10 g $L^{-1}$ $h^{-1}$ | (G. H. Wegner, 1990) |
| ***Methylophilus methylotrophus*** | Imperial Chemical Industries (Pruteen) | Industrial Process | Protein content: 70% Production capacity: 50,000-75,00 t/y | (Ritala et al., 2017) |
| ***M. extorquens*** | KnipBio (KnipBio Meal) | Industrial Process | NA[a] | (Ritala et al., 2017) |
| ***Methylomonas clara*** | Hoeschst-Uhdegelsenberg (Probion) | Discontinued Industrial Process | Production capacity 1000 t/y | (Wagner & Sahm, 1976) (Goldberg, 1985) |
| ***Methylomonas methanolica*** | Norsk Hydro and Ab Marabou (Norprotein) | Discontinued Industrial Process | Capacity 4500 liter Protein content: 60-70% | (Goldberg, 1985) |
| ***Hansenula polymorpha*** | Nanjing U. of Technology | Patent | Protein content: 68% | (Min et al., 2016) |
| ***K. phaffii*** | Coal Biochenmical High Tech Engineering Co. Ltd. | Patent | Protein content: 55% | (Guohou et al., 2013) |
| ***Methylovorus glucosotrophus*** | Henan Coal Chemical Ind. Group Inst. Co | Patent | Protein content: up to 77% | (Yanping et al., 2013) |

[a]NA – Not available information

# Chapter 3

# Materials & Methods

## 3.1 Pure cultures

*Methylorubrum extorquens* DSM 1338, previously known as *Methylobacterium extorquens*, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and *Komagataella phaffii* ATCC 76273 from the American Type Culture Collection (ATCC, Virginia, United Sates). Both were stored at -80°C prior to use.

## 3.2 Media and substrate

The following media were used during the experiments: Ammonium Mineral Salts (AMS), Methylotrophic Mineral Medium (MMM), Methylotrophic Mineral Medium with Vitamins (MMMV), DSMZ1629 and Nutrient Broth (NB). NB was only used as pre-culture growth medium. The full recipes for these media can be found in Appendix A.1.

AMS, MMM and MMMV were prepared twice concentrated through a combination of stock solutions that were autoclaved separately to prevent mineral precipitation, before being cooled down for use. The stock solutions for AMS were salts stock, FeNaEDTA stock, a basic buffer, an acid buffer and a trace metals solution, whereas for MMM, stocks solutions of phosphorus, nitrogen source, calcium chloride solution, magnesium chloride solution and trace metals solution were prepared. AMS and MMMV specifically contained vitamins which were filtered (0.2 μm Minisart® filters, Sartorius Stedim Biotech) instead of autoclaved. DSMZ1629 was also prepared twice concentrated directly with all components before being autoclaved.

The carbon source used was methanol (MeOH), which was prepared as a 50% stock solution (v/v) by diluting MeOH (≥ 99.9%, Carl Roth) in Milli-Q water using volumetric flasks. Before use, it was filter-sterilized (0.2 μm Minisart® filters, Sartorius Stedim Biotech) before diluting it for the intended concentration in autoclaved demi-water.

## 3.3. Culture Preparation

The pure cultures were firstly grown in plates of either Sabouraud Dextrose Agar (SDA, Appendix A.1.1), for the yeast, or Nutrient Agar (NA, Appendix A.1.2) + 1% MeOH solution, for the bacterium.

The platted cultures were grown at 28 ºC and then stored in the fridge at 4 ºC for later use after sufficient growth was achieved.

Throughout the experiments, adjustments were made on the preparation of the pre-cultures and inoculum to minimize biomass losses and the effect of different carbon sources/media used compared to the conditions in the final media. However, in all cases *M. extorquens* and *K. phaffi* were grown individually through single-colony inoculation from the plates (2 days, 28ºC), optional recultivation (2 days, 32ºC) and washing (5 min, 5000 rpm) with phosphate buffer solution (PBS, Sigma-Aldrich), and adjustment of OD to final value.

Table 3 shows how this preparation was accomplished for different experiments. It is important to note that all pre-cultures grown between 4-20 mL were done using tubes, and above 20 mL using Erlenmeyer flasks.

**Table 3:** Summary of culture preparation for preliminary tests and bioreactor batch experiments.

| **Experiment** | **Microorganism** | **Medium** | **Replicate** | **Pre-cultures** | | **Inoculum** | |
|---|---|---|---|---|---|---|---|
| | | | | **Inoculation** | **Recultivation** | **Wash** | **Initial OD** |
| **Preliminary tests** | *M. extorquens; K. phaffii* | AMS | All | 4 mL of NB+1%MeOH (v/v) | 0.4 mL+3.6 mL NB+1%MeOH (v/v) | Yes | 0.825 |
| | | MMMV | All | 20 mL MMMV+0.5% MeOH (v/v) | - | No | 1.5 |
| | | AMS, MMM, MMMV, DSMZ1629 | All | 20 mL NB+1%MeOH (v/v) | - | Yes | 0.260 |
| | | AMS, MMM | All | 4 mL of NB+1%MeOH (v/v) | - | No | 0.130 |
| **Bioreactor experiments** | *M. extorquens* | AMS | 1 | 4mL of NB+1% MeOH (v/v) | 200 mL of NB+1%MeOH (v/v) | Yes | 0.825 |
| | | | 2 | | | | |
| | *K. phaffii* | MMMV | 1 | 100 mL AMS+0.2% MeOH(v/v) | 100 mL MMMV + 0.5%MeOH (v/v) | Yes | 1.5 |
| | | | 2 | 4 mL NB+1%MeOH (v/v) | 100 mL MMMV + 0.5% MeOH (v/v) | No | |
| | *M. extorquens* | MMMV | 1 | 4-10 mL of MMMV+ 0.5%MeOH (v/v) | | | |
| | | | 2 | | | | |
| | *M. extorquens; K. phaffi* | MMMV | 1 | | | | |
| | | | 2 | | | | |

## 3.4 Preliminary Tests

### 3.4.1 Plate preparation

Sterile 96-well plates were prepared by filling the outer wells with 200 μL of PBS to prevent evaporation. The inner wells were prepared also for a total volume of 200 μL, with 50% (v/v) of twice concentrated medium, 40% (v/v) of MeOH and 10% (v/v) of either inoculum or PBS for control.

### 3.4.2 Flask preparation

Autoclaved 250 mL Erlenmeyer flasks were used to grow the cultures prior to the experiments. A final working volume of 110 mL was used by adding 50 mL of twice concentrated medium, 50 mL of MeOH and 10 mL of inoculum was added.

### 3.4.3 Effect of temperature on microbial growth and protein content

Two tests were performed on 96-well plates to determinate the optimal temperature for growth (based on OD) and protein production (based on protein content) obtained for *M. extorquens, K. phaffi* and the combination of the two strains. Each condition, including the control, had fifteen replicates.

The first experiment was performed using AMS and 2 temperatures: 28 and 30ºC. The second experiment used MMM medium and three temperatures: 28, 30 and 32ºC. This was based on optimal growth temperatures for the strains used: 28 ºC for *K. phaffii* and 30 ºC for *M. extorquens*, according to the product sheet of the suppliers. In both cases, the final concentration of MeOH was 0.2%(v/v).

Over the course of 3 days, OD was measured and, at the end of the experiment, pH and protein content were also measured.

### 3.4.4 Effect of methanol concentration on microbial growth and protein content

To determinate non-toxic MeOH concentrations to be used in the bioreactor experiments, three 96-well plates were incubated at 32°C with either *M. extorquens, K. phaffii* or their combination with different concentration tested: 0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 0.7, 0.9 and 1% (v/v), based on previous literature review on MeOH toxicity, as mentioned in Section 2.2. Each condition had 4 replicates and 2 respective controls. Plates were analyzed as described in the previous section.

### 3.4.5 Effect of medium composition on microbial growth

To test the best-performing medium to use in the bioreactor experiments, four media were tested for microbial growth as described below: AMS, MMM, MMMV and DSMZ1629. AMS was chosen based on previous research at CMET (Sakarika et al., 2020). MMM is an optimized medium for *M. extorquens* AM1 as described in (Delaney et al., 2013) without the PIPES buffer, due to lack of availability. Since vitamins promote yeast growth (Matthews et al., 2018), another MMM with

additional vitamin solution was used. Finally, DSMZ1629 is a minimal mineral medium that has also shown positive results for *M. extorquens* AM1 (Belkhelfa et al., 2019).

A first experiment was done by inoculating *M. extorquens* and *K. phaffii* individually in triplicated flasks containing either AMS or MMM medium with a final MeOH concentration of 0.2% (v/v). These flasks were incubated at 32 ºC on a continuously rotating plate (120 rpm), while OD and pH were followed for 85 hours by sterile sampling of 1-2 mL of culture.

Another experiment was performed on a 96-well plate testing all four media mentioned above, with each condition having four inoculated replicates for *M. extorquens* and *K. phaffii*, and three controls. The final concentration of MeOH in each well was 0.4% (v/v). The plate was incubated at 30 ºC and OD continuously monitored for the following three days. At the end of the experiment, pH was also measured.

## 3.5 Bioreactor batch experiments

To determinate biomass growth, SCP composition and quality throughout time, batch experiments using *M. extorquens* and *K. phaffii*, individually and combined, were done on a 2 L Biostat® B (B. Braun Biotech International, Germany) bioreactor with a working volume of 1 L. Each experiment was done in duplicate.

### 3.5.1 Bioreactor set-up

The Biostat® has a heating jacket incorporated in double glass, which was connected to a warm water bath to keep temperature inside at 30 °C. The pH was controlled through the Biostat® control unit that was set automatically to add base when the pH got lower than around 7.00, using a pH electrode for measurement and a peristaltic pump for base addition (5 M NaOH) from a graduated cylinder. This enabled reading of volume used for pH correction. Antifoam was also initially added and when considered necessary through visual observation.

Oxygen was first filter-sterilized through a Millipore 0.2 μm air filter before being supplied at 2 vvm through a ring sparger at the bottom of the reactor. Finally, mixing was set at 500 rpm using internal impellers. An extra bottle was also connected to the Biostat® to prevent overpressure. The complete setup is shown in Figure 4.

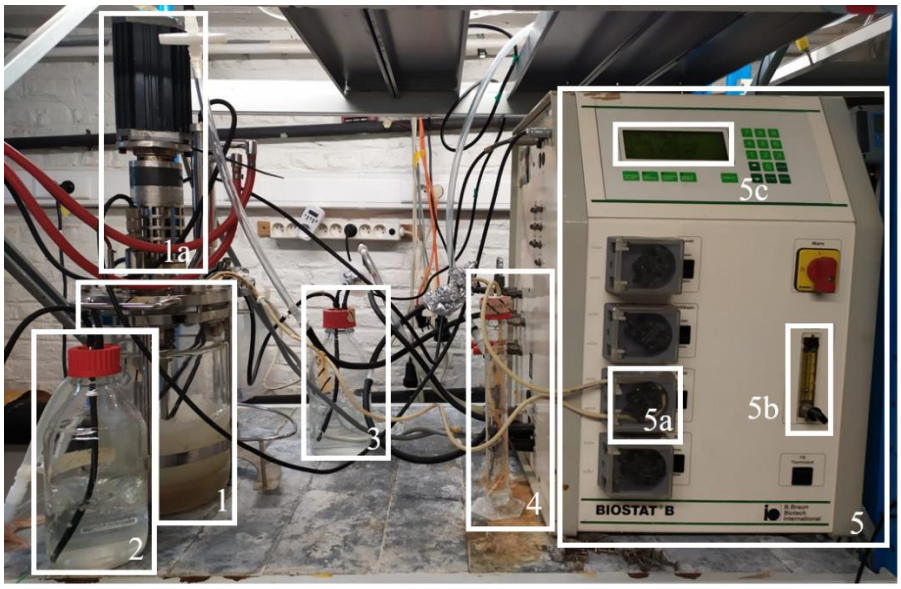


**Figure 4:** Reactor set-up. (1) is the Biostat® with (1a) being the mixer. (2) is the aeration bottle, (3) an auxiliary venting bottle and (4) the cylinder containing the base. (5) shows the Biostat® regulation device with (5a) being the peristaltic pump for base addition, (5b) the airflow regulator and (5c) the display.

The final medium for these experiments contained 50% (v/v) of either AMS or MMMV (twice concentrated), 40% (v/v) of MeOH with a final concentration of 0.5%, and 10% (v/v) of inoculum. Addition of the final medium was accomplished through pumping to the reactor at the start of the experiment.

### 3.5.2 Sampling conditions

The bioreactor experiments were started in the afternoon (18h), when the first sample was taken, and consequent samples were taken the following 2-3 days at 8h30, 13h30 and 18h to obtain the full growth curve until stationary phase was reached. Through the Biostat® controller, it was possible to measure the partial pressure of $O_2$ ($pO_2$) and the pH at these timepoints. Immediately after sampling around 10 mL of culture from the bioreactor, the $pH_{out}$ and OD at 600 nm were also measured. 4 mL of these sample were then centrifuged (5 min, 5000 rpm) in 2 mL Eppendorf tubes to obtain the supernatant, that was filtered (0.2 µm PVDF filters, Chromafil®) and stored at 4 ºC for further analysis of organic acids, anions, cations, and control values for protein content. For the flow cytometry analysis, only the samples at 8h30 and 18h were measured, according to section 3.8. The remaining sample was stored also at 4 ºC for protein, amino acid and carbohydrates analysis.

## 3.6 Biomass harvesting through acid precipitation

To harvest the biomass from *K. phaffii* obtained throughout the experiments, an acid precipitation assay using formic acid (HCOOH, 95%) and hydrochloric acid (HCl, 5 M) was done. 200 mL of culture were set at pH 2.5, 3, 3.5, 4 and 4.5 with either formic acid or HCl (Table 4). A control with

Milli-Q (MQ) water was also performed. The bottles were divided in four 50 mL falcon tubes (4 replicates) and OD in each (600 nm) was followed for 3 hours using plate readers.

**Table 4:** Volume of acid solutions added to culture of *K. phaffii*.

| **pH** | **HCl bottle** | | **HCOOH bottle** | | **MQ bottle** |
|---|---|---|---|---|---|
| | $V_{HCl}$ (µL) | $V_{MQ}$ (µL) | $V_{HCOOH}$ (µL) | $V_{MQ}$ (µL) | $V_{MQ}$ (µL) |
| **2.5** | 354 | 590 | 945 | 0 | 945 |
| **3** | 200 | 0 | 155 | 45 | 200 |
| **3.5** | 140 | 0 | 63 | 77 | 140 |
| **4** | 110 | 0 | 25 | 85 | 110 |
| **4.5** | 90 | 0 | 15 | 75 | 90 |

## 3.7 Analytical Techniques

### 3.7.1 OD

$OD_{600}$ was measured for the samples taken from the bioreactor using a Spectronic™ 200 spectrophotometer (Thermo Fisher Scientific, Belgium). Samples from the precipitation assay were measured using Tecan's Infinite® f50 plate reader whereas the samples from the 96-well experiments were measured using an Infinite® 200.

### 3.7.2 pH

Throughout the experiments, the pH was measured using a Consort C532 pH meter.

### 3.7.3 Calibration curve correlating OD with TSS and VSS

To obtain the total suspended solids (TSS) and the volatile suspended solids (VSS), which are expressed in cell dry weight (CDW) per volume of growth medium, a calibration curve was done to link the OD measured during the sampling with these two parameters, based on (APHA, 1992). This calibration curve decreases the workload of measuring TSS and VSS for every sample individually.

The OD was firstly measured at 600 nm ($OD_{600}$) in two different ways: in duplicated 1,5 mL cuvettes, and in triplicated wells in a 96-well plate. Afterwards, TSS was measured based on the portion of total solids that are retained on a glass-fiber filter of 0.7 µm nominal por size after drying at 105 ºC, and VSS as the portion which are retained on the filter after drying at 550 ºC.

### 3.7.4 Protein content

Total (crude) protein was obtained by using Pierce™ BCA Protein Assay Kit (ThermoScientific). Non-sterile 96 well plates were used for this procedure. 25 µL of each standard solution or unknown sample was transferred to a well and analyzed in triplicate. The standard solutions were used from Pierce™ Bovine Serum Albumin Standard Pre-Diluted Set. Afterwards, 200 µL of BCA working reagent were added to each well and mixed. The working reagent was prepared by mixing Reagent A and Reagent B from the kit with a 50:1 ratio. The plate was covered and incubated at 37°C for 30

minutes. After cooling down at room temperature, the absorbance at 562 nm was measured using Tecan's i-control software.

### 3.7.5 Carbohydrates

For total carbohydrates analysis, the method described by Josefsson (1976) was used, with each bioreactor sample being measured once without any pre-treatment.

The reagent used in this analysis was prepared by adding 25 g $H_3BO_4$ gradually over 2-3 hours to 1 L of $H_2SO_4$ (98%). Afterwards, 5 g L-tryptophan was also gradually added. The reagents were mixed for a minimum of 6 hours and then stored overnight at 4ºC in a dark bottle. For analysis, 1 mL of diluted sample and 2 mL of the reagent were added to a vial, vortexed and heated at 100ºC for 20 min. The sample were cooled down to room temperature and absorbance measured at 520 nm.

To obtain the carbohydrates concentration is also necessary to have a calibration curve that follows a linear correlation between absorbance and concentration. This curve was prepared by measuring the absorbance at 520 nm of gradual concentrations (5, 10, 20, 40, 70 and 90 mg/L) of a glucose monohydrate stock solution (1g/L) using distilled water for dilutions.

### 3.7.6 Organic acids

Filtered samples (0.2 μm PVDF filters, Chromafil®) were submitted to HPLC (Prominence-i LC 2030 Plus, Shimadzu) analysis to measure organic acids and residual MeOH concentration. This HPLC is equipped with a refractive index detector (RID-20A, Shimadzu) and an Aminex HPX-87H column with 9 μm particle size (7.8 x 300 mm, Bio-Rad), at a column temperature of 41°C. The eluent used was sulfuric acid (5 mM $H_2SO_4$) at a flow rate of 0.6 mL/min.

### 3.7.7 Anions and cations

Filtered samples (0.2 μm PVDF filters, Chromafil®) were submitted to Ion Chromatography (930 Compact IC Flex; Metrohm, CH) analysis to measure anions ($CL^-$, $NO_2^-$, $NO_3^-$, $SO_4^-$, $PO_4^-$) and cations ($Na^+$, $NH_4^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$) concentration. Both IC machines are equipped with a conductivity detector and a Metrosp A Supp 5-150/4.0 column. The eluent is either $HNO_3$ or dipicolinic acid (DPCA) at a concentration of 1.7 mM, in the case of cations analysis, and either $NaHCO_3$ or $Na_2CO_3$, in the case of anions analysis. In both cases, the elution rate was 0.7 mL/min.

## 3.8 Intact and damaged cell quantification

For the analysis described below, an Attune NxT Flow Cytometer (Thermo Fisher Scientific, Belgium) was used, set to a Blue-Red configuration with six fluorescence detectors (530/30, 615/25, 670/14, 695/40, 720/30 and 780/60 nm), two scatter detectors, one 50-mW 488-nm laser (blue) and a 100-mW 637-nm laser (red).

Immediately after sampling, cell suspension from the bioreactor was diluted to around $10^4$ cells/µL using PBS in tubes containing a final volume of 500 µL, and then stained with 1% of a combination of SYBR green I and propidium iodide (SGPI, 100x concentrate SYBR®Green I, Invitrogen, and 50x 20 mM propidium iodide, Invitrogen, in 0.22 µm-filtered dimethyl sulfoxide) for intact and damaged cell quantification. The stained samples were incubated for 20 min in the dark at 37 ºC and analyzed immediately afterwards. The threshold used was the green fluorescence in the BL1 (530/30BP) channel. SYBR Green I was detected on BL1 (530/30BP) while propidium iodide was detected on BL3 (695/40BP).

## 3.9 Data analysis

### 3.9.1 Calculations

To calculate the biomass ($Y_{X/S}$) and protein yields ($Y_{P/S}$), the substrate concentrations were first determined in terms of Chemical Oxygen Demand (COD) according to the method defined by Henze et al. (2008) that a compound with $C_nH_aO_bN_c$ is oxidized according to the equation 4:

$$C_nH_aO_bN_c + \left(n + \frac{a}{4} - \frac{b}{2} - \frac{3}{4}c\right)o_2 \rightarrow nCO_2 + \left(\frac{a}{2} + \frac{3}{2}c\right)H_2O + cNH_3 \quad (eq.4)$$

Therefore COD ($g_{COD}$/L) of a compound can be calculated from its concentration (C) in g/L, using the stoichiometric coefficients (n, a, b, c), its molecular weight ($MW_C$) and of oxygen ($MW_{O2}$) and the compound by equation 5:

$$COD\ \left(\frac{g_{COD}}{L}\right) = \frac{C\ \times \left(n + \frac{a}{4} - \frac{b}{2} - \frac{3}{4}c\right) \times MW_{O_2}}{MW_C} \quad (eq.5)$$

The yields were then determined according to equation 6 and 7:

$$Y_{X/S}\left(\frac{g_{CDW}}{g_{COD}}\right) = \frac{X_{final} - X_{initial}}{S_{final} - S_{initial}} \quad (eq.6)$$

$$Y_{P/S}\left(\frac{g_{protein}}{g_{COD}}\right) = \frac{P_{final} - P_{initial}}{S_{final} - S_{initial}} \quad (eq.7)$$

Where $X_{final}$, $P_{final}$ and $S_{final}$ are the biomass concentration ($g_{CDW}$/L), protein concentration ($g_{protein}$/L) and substrate concentration ($g_{COD}$/L), respectively, obtained when the culture reached stationary phase ($g_{CDW}$/L), and $X_{initial}$, $P_{initial}$ and $S_{initial}$ are the biomass concentration ($g_{CDW}$/L), protein concentration ($g_{protein}$/L) and substrate concentration ($g_{COD}$/L), respectively, at the start of the experiment.

The yield of protein in terms of nitrogen consumption ($Y_{P/N}$) was also calculated according to equation 8:

$$Y_{P/N}\left(\frac{g_{protein}}{g_{COD}}\right) = \frac{P_{final} - P_{initial}}{N_{final} - N_{initial}} \qquad (eq.8)$$

Where $N_{final}$ is the nitrogen concentration when the culture reached stationary phase ($g_{COD}$/L) and $N_{initial}$ is the nitrogen at the start of the experiment ($g_{COD}$/L)

### 3.9.2 Statistical analysis

Statistical analysis was performed using R (v3.6.1) based on a confidence level of 95% ($p<0.05$).

For the preliminary tests, the effect of temperature and methanol concentration on protein content was analyzed using a one-way analysis of variance (ANOVA). If a statistical significance was found, a post-hoc analysis (Tuckey Test) was performed. The same analysis was done to test the effect of pH, presence and type of acid was evaluated based on biomass recovery for the acid precipitation assay, which was calculated through the difference between the initial and final CDW in the supernatant.

For the batch experiments, analysis of the effect of the culture, medium and growth phase on the protein content and protein productivity was done in categories (lag, exponential, and stationary growth phase) using a three-way ANOVA analysis with a linear mixed effects model (lmer, R package lme4, optimized with restricted maximum likelihood (REML)), setting time as a random effect. Growth phases were defined based on the batch experiments performed: the lag phase ending when a steady exponential increase started, and the exponential phase ending when growth was stable after two consecutive timepoints, both which were considered stationary phase.

# Chapter 4

# Results

## 4.1 Effect of temperature on microbial growth and protein content

The effect of temperature (28-32ºC) was tested on growth and protein content of *M. extorquens*, *K. phaffii* and their co-culture, grown in either MMMV or AMS medium containing 0.2% (v/v) MeOH in 96-well plates. Results for *K. phaffii* and the co-culture grown on MMMV are not shown since the yeast was unable to grow in these conditions, likely related to oxygen limitation in the plate. Furthermore, most replicates for the experiments using AMS medium at 28ºC had to be removed since the OD was constanly peaking or falling abruptly due to evaporation and condensation in the plates. The remaining replicates revelead no clear effect of temperature on growth of *M. extorquens* and the co-culture (Figure 5a, Figure 5c and Figure 5d). Growth was found higher for *K. phaffii* at 28ºC but it was still very inconsistent (Figure 5a), showing a peak in CDW followed by a steady decrease. This does not correspond to the typical growth curve that follows a lag, exponential and stationary phase that was observed for other conditions.

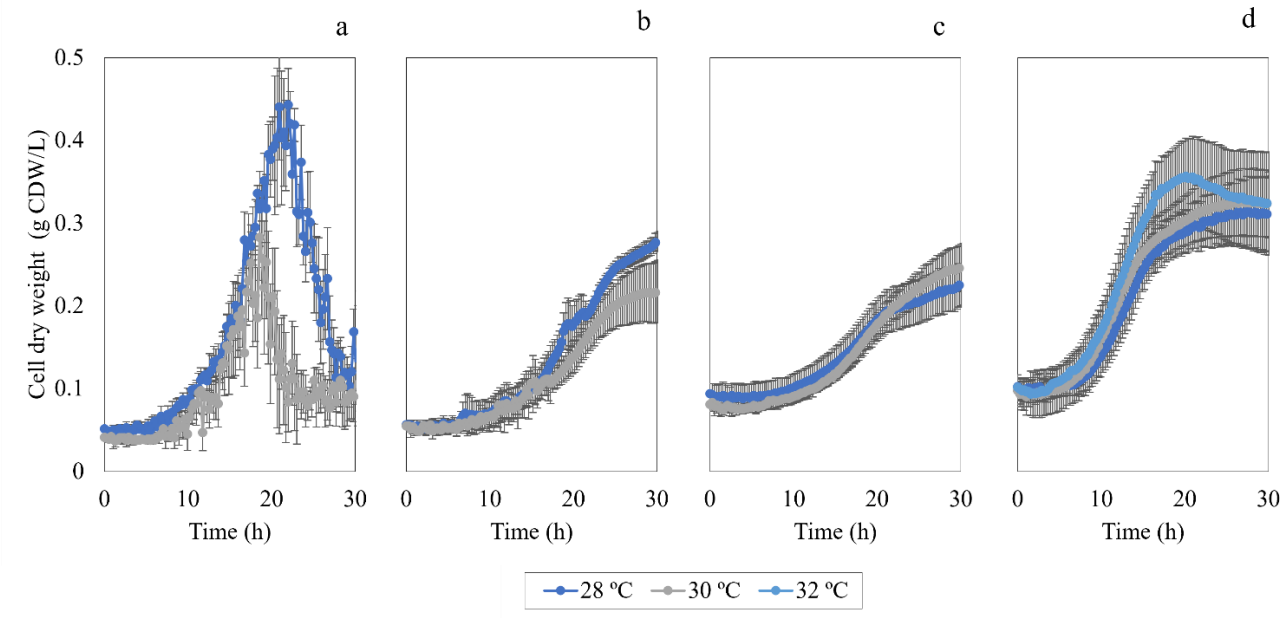


**Figure 5:** Growth curves of *K. phaffii* (a), co-culture (b), *M. extorquens* grown on AMS (c) and MMMV (d) grown at different temperatures (28, 30, 32 ºC).

Besides the CDW, another parameter evaluated to chose the optimal temperature for high SCP quality was protein content ($\%_{CDW}$) (Figure 6). Since the yeast had an abrupt drop in CDW, the maximum

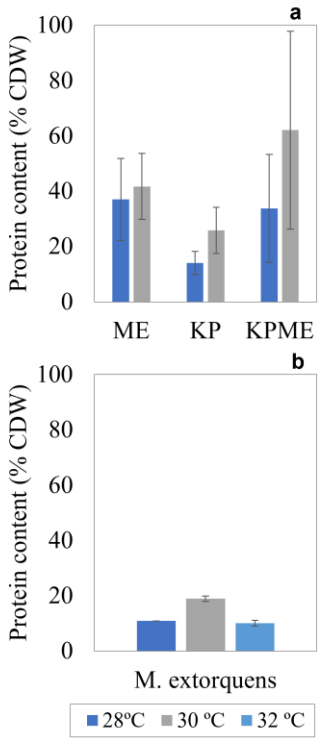


**Figure 6:** Protein content (%$_{CDW}$) for all tested temperatures and cultures on AMS (a) and MMMV (b).

value achieved was used in the calculations instead of the final, considering that the drop was due to deposition of the yeast in the bottom of the well.

In the experiments using AMS medium, the protein content was found to be non-dependent of temperature ($p=0.32>0.05$) but the type of culture had a significant effect ($p<0.01<0.05$) on the protein content. However, it is possible to see from **Figure 6** that the average protein content is higher for 30ºC in all cultures. This is statistically confirmed in the case of the experiments using MMMV medium in which the protein content of *M. extorquens* was dependent of temperature ($p<0.01<0.05$), with samples at 28ºC and 32ºC ($p=0.80>0.05$) having similar protein contents but statiscally lower than at 30ºC ($p<0.01<0.05$). For these reasons, 30ºC was chosen as the temperature in the Biostat® reactor.

## 4.2 Effect of methanol concentration on microbial growth and protein content

The effect of MeOH concentrations between 0.025-1% (v/v) was tested on growth and protein content in *M. extorquens*, *K. phaffii* and their co-culture, grown on AMS medium at 32ºC. Growth curves obtained for the monocultures are shown in Figure 7 where it is possible to see that none of the concentrations tested were toxic for *M. extorquens*. In the case of *K. phaffii*, growth curves in the plate were again inconsistent. Although higher concentrations of MeOH led to the decrease in growth of this strain, final CDW on 0.025% (v/v) was only 1.3 times higher than 1% (v/v). Moreover, for both *M. extorquens* and *K. phaffii*, growth was also observed in the wells without the carbon source (0% v/v). Since CDW was low for both cultures, this might be related to evaporation increasing the concentration of the initial inoculum and leading to the transfer of MeOH from other wells. A more likely explanation is that traces of substrate used in the pre-inoculum were still present and being consumed.

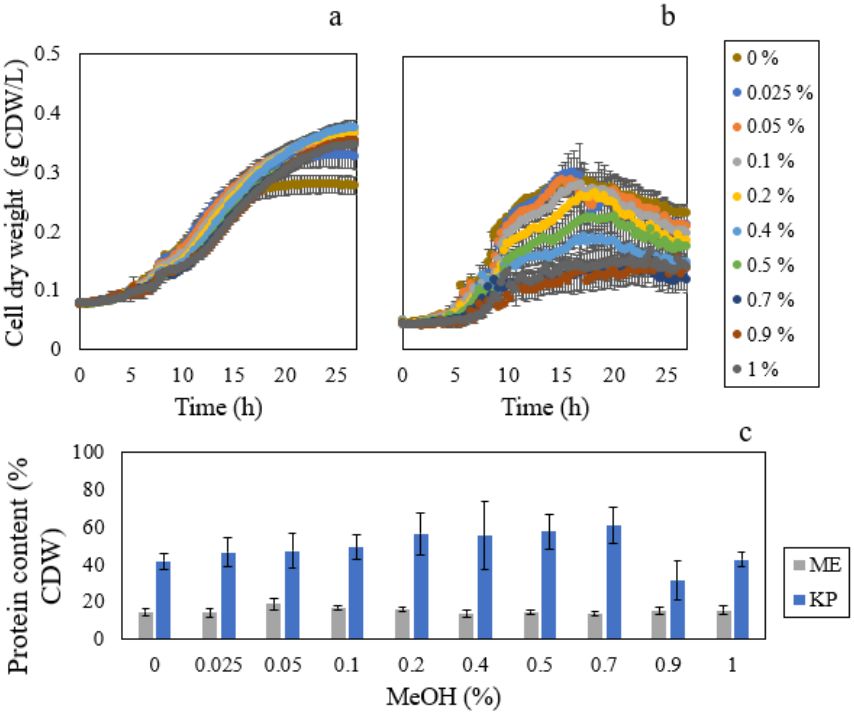


**Figure 7:** Growth curves for *M. extorquens* (a) and *K. phaffii* (b) on different MeOH concentrations (0-1%) and final protein content (%$_{CDW}$) for all tested conditions (c).

Regarding protein content (%$_{CDW}$, Figure 7c), *M. extorquens* had lower values than observed in *K. phaffii* with no correlation with the concentration of MeOH ($p=0.133>0.05$). For *K. phaffii*, the protein content was higher for the concentrations where intermedium growth was observed but the only statistical difference found was between the 0.9% (v/v) MeOH samples and all the other conditions except 1% (v/v) MeOH samples. Therefore, no particular pattern was found, but it was possible to assume 0.5% (v/v) as an intermedium MeOH concentration that provides non-toxic growth for both *M. extorquens* and *K. phaffii* and could be thus used in the bioreactor experiments.

## 4.3 Effect of medium composition on microbial growth and SCP quality

### 4.3.1 Preliminary tests

An initial experiment performed in Erlenmeyer flasks at 30ºC evaluated the growth of *M. extorquens* and K. *phaffii* on two different media: MMM and AMS, both containing 0.2% (v/v) MeOH. The effect of medium composition on growth was not found significant (Figure 8). Visually, the bacterial culture was different between the two media used (Appendix A.3.2). *M. extorquens* grown on AMS showed a brighter pink color and aggregations. On the other hand, *M. extorquens* grown on MMM was light pink and formed a more homogenous suspension.

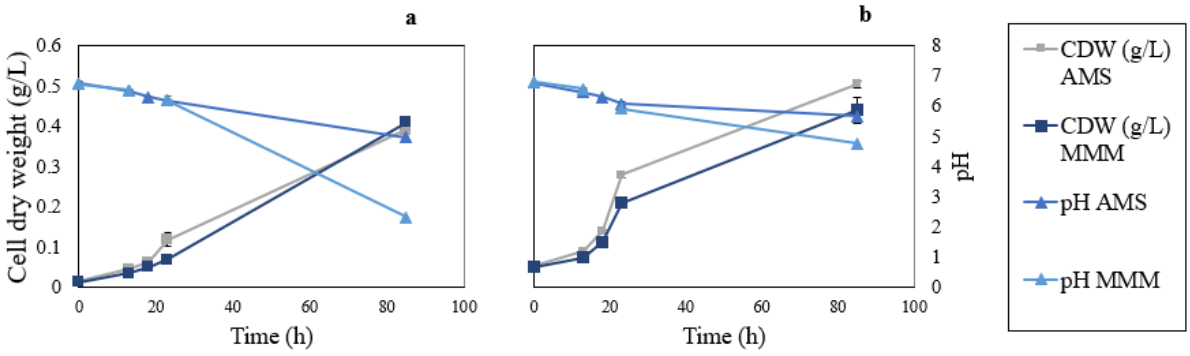


**Figure 8:** Growth curves and pH for *K. phaffii* (a) and *M. extorquens* (b) grown on MMM and AMS.

To further confirm these observations and test other media (DSMZ1629, MMMV), a second experiment was performed by growing the monocultures in a 96-well plate. This experiment provided more information on the optimal medium for culture growth, with clear distinctions of growth between the media tested (Figure 9). For *M. extorquens*, DSMZ1629 did not provide any significant growth. Furthermore, growth of both *K. phaffi* and *M. extorquens* on AMS was similar to the low values observed in other preliminary tests, with CDW not exceeding 0.4 g CDW/L. MMM medium had 2 times higher final CDW than AMS for *M. extorquens* growth and was similar to AMS regarding the yeast. However, for both cultures, MMMV medium proved to be the best-performing one in terms of cell growth. For *K. phaffii*, this difference was very clear, with this medium having up to 8 times higher final CDW. Therefore, this medium was used in the bioreactor experiments comparing the mono and co-cultivation of these strains (see Section 4.4).

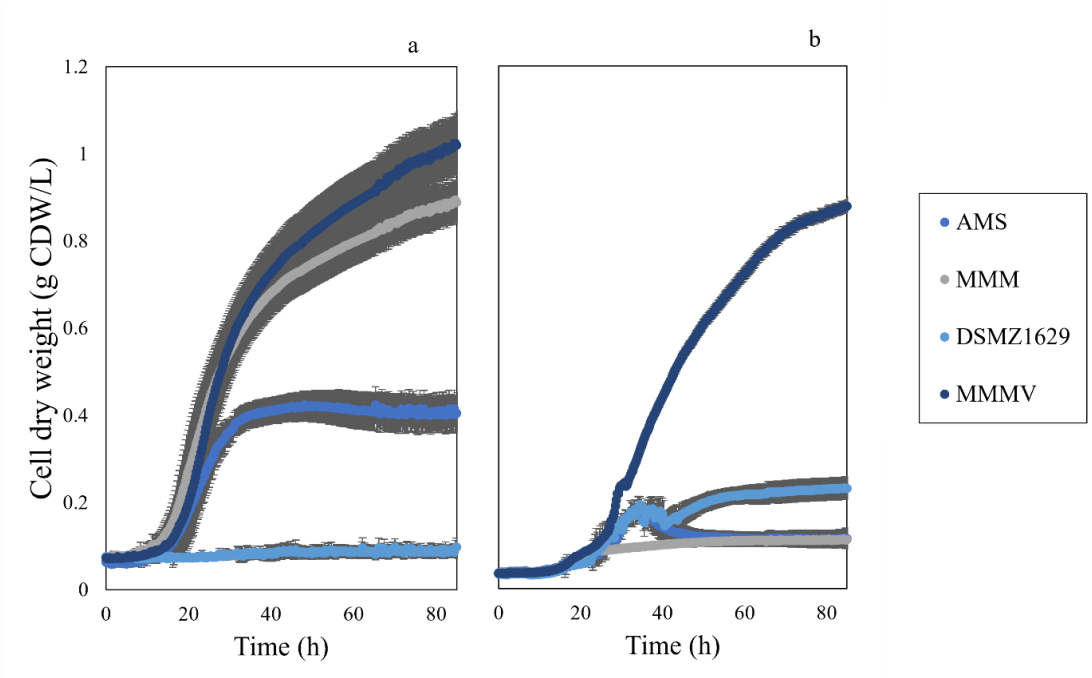


**Figure 9:** Growth curves for *M. extorquens* (a) and *K. phaffii* (b) grown on different media.

### 4.3.2 Bioreactor experiments

The effect of medium composition on *M. extorquens* growth using AMS and MMMV medium with 0.5% MeOH was also investigated via bioreactor experiments operated in batch at 30 ºC. The average CDW of *M. extorquens* grown on AMS medium was 3 times higher than when grown on MMMV (Figure 10) and the average protein productivity (1.0±0.8 mg $L^{-1}$ $h^{-1}$) was 4 times higher than in the MMMV-grown bacterium (Appendix A.4.2). Although medium composition was shown to significantly affect the protein productivity ($p=0.02<0.05$), it did not have a significant effect on protein content ($p=0.29>0.05$), which ranged from 7-14%$_{CDW}$ for MMMV-grown bacterium and 1-25%$_{CDW}$ for AMS-grown bacterium.

Moreover, growth phase affected the protein content only in the AMS experiments, with lag phase showing significant differences to the exponential ($p<0.01<0.05$) and stationary phase ($p<0.01<0.05$), and it did not significantly affect the protein productivity ($p=0.07>0.05$). This is seen in Figure 10, in which the protein content increased throughout time in the AMS-grown bacteria, reaching a maximum of 25%$_{CDW}$ on the stationary phase, whereas MMMV-bacterium stays within the low range mentioned before, with the initial timepoint displaying an average protein content of 7%$_{CDW}$.

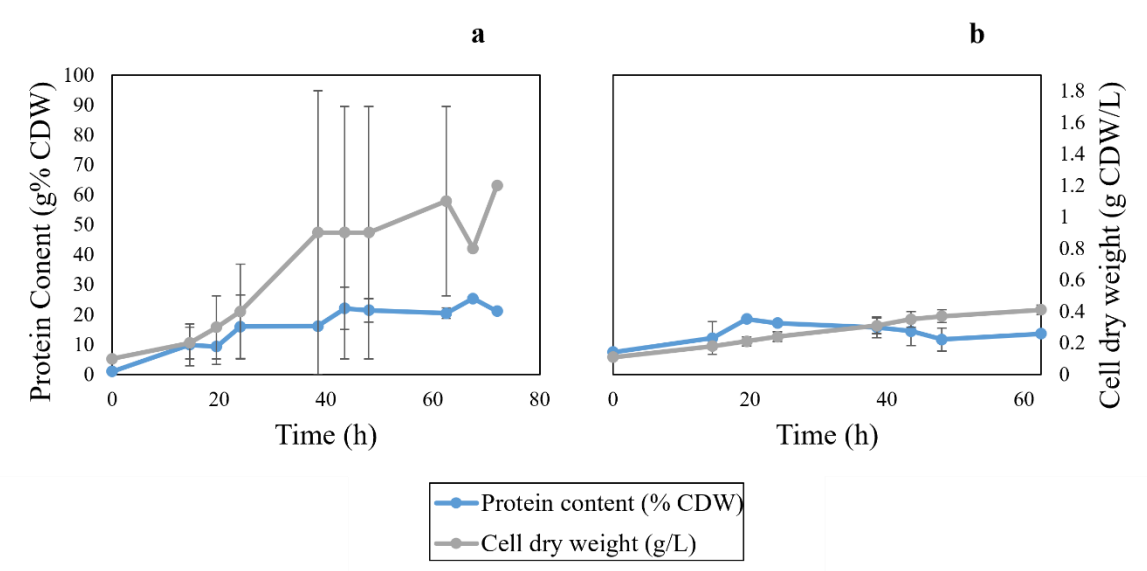


**Figure 10:** Growth curves in CDW (g/L) and protein content (%$_{CDW}$ ) for *M. extorquens* grown in either AMS (a) or MMMV (b).

It is important to note that the two replicates using AMS medium showcased very different growth behaviors, leading to high standard deviations (Appendix A.4.1). One of the replicates exhibited high CDW and reached the stationary phase at 62.5 hours. The other replicate had a long lag phase that led to 72 hours of growth till stationary phase, with lower CDW. This could be attributed to oxygen limitation provoked by clogging of the aeration filter.

Besides protein content, the total biomass composition was analyzed (Figure 11). For both media tested, the ash content decreases throughout time whereas carbohydrate concentration did not present a clear comon pattern. However, carbohydrate concentration had a higher average in MMMV medium (34%$_{CDW}$) than AMS (25%$_{CDW}$). At the end of the experiment, the most significant component of the biomass grown on MMMV medium were carbohydrates whereas bacteria grown on AMS showed higher content of the remaining components not directly measured (e.g. lipids, PHB).

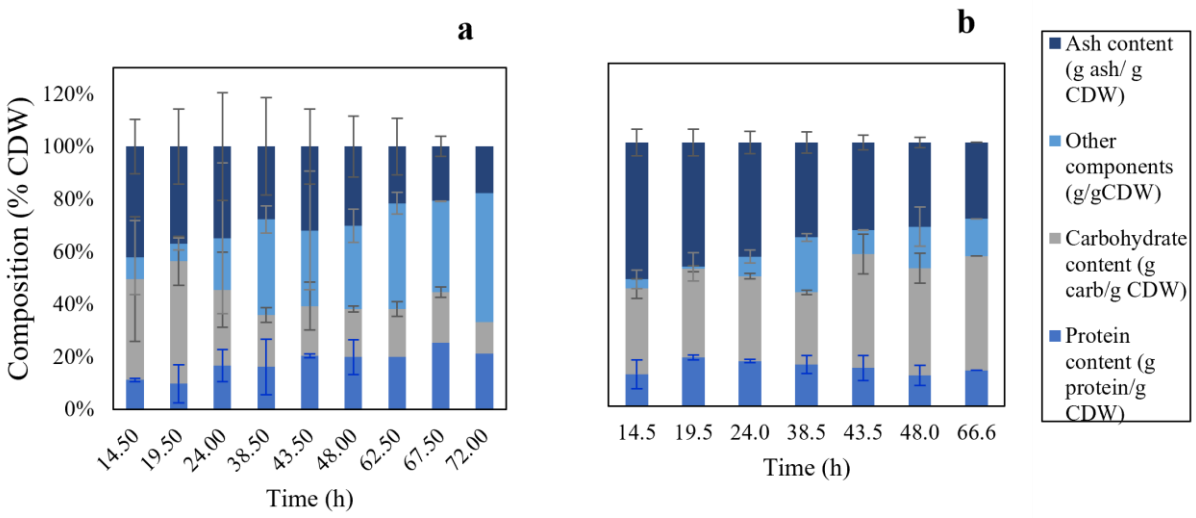


**Figure 11:** Biomass composition (%) for *M. extorquens* grown in AMS (a) and MMMV (b).

## 4.4 Effect of culture on SCP production rate and quality

The effect of culture on microbial growth and SCP quality was tested by growing *M. extorquens* and *K. phaffii*, individually and combined in MMMV medium with 0.5% (v/v) MeOH in a bioreactor ran in batch at 30ºC. The type of culture had a significant effect on the protein content ($p<0.01<0.05$) and protein productivity ($p<0.01<0.05$). Compared to the bacterial monoculture, final CDW (g/L) of the co-culture was 2.8 and 3.1 times higher for the co-culture and *K. phaffii*, respectively, and final protein content (%$_{CDW}$) followed a similar trend, with 2.9 and 3.2 times higher values. This was expected since the co-culture's CDW was calculated through the mass ratio between the two cultures, in each *K. phaffii* was the dominant species with an average of 81% (w/w) (Appendix A.2.2). Furthermore, all cultures displayed the highest protein content in the exponential phase (Figure 12), after which protein productivity significantly decreased ($p=0.03<0.05$).

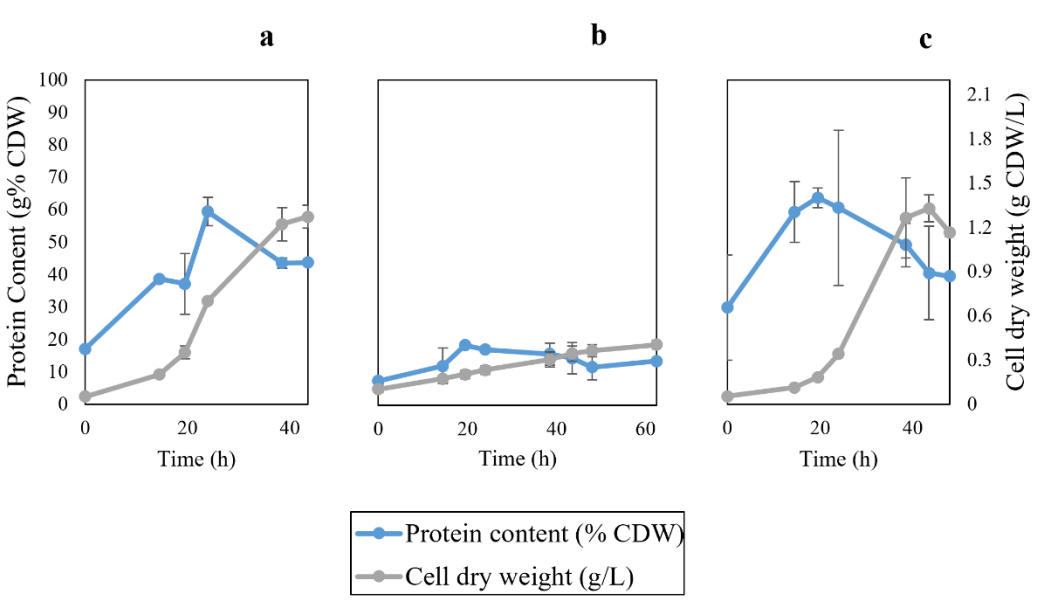


**Figure 12:** Growth curves in CDW (g/L) and protein content (%$_{CDW}$) for *K. phaffii* (a), *M. extorquens* (b) and their co-culture (c) grown on MMMV.

Regarding biomass composition (Figure 13), ash content was not measured for *K. phaffii* and consequently, the co-culture since the calibration curve for the VSS (g/L) was not optimal. Although it is not possible to compare the ash content, the carbohydrates for these two types of cultures decreased throughout time, which is the opposite pattern observed for the bacterial monoculture. The remaining components increased over the course of the experiment, and by the end *K. phaffii* still had protein as its main component (44%$_{CDW}$) while the co-culture had equal percentage (39%$_{CDW}$) of both protein and the remaining components. However, it is important to note the negative values of the remaining components (%$_{CDW}$) in the first timepoints of the co-culture (-10%$_{CDW}$ and -13%$_{CDW}$ at 14.5h and 19.5h, respectively), which were consequentially removed from Figure 13. This could have been related to either the calibration curve or the dilution for carbohydrate analysis not being the most suitable on these timepoints.

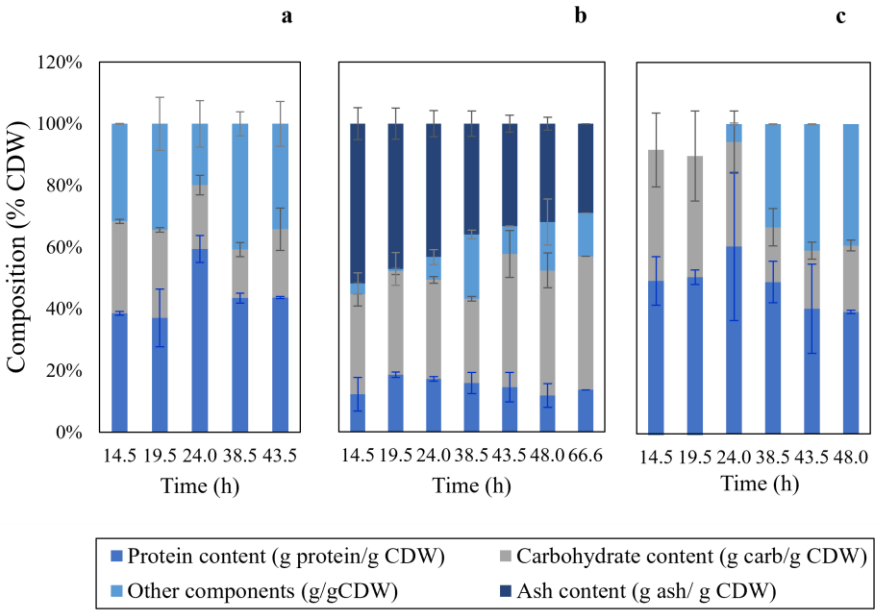


**Figure 13:** Biomass composition (%) for *K. phafii* (a), *M. extorquens* (b) and their co-culture (c) grown on MMMV medium. Negative values of the first timepoints of the co-culture were removed from this figure.

## 4.5 Estimation of the intact and damaged cell count of batch tests

To understand the effect of the co-cultivation of the yeast and bacterial strain and to further characterize the monoculture growth, influence of the medium, existence of possible subpopulations and contaminations, flow cytometry analysis was performed to estimate the intact and damaged cell population throughout time.

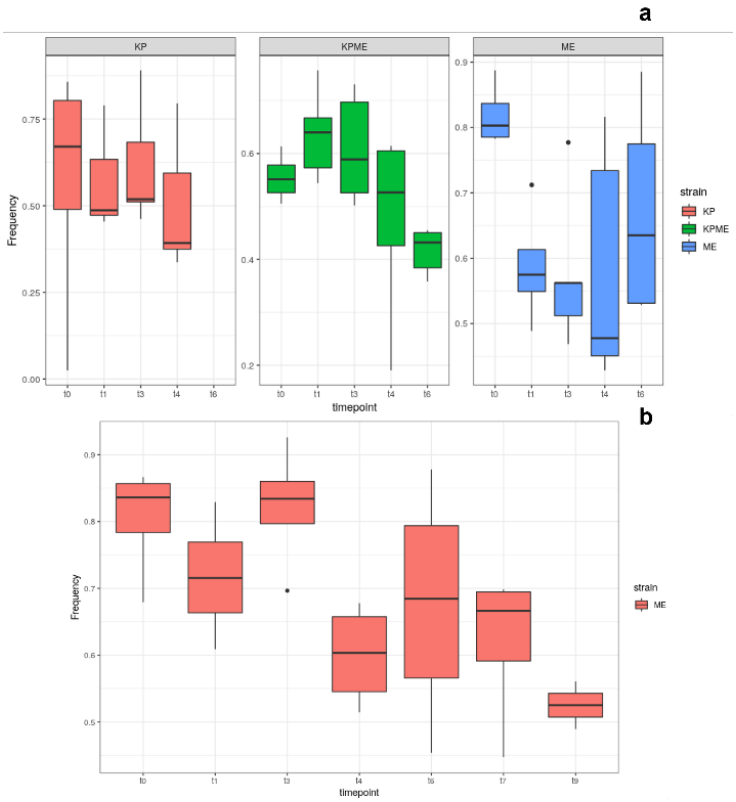


**Figure 14:** Frequency of singlets in regarding to total events for experiments conducted in MMMV (a) and AMS (b).

The first step was removing from the flow cytometry results the counts related to aggregates and buddying yeast population that are expected to occur as time passes, due to cell growth, natural aggregation of the yeast population and the tendency of AMS-medium to lead to flocs. Therefore, the only replicates that did not have a continuous decrease of singlets regarding total events were MMMV-grown bacteria (Figure 14).

The second step was gating of the yeast and bacterial population within the singlet population. This was done through three further sequential steps. Firstly, a primary gating was performed based on the two scatter detectors: forward scatter (FSC), which broadly correlates with cell size, and side scatter (SSC), which indicates the granularity or optical complexity of cells. Since bacteria have generally a smaller size than yeasts, it was possible to have two separate gates for yeast and bacterial populations based on the monocultures grown in MMMV-medium, with minimal overlap for all timepoints. By filtering using the primary gating, it is possible to obtain a second gate, which related the fluorescence signal of SYBR Green (used as a proxy for the intact cells) to SSC. Both steps can be seen in more detail in Appendix A.2.2. Consequently, the differentiation between *K. phaffii* and *M. extorquens* present in the co-culture was possible (Figure 15). Besides this differentiation, the formation of subpopulations is observable in the yeast gate and bacterial gate as time passes (Figure 15b).

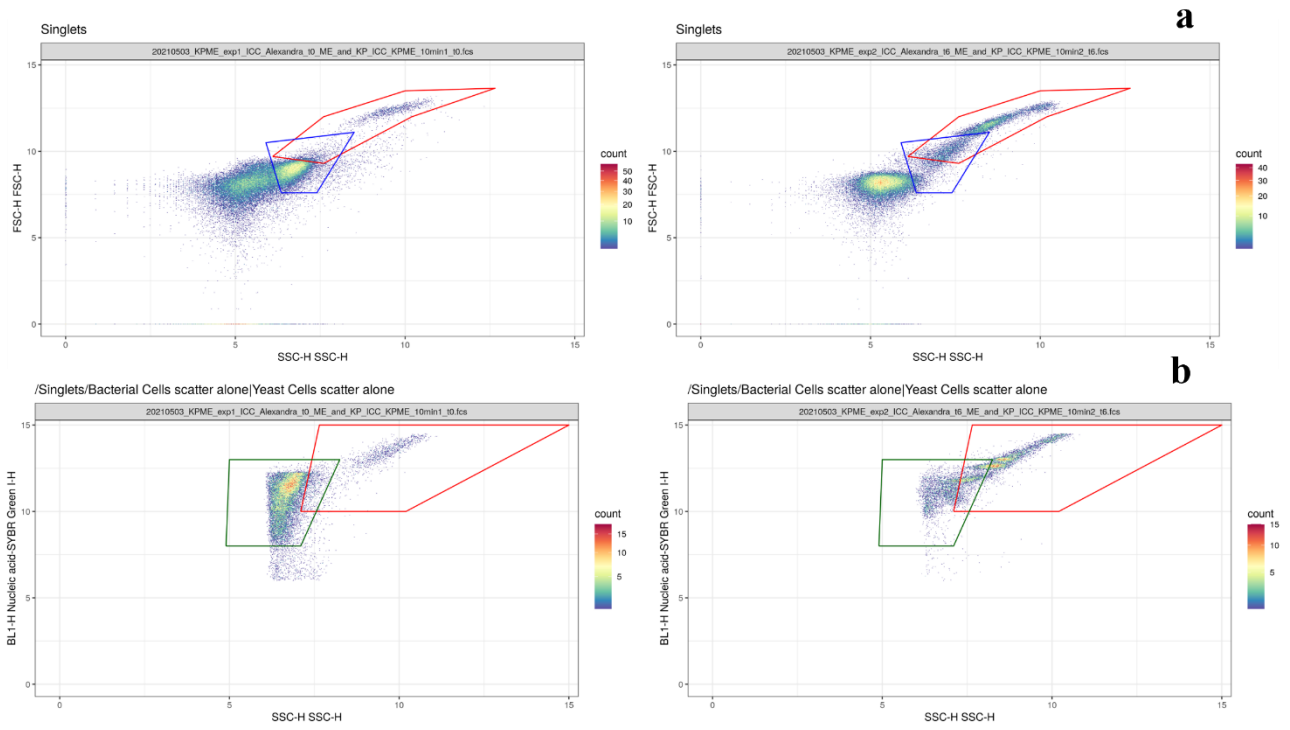


**Figure 15:** Examples of primary gating (a) and secondary gating (b) of the co-culture replicates in the initial timepoint (left) and after 48.5 h (right). The red gate represents the result of yeast gating and the blue (primary gating) or green (secondary gating) represents the bacterial gating.

These same gates were also used in the AMS-grown bacteria samples to highlight the visual differences in cultures grown on different media, scan for possible contaminations and further validate the gating performed for the co-culture. Although different cellular or noise placement were expected since AMS-grown bacteria had shown visual differences to MMMV-grown bacteria (Appendix A.3.2), the presence of cells in the yeast gate at high intensity (Figure 16), moving from a

sort of a “tail” starting from the bacterial gate population to two very distinct populations (similar to the co-culture results) raised concern for contamination.

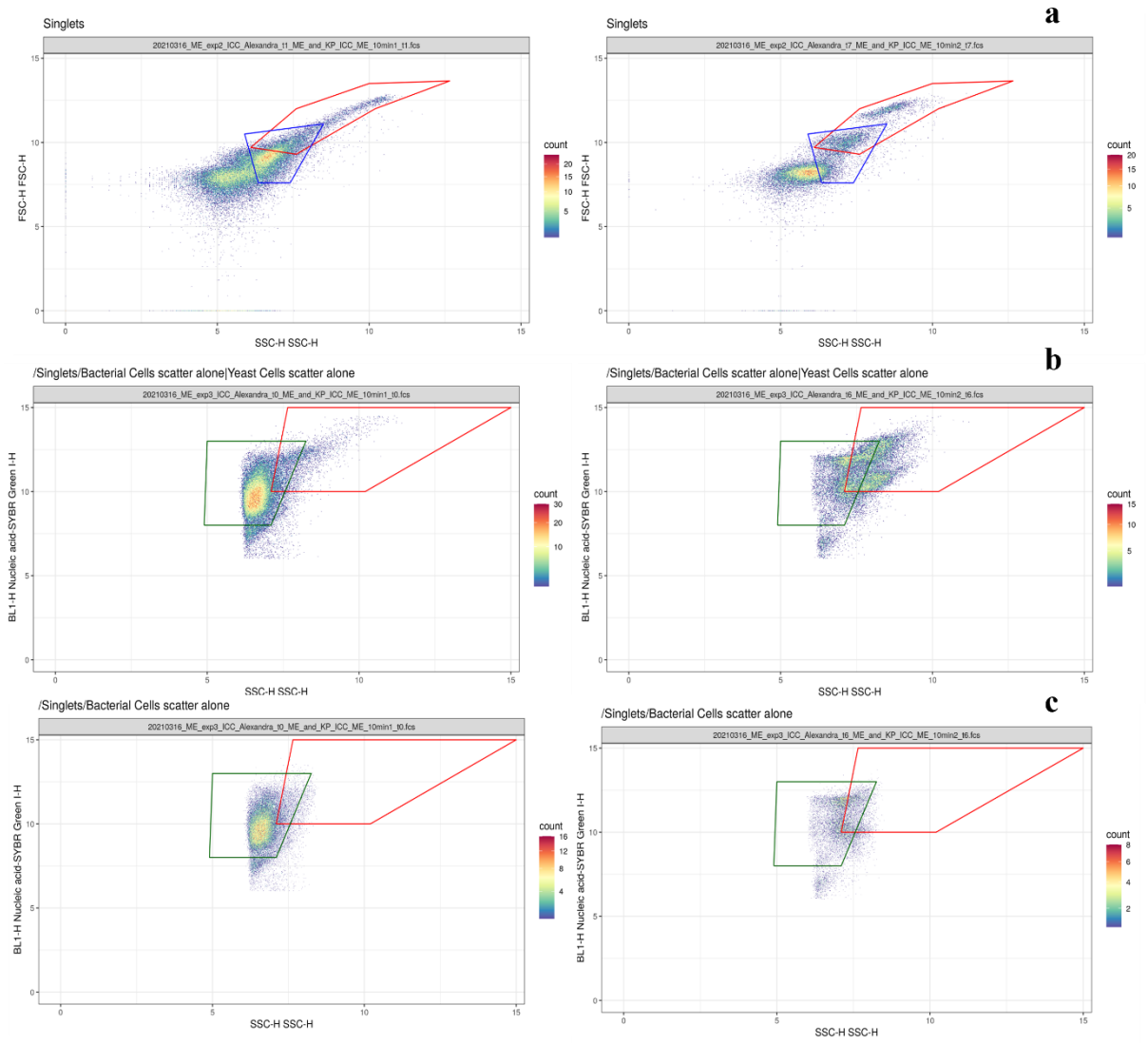


**Figure 16:** Examples of primary gating (a) and secondary gating before (b) and after (c) filtering using the primary bacterial scatter gating in AMS-grown bacteria.

After these two gating strategies allowed for sufficient filtering, the next and last step of the gating process was to distinguish between intact and damaged cells for each condition (Appendix A.2.2). Hence, the sequentially filtering allowed for assessment of the cell counts of each strain (Figure 17), including within the overlapping areas.

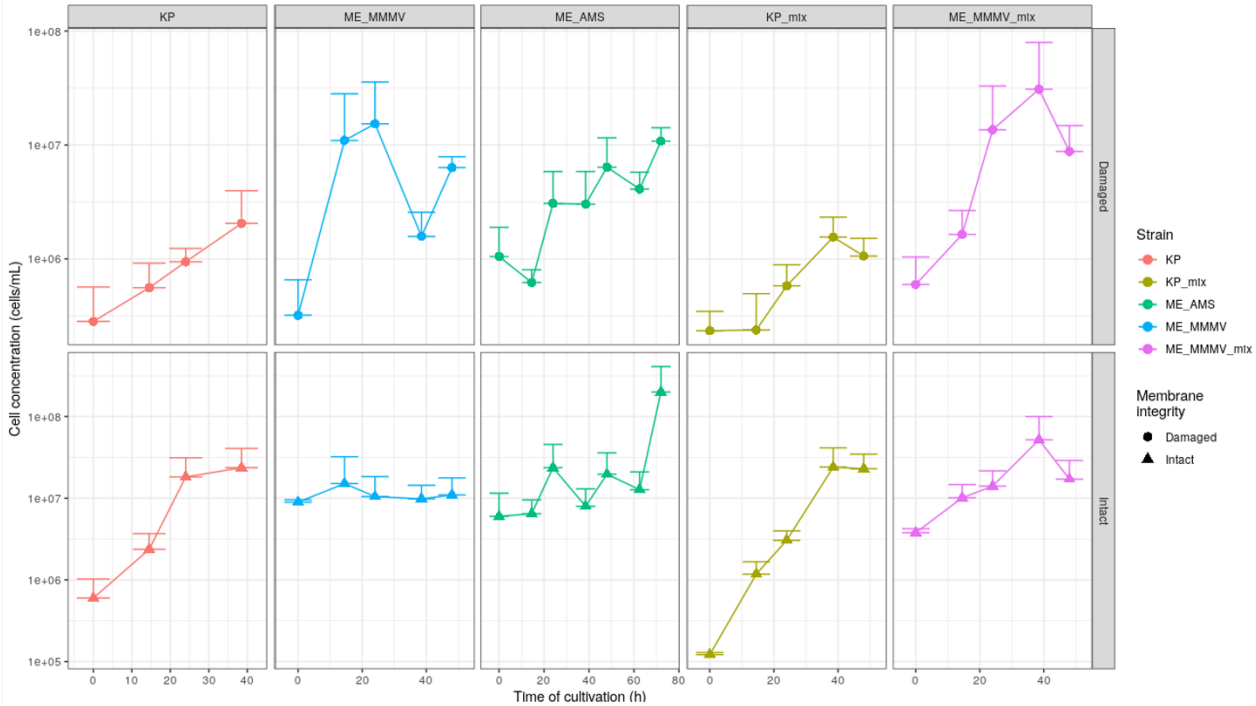


**Figure 17:** Intact and damaged cell counts for *K. phaffii* monoculture (KP), *M. extorquens* monoculture in AMS (ME_AMS) and MMMV (ME_MMMV), and *K. phaffii* (KP_mix) and *M. extorquens* (ME_MMMV_mix) in the co-culture.

Regarding MMMV-grown bacteria, while the damaged cells increased in number throughout time, the same does not happen for intact cells, with the final count being only 1.22 times higher than the initial. This was first seen in the low CDW for this condition (Figure 10). When comparing the percentage of intact versus damaged cells (Figure 18) it is also possible to see how quickly damaged cells grow in this case compared to AMS-grown bacteria and to the other cultures grown on MMMV, reaching up to 60% of the population. Furthermore, the AMS-grown bacteria also did not show a steady increase in intact cells population which could be related to (1) the variances observed between replicates, (2) the fact that the gate was not appropriate for this medium potentially due to different phenotypes or (3) the presence of a contamination. However, the damaged cell population was significantly lower, only reaching up to 28% of the population (Figure 18).

Regarding the co-culture's cell counts, it is not surprising that initially there is significantly less yeast cells, related to the fact that the inoculum is based on OD and not cell count. However, the yeast quickly grew to reach values similar to its monoculture. Furthermore, bacteria in the co-culture grow up to 14 times more than the initial concentration, which indicates that the presence of the yeast improved its growth in MMMV. However, the damaged bacterial cells still represented up to 44% of the population and it's likely the yeast would have dominated more the co-culture cell counts had it started with an equally number of cells.

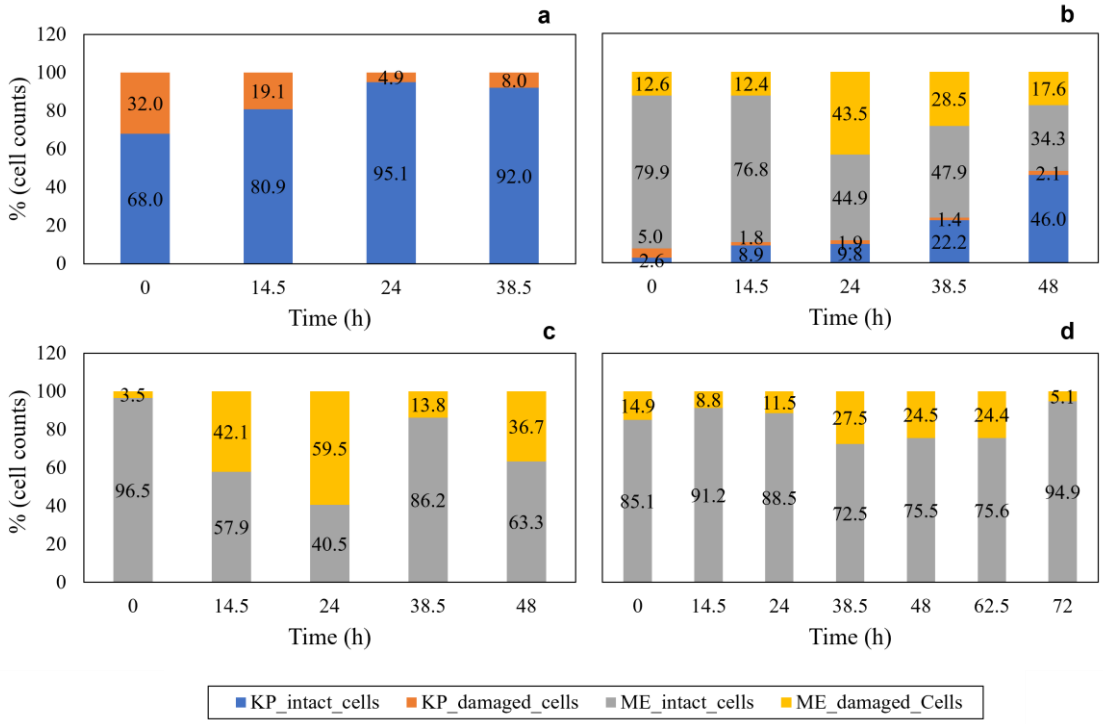


**Figure 18:** Intact and damaged cells (% cell counts) of *K. phaffii* (KP) and *M. extorquens* (ME) in the yeast monoculture (a), co-culture (b) and the bacteria monoculture grown in either MMMV (c) or AMS (d).

## 4.6 Evaluating the SCP production process in batch mode

### 4.6.1 pH control and $pO_2$

For all batch experiments mentioned above, $pO_2$ and pH changes were followed throughout time. Since all the cultures have an aerobic metabolism, dissolved oxygen is consumed, leading to decrease in $pO_2$. This was observed for all experiments except in the case of the co-culture grown on MMMV (Figure 19), due to the malfunction of the Biostat® control unit that occurred in the second replicate and prevented the reading of the results. Furthermore, differences observed in the initial $pO_2$ between experiments might be related to the immediate consumption of dissolved oxygen by the cultures after inoculation, which means slight timing differences associated with the transfer of the cultures and setting up the bioreactor play a significant role in this value.

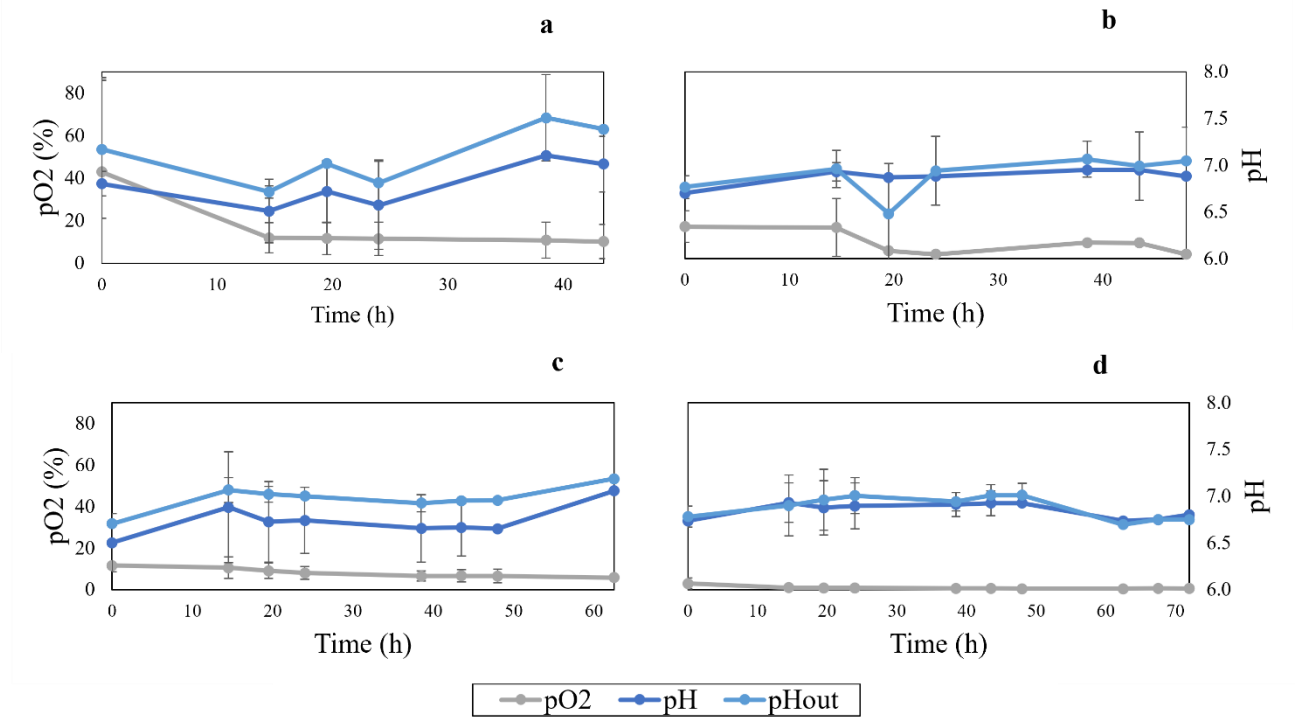


**Figure 19:** $pO_2$, pH in the bioreactor and pHout for *K. phaffii* (a), the co-culture (b), *M. extorquens* grown on AMS (c) and MMMV (d).

MeOH metabolism also leads to formation of organic acids, decreasing the pH, which was kept around 7.0 through the Biostat® control unit in all conditions (Figure 16), expect in the second replicate of the co-culture due to the malfunction mentioned above. Overall, the average pH was 7.1±0.3 for *K. phaffii*, 6.9±0.2 for the co-culture, 7.0±0.1 for *M. extorquens* grown on MMMV and 6.9±0.1, for growth on AMS.

The pH control was possible through the manual base addition (NaOH, 5 M) in the case of the second replicate of the co-culture, and automatic addition for all the other conditions. *K. phaffii* had the highest base consumption (2.8±0.4 mL), followed by *M. extorquens* (1.5±0.7 mL on MMMV medium; 1.5±0.0 mL on AMS medium) and the co-culture (0.9±0.8 mL) (Figure 20a). The high standard deviation observed for the co-culture is related to the manual addition of base, which was done only when it was necessary (when pH<6.85) to prevent culture stress. Besides base addition, around 0.3 mL of acid (HCl, 5 M) was added at 38.5 h for one of the yeast replicates since the pH rose above 7. This was due to the formation of organic acids (acetate and formate) that were consumed throughout time, raising the pH (Figure 20b)

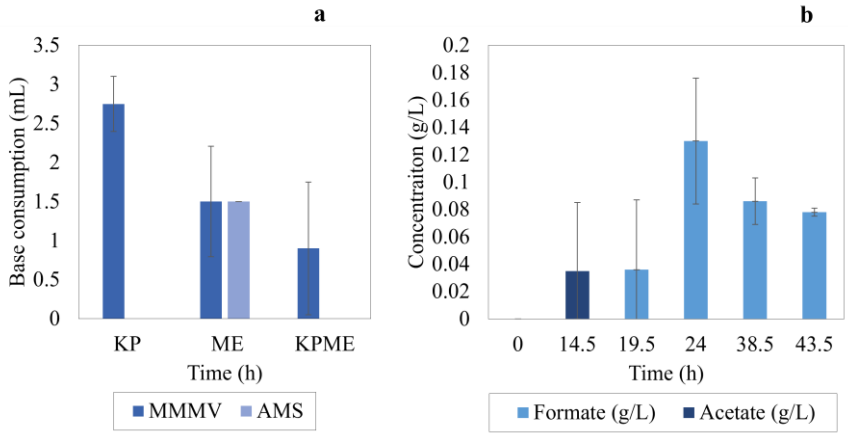


**Figure 20:** Base consumption (mL) for all tested conditions (a) and residual organic acids concentration (g/L) throughout time in *K. phaffii* cultures (b).

### 4.6.2 Consumption of essential nutrients

Other important parameters to follow during the batch experiments are the consumption of carbon and nitrogen sources throughout time (Figure 21). MeOH was fully consumed by the end of the experiments regardless of the type of medium or culture. However, MeOH was not the only carbon source observed during the experiments. Ethanol was used during the assembly and sampling of the bioreactor (to ensure sterility) which was detected in the bioreactor in all experiments and consumed fully throughout time. This was taken into consideration for the calculation of biomass and protein yields by using the total COD concentration, instead of only MeOH. On the other hand, nitrogen was only partially consumed, or even not clearly consumed, in the case of *M. extorquens* grown in MMMV medium, which means it was not a limiting nutrient for growth. Therefore, there was only carbon limitation.

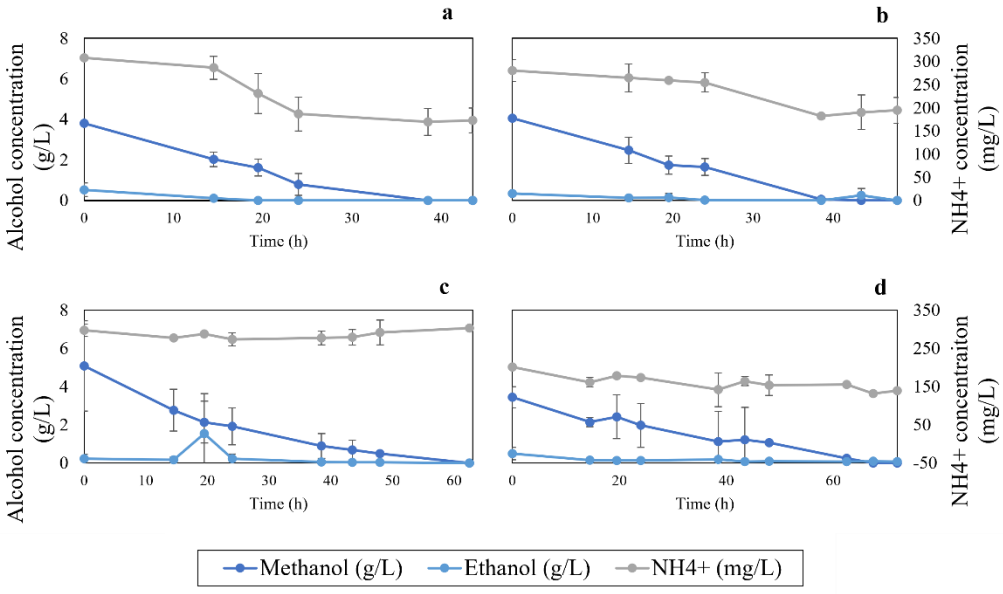


**Figure 21:** Nitrogen and carbon source concentration (g/L) for *K. phaffii* (a), the co-culture (b) and *M. extorquens* grown on MMMV (c) or AMS (d) throughout time.

A common point between in all the bioreactor experiments is the difference observed in the initial MeOH concentration that should theoretically be 0.5% (v/v), or 5 g/L but can suffer changes due to the imprecise solution preparation. This also applies for the differences observed in initial cations and anions concentration between cultures using the same medium (Appendix A.4.3). Another reason why this can occur is due to evaporation and sampling of the bioreactor which changes the final working volume of 1 L. Evaporation was observed to be around 150 mL for all the experiments.

### 4.6.3 Protein and biomass yields

Biomass and protein yields were calculated based on COD (total organic compound concentration, including alcohols and organic acids) and nitrogen consumed (Figure 22). Regarding *M. extorquens*, the experiments using the AMS medium displayed higher yields (up to 7.4 times) than MMMV medium, although with high standard deviations due to the different behavior of the replicates mentioned before. Nevertheless, the higher yields were shown to be statistically significant for both biomass ($p=0.01<0.05$) and protein ($p<0.02<0.05$) according to COD consumed. Furthermore, the protein yield according to consumed nitrogen was negative in MMMV (-0.7±2.3) since there was no visible nitrogen consumption in one of the replicates for this medium. This is most likely due to a combination of low CDW obtained in this medium with the evaporation that occurs significantly in the bioreactor.

Between cultures grown in the same medium (MMMV), the yields were up to 11 times higher for the co-culture and the yeast monoculture compared to *M. extorquens*. This was shown to be statistically significant for both biomass ($p=0.02<0.05$) and protein yield ($p<0.01<0.05$) according to COD consumed. Moreover, biomass yield of the co-culture was found to be 1.4 times higher than the yeast.

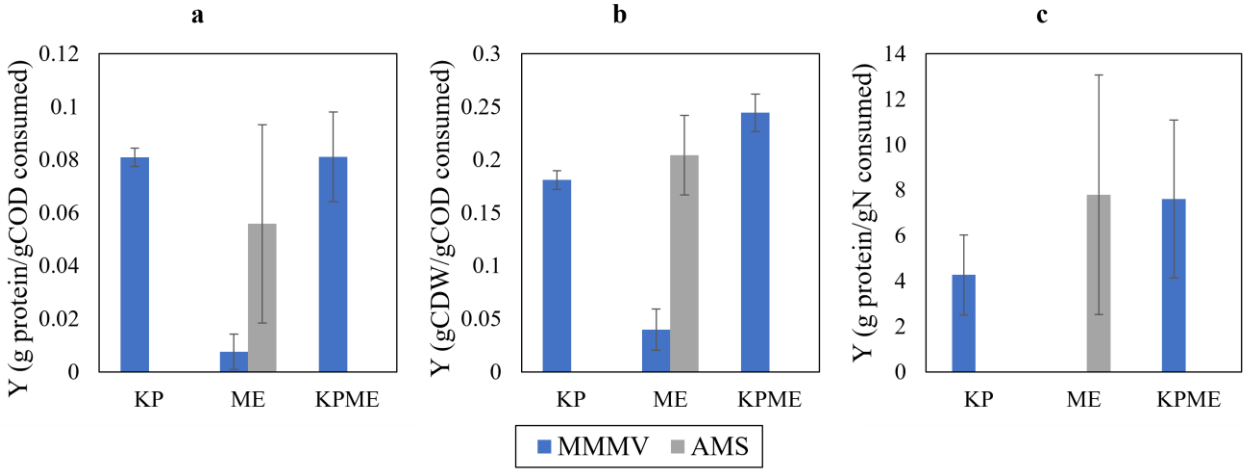


**Figure 22:** Protein yield (a) according to COD consumed ($g_{protein}/g_{COD}$), biomass yield (b) according to COD consumed ($g_{CDW}/g_{COD}$) and protein yield (c) according to nitrogen consumed ($g_{protein}/g_{N}$) for *K. phaffii* (KP), *M. extorquens* (ME) and their co-culture (KPME). Negative value for nitrogen yield in MMMV-grown bacteria was removed.

## 4.7 Biomass harvesting through acid precipitation

Acid precipitation was performed for 3 hours on a culture of *K. phaffii* by adding HCOOH (95%), HCl (5 M) and MQ (as a control) to achieve pH values between 2.5-4.5. The recovered biomass in the pellet (%$_{CDW}$) after 3 h is shown in Figure 23.

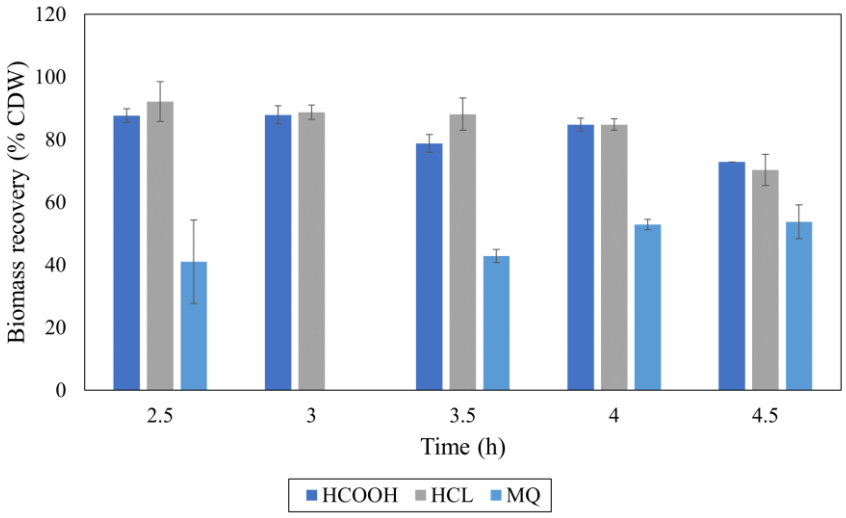


**Figure 23:** Biomass recovery in the pellet (%$_{CDW}$) of *K. phaffii* by acid treatment.

The higher biomass recovery was shown for both HCOOH and HCL in all tested pH values (2.5, 3, 3.5, 4 and 4.5) compared to the MQ controls and was found to be statistically significant ($p<0.05$) (Appendix A.6). Maximum values for biomass recovery (%$_{CDW}$) in the pellet were 54%$_{CDW}$ for MQ controls and 92%$_{CDW}$ for acid samples, which is almost twice the amount. Furthermore, all control biomass recovery values can be considered the same, except between conditions tested at pH 4 and 2.5 ($p<0.01<0.05$) and excluding the fact that the samples for the last timepoint for pH 3 were considered outliers and thus not presented in this thesis. The difference between using formic acid and hydrochloric acid was only significant at pH 3.5 ($p<0.01<0.05$), in which hydrochloric acid worked 9% better.

Regarding the difference in biomass recovery observed between different pH values, the recovery was found to be similar between pH 2.5 and 4 ($p=0.45>0.05$), 2.5 and 3 ($p=0.94>0.05$), and 4 and 3 ($p=0.87>0.05$), with values between 85%$_{CDW}$ and 92%$_{CDW}$ of biomass recovery. The remaining conditions (3.5 and 4.5) were shown to be significantly different but there does not seem to be a trend of which pH (higher or lower) works better. However, pH 4.5 was shown to have significantly less biomass recovery compared to all other pH values, with maximum value of 73%$_{CDW}$ biomass recovery. This indicates that acid precipitation was most efficient between pH 2.5-4.

Time was also shown to play a part in CDW (g/L) measured in the suspension (Figure 24). It is possible to see that precipitation is faster in the beginning and slowly decreases strength. For example,

less than 5%$_{CDW}$ precipitation happens between 75 min and 90 min for pH 2.5 and 3.5, and between 90 and 105 min for pH 3 and pH 4. For 4.5, precipitation happens slower, more similar to what observed in the control samples. Furthermore, between the last two timepoints in which sampling took place (75 min apart), between 4-24%$_{CDW}$ of the observed biomass recovery happened in cases of acid precipitation whereas for MQ water between 34-70%$_{CDW}$. Since stabilization was seen before this last sampling points for acid precipitation samples, it is likely that this last recovery was not an effect of the acid itself, but rather the natural sedimentation that happens throughout time as well.

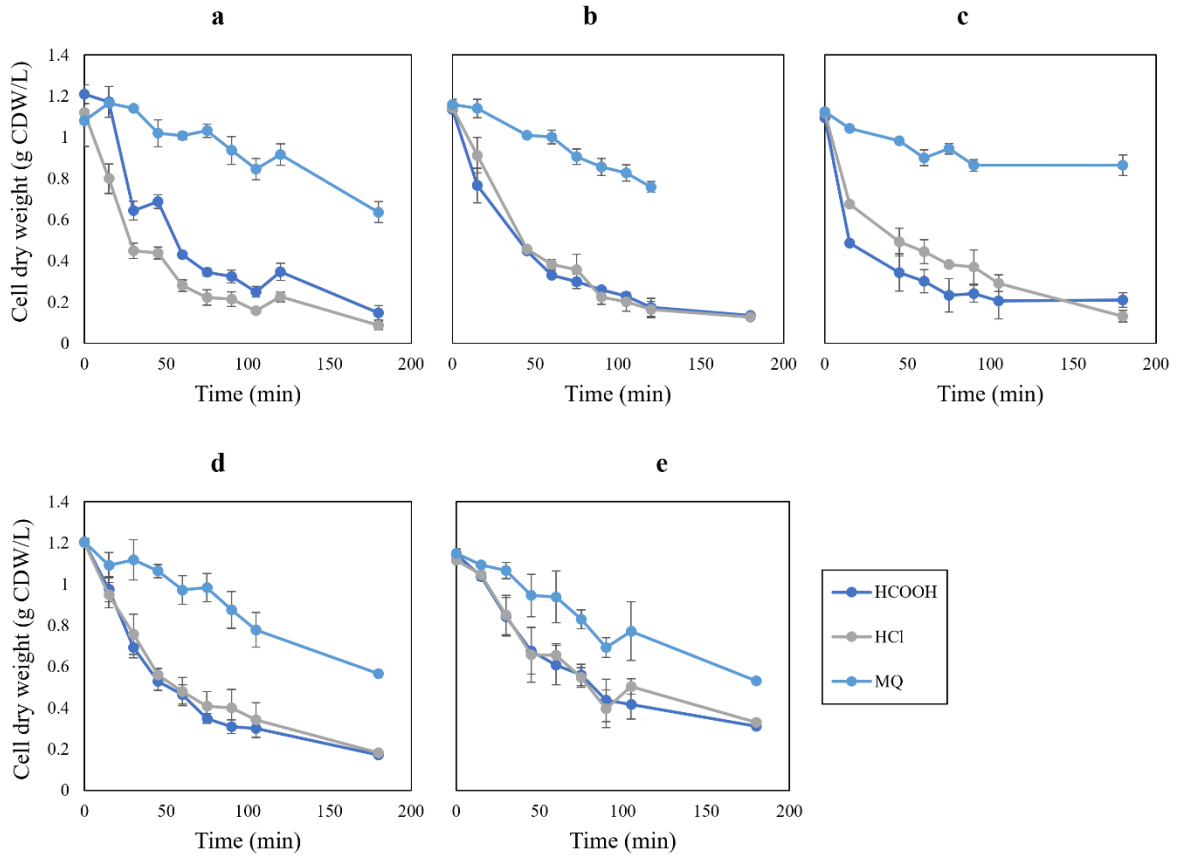


**Figure 24:** CDW (g/L) in the suspension throughout time using acid treatment and respective controls at pH 2.5 (a), 3 (b), 3.5 (c), 4 (d) and 4.5 (e).

Chapter 5

# Discussion of results

## 5.1 Optimal temperature and MeOH concentration for microbial growth and protein content

The preliminary tests performed on 96-well plates provided information on the temperature (28-32 ºC) and MeOH concentration (0-1% v/v) to be used in the bioreactor experiments for all types of culture (*M. extorquens*, *K. phaffii* and their co-culture). Regarding temperature, 30 ºC was the optimal temperature for growth and protein content in *M. extorquens* grown on MMMV medium. Although this not statistically observed in the experiments using AMS medium, the average protein content was higher for all cultures at 30 ºC (Figure 6).

Besides this temperature being the recommended optimal growth temperature for the *M. extorquens* strain used in this study, a literature review on its cultivation for SCP production in lab and industrial scale has also shown the common use of this temperature (Feinberg & Marx, 2014; Saville et al., 2015; Tlusty et al., 2017a). Regarding *K. phaffii*, although the recommended optimal temperature for growth is 28 ºC, this species is known for having great thermo- and osmo-tolerance (Bernauer et al., 2021) and various strains have been industrially grown at 30 ºC leading up to high cell densities (E. H. Wegner, 1983). This further demonstrates that this temperature does not negatively affect cell growth compared to 28 ºC. However, not always high growth rates are followed by high protein content (Klumpp et al., 2009) and not enough studies are investigating the effect of cultivation temperature on the protein content of microorganisms.

The substrate concentration also plays an important role in microbial growth and protein content since MeOH can be toxic. The results observed in this thesis did not show toxicity for *M. extorquens* grown on MeOH concentrations between 0-1% (v/v) or a statistically significant difference in protein content. For *K. phaffii*, growth decreased as MeOH concentration increased, with protein content being the lowest for 0.9% (v/v) followed by 1% (v/v). However, growth was also observed for replicates without MeOH which means conclusions from this assay should be taken critically. Due to this problem, decision on the MeOH concentration to be used in the bioreactor experiments was also based on literature review. Research has shown that growth of *M. extorquens* AM1 is completely inhibited on concentrations above 5% (v/v) (Cui et al., 2018). Another study defines optimal growth of *M. extorquens* at 1% (v/v) (Belkhelfa et al., 2019). Industrially, growth of this strain is commonly

done using 0.5% (v/v) of MeOH (Feinberg & Marx, 2014; Saville et al., 2015; Tlusty et al., 2017a). On the other hand, methylotrophic yeasts are not as resistant to high methanol concentrations. The inhibitory minimal concentration differs between studies, with some suggesting that MeOH concentration should not exceed 0.5% (v/v) (Abou-Zeid & Baghlaf, 1983) and others stating it should be between 0.4-3% (v/v) (Stratton et al., 1998). For maximum protein concentration, another study has suggested concentrations of between 0.005 to 0.5% (v/v) for *K. phaffii* (E. H. Wegner, 1983). Having in mind these results and the fact that intermediate concentrations also led to higher protein content in the yeast strain (although not statistically significant), 0.5% (v/v) was chosen as the MeOH concentration to be used in the bioreactor experiments for all cultures.

As mentioned before, it is important to look at these conclusions critically since there were several problems in using 96-well plates. Problems with mixing, evaporation, condensation, cross-contamination, and differences in results between inner to outer wells due to different oxygen transfer rates are common in 96-well plates experiments (Auld et al., 2004), leading to limited and oscillating growth throughout time. Particularly since MeOH is a highly volatile substrate, in future research it is recommended that preliminary tests should be performed on glassware such as Erlenmeyer flasks to decrease evaporation and oxygen limitation. Furthermore, although clear growth curves were observed for one of the cultures (*M. extorquens*), it is important to mention the low CDW (up to 0.4 g CDW/L) obtained, which also indicates the difficulty of microbial growth in these plates. Research has shown low and inconsistent growth of this bacterium on 96-well plates and has seen the choice of medium playing a crucial role in decreasing the OD oscillating values (Delaney et al., 2013). This was an important finding that motivated the investigation of other media in the microbial growth of the cultures used.

## 5.2 Role of medium composition on microbial growth and protein content

Different media (DSMZ1629, MMM, MMMV and AMS) were tested in *M. extorquens* and *K. phaffii* cultures using either 96-well plates, Erlenmeyer flasks or a bioreactor. Analysis of flow cytometry results from the bioreactor samples was also able to provide some insight on this effect between AMS and MMMV medium.

The results showed that *M. extorquens* was unable to grown on DSMZ1629, even though it has been a standard medium used for the *Methylobacterium sp* and *M. extorquens* (Belkhelfa et al., 2019). This might be related to the high concentrations of phosphate buffer and EDTA chelator, both which have previously shown to inhibit *M. extorquens* growth (Delaney et al., 2013). Another conclusion is that MMMV performed better than MMM, which is likely related to the addition of vitamins, that are

known to stimulate yeast growth (Matthews et al., 2018) and of some methylotrophic bacteria (Iguchi et al., 2011). Besides these conclusions, the media behavior varied significantly across the multiple tests.

The first experiment performed in the flasks showed no significant difference on microbial growth between MMM and AMS medium for both yeast and bacteria, but MMM provided 2 times increased growth in *M. extorquens* in the 96-well plates. Furthermore, final CDW (g CDW/L) was up to 7.6 times higher for MMMV in both strains in 96-well plates compared to AMS, but the opposite was seen in the bioreactor experiments using *M. extorquens*, in which AMS provided 3 times high CDW and significantly less damaged cells population (Figure 18). It is also important to mention that between bioreactor replicates of AMS-grown *M. extorquens*, the behavior was also vastly different (Appendix A.4.1). Although it was mentioned before that growing the microorganisms at a smaller scale causes many difficulties and that inconsistent behavior is not uncommon on the bacterial strain used (Carroll et al., 2014; Delaney et al., 2013), the medium composition is something that could have enhanced the issues of disparate growth.

The major difference between AMS and MMMV medium is the use of EDTA as a chelator of metal ions. This compound is known for inhibiting growth of *M. extorquens* by making these cations inaccessible for the cells (Delaney et al., 2013). These cations, particularly metal ones, are known cofactors of proteins and used for a variety of functions, such as aiding in the formation of the correct protein fold or forming the active site of an enzymatic reaction (Smith, 2015). The fact that AMS-grown bacteria were visually aggregated in the flasks (Appendix A.3.2), and the final singlets frequency was lower (about 50% of the population) compared to the MMMV-grow bacteria (up to 80%) further points at stress conditions provoked by the medium composition.

Another factor that could support this theory is the biomass composition of AMS-grown bacteria. Not only was protein content low (up to 25%$_{CDW}$) but the unmeasured components (e.g., lipids; PHB) were shown in highest percentage (49%$_{CDW}$) by the end of the experiment. Among these potential unmeasured components, PHB are intracellular storage compounds accumulated as energy reserve by some microorganisms under stress, generally induced when there is carbon in excess in the system (which happened till the very end of the experiments) but limitation of other nutrients such as nitrogen, sulphate, oxygen (Korotkova et al., 2002) and trace metals (Al Rowaihi et al., 2018). Besides the likely inaccessibility of metal ions mentioned before, there were problems with clogging of the aeration filter (especially in the replicate that lasted longer), which could have made oxygen unavailable to the cells as well.

High concentrations of PHB could also explain the flow cytometry gating results for AMS-grown bacteria, in which there was a seemingly yeast contamination (Figure 16). The distinction between strains was largely based on size. The average and maximum reported size of *M. extorquens* is 3.2 and 5.5 µm (Tim J. Strovas et al., 2007), respectively, whereas the average size of *K. phaffii* is between 4-6 µm (Pekarsky et al., 2018) but the length can go up to 8 µm (Suh et al., 2006). This does explain some possible overlaps but not entirely the signals observed in the higher intensities of the graphs. On the other hand, it is interesting that centrifuging the culture of AMS-grown bacteria did not show this possible yeast contamination while the co-culture final biomass exhibited a petal-like pellet of pink and yellow (Appendix A.4.5). Therefore, this observation could be ascribed to the naturally heterogenous bacterial populations which, even with genetical identical cells, can vary in biomass composition, leading to the co-existence of different phenotypes and metabolic specialization (Davis & Isberg, 2016; Karmann et al., 2017). This means that, while some bacteria were focused on growth and subsequent division, others could have been producing high quantities of PHB, significantly increasing their size, and moving the population up the gate. Either way, this theory can only be proven by examining the PHB content in the cells (e.g., using flow cytometry) and scanning for contamination (e.g., using PCR, microscopy).

Although this might explain the results obtained in AMS medium, the same cannot be said for the inconsistency in MMMV. In the reactor experiments, bacteria displayed a low range of protein content (7-14%$_{CDW}$), and barely observable increase of intact cell population, while the damaged cell population generally increased, reaching up to 60% of the population (Figure 18). Cell metabolism was occurring since full consumption of MeOH was observed (Figure 21), base was also consumed (Figure 20) and $pO_2$ decreased throughout time (Figure 19). Furthermore, the estimation of intact population was based on three gating strategies, in which some difficult was encountered in distinguishing between noise and bacteria (Appendix A.2.2), possibly overcounting initial noise. However, the rate of reproduction was undoubtedly lower than the accumulation of damaged cells. This was also observable in the consumption of nitrogen throughout time, which was almost undetectable (Figure 21), leading to negative nitrogen yields, that cannot be solely explained by the evaporation happening in the bioreactor. Not only can the lysis of bacterial cells lead to the release of inhibitory compounds (Hamer, 2011), it could also release some of the fixated nitrogen through deamination. Therefore, growth in this medium seems to have been unsuccessful at a larger scale.

From these experiments, it is not possible to know exactly what could have happened. While it can be attributed to the inconsistency of this strain, other experiments testing individual components of medium in the same culture would prove insightful to answer what is preventing this culture from

growing consistently and in what way can the medium optimized. Previous work has shown that concentrations of components such as calcium, phosphate, sodium, potassium, and magnesium influence cell growth (Kim et al., 2003). For example, MMMV had lower concentration of $Ca^{2+}$ (Appendix A.4.2) which aids in substrate oxidation by ensuring correct configuration of MeOH dehydrogenase (Zheng & Bruice, 1997). Furthermore, none of the media tested in this study had lanthanides, which have been associated with methanol oxidation, increasing MeOH dehydrogenase activity up to 10-fold (Semrau et al., 2018), and have been known to increase growth rate (up to 22%) and yields (up to 15%) (Good et al., 2018). Therefore, its addition could improve the results shown here.

This future work would prove essential to improve the extremely low protein content (up to 25%$_{CDW}$). Bacteria have reported protein concentrations of 50-80% (Table 1), while bacterial methylotrophs specifically (Table 2) have contents between 60-70%, which is up to 4 times higher than reported here. It is important to mention that this low protein content is also related to the protein analysis method performed, which is very susceptible to errors related to inefficient mixing and low CDW. Further analysis of the samples, using methods such as flow cytometry, would prove essential to have a more accurate assessment of the biomass composition.

Although the intention in this thesis was to evaluate parameters to maximize this protein content (temperature, MeOH concentration, medium composition) a more ambitious route would be to use genetically modified strains. However, studies still need to provide more information on long-term animal feeding of such GMOs and evaluate impacts on human health (Hilbeck et al., 2015). This would be fundamental to speed up the long process of approval, not only by certified agencies but also by the public.

## 5.3 Comparison between mono and co-cultures for SCP production

Batch experiments showed similar behaviors between the co-culture and *K. phaffii* regarding all parameters (protein content, CDW and biomass composition), with protein content and final CDW being up to 3.2 times higher than the *M. extorquens* monoculture grown on the same medium. Growth phase influenced protein productivity, with all cultures displaying average values of protein content higher in the exponential phase than the stationary phase (Figure 12). This decrease in protein content also coincides with the depletion of MeOH from the medium (Figure 21) which might indicate amino acid catabolism in the face of carbon limitation (Ljungdahl & Daignan-Fornier, 2012) and the proteolytic degradation of proteins from the increasing damaged cell population.

By the end of the experiment, *K. phaffii* had the highest protein content (44%$_{CDW}$), followed by the co-culture (39%$_{CDW}$) and finally *M. extorquens* (14%$_{CDW}$). Regarding the yeast strain results, this is similar to reports of protein content between 45-55%$_{CDW}$ for yeast (Table 1) and not far from industrially grown *K. phaffi* values (55%, Table 2). Furthermore, although the protein content of the co-culture was lower than *K. phaffii*, this was not statistically significant. Research on this is unclear: there has been reports of up to 4 times increase in protein content compared to individual cultures (Goldberg, 1985; Rajoka et al., 2012) but there is also been reports of similar protein content to one of the individual strains (Arkronrat et al., 2016). This is very dependent of the strains used and what is the behavior between them. Microorganisms can either (1) compete both for the same substrate, (2) one of the strains can grow on the primary substrate and produce organic compounds that serve as carbon source for the remaining strains, (3) can have mutual supplementation of nutrients or (4) antagonism.

In this case, possibly three of these theories can be observed, except for antagonism. First, both strains compete for the same substrate (MeOH) but does not seem to negatively impact their growth. Although bacteria originally dominate numerically the inoculum, the yeast quickly catches up, reaching the same final intact cells number than observed in the monoculture (Figure 17). Furthermore, bacteria grow 14 times more than their initial concentration in the co-culture, which means that the presence of the yeast likely aided in their growth. Acetate and formate were present in the yeast mono-culture replicates but non-detectable in the co-culture results. This might have indicated that *M. extorquens* was consuming these compounds and using them as substrates, facilitating a cooperation relationship at later timepoints. A carbon shift between C1 compounds (methanol/formate) and C2 compounds metabolism (ethanol/acetate) can lead to different subpopulations in *M. extorquens AM1* (Strovas & Lidstrom, 2009) and could potentially have stressed-induced *K. phaffii* into different phenotypes (Holland et al., 2014), which was seen in the secondary gating of the co-culture samples (Figure 15).

By the end of the experiment, the strains were in almost equal percentages (Figure 18), although the bacteria still showed high levels of damaged cells that might have contributed to the lower levels of the final protein content of the co-culture.

## 5.4 Evaluating SCP process efficiency and sustainability

For all cultures and media used in the batch experiments, the carbon source was fully consumed by the end of the experiment, indicating carbon limitation, whereas nitrogen was only partially consumed. This is the ideal scenario in the context of SCP since nitrogen is needed for protein

production. However, the nitrogen concentration in the medium can be further reduced to minimize waste in the overall process while assuring an optimal C:N ratio for growth and protein content. This parameter is especially relevant in SCPs from yeasts, in which the recommended ratio for high protein concentration should be between 7:1 and 10:1 (Upadhyaya et al., 2016). Since the ratio was 7:1 in the *K. phaffii* experiments using MMMV medium, there is a possibility to reduce the nitrogen concentration.

It is also important to mention that the presence of ethanol coming from sterile sampling and bioreactor handling. This compound was also used as a carbon source since both methylotrophs can metabolize it into acetaldehyde and acetate for acetyl-CoA synthesis. Even though it supports lower growth rates for methylotrophs, it is quickly consumed when present in the system. A study with mixed feed of ethanol and MeOH on methylotrophic yeast showed that ethanol was firstly steadily consumed and only after MeOH was utilized (Sibirny et al., 1991). Although it would be interesting to test the effect of using both substrates in SCP production, this goes beyond the scope of this thesis. For future research at lab scale using pure cultures grown on MeOH, careful handling of the bioreactor is required to prevent ethanol dosing.

While MeOH is consumed, the pH drops and NaOH is dosed to maintain pH around 7.0. This was successfully done through all experiments, with *K. phaffii* consuming more base (3 times more than the co-culture, and 2 times more than the bacteria) due to its faster growth. An interesting approach for further studies would be to substitute the use of external chemicals (in this case, NaOH) with other bases that could also serve a double purpose. For example, by using $NH_4OH$ it could be possible to regulate the pH and use it as a nitrogen source. This is not uncommon in microbial fermentation: carbon and nitrogen sources are often also used as pH regulators (García-Arrazola et al., 2005; Yin et al., 2019).

By following the consumption of the carbon sources and nitrogen, biomass and protein yields were also calculated. As expected, MMMV-grown bacteria performed poorly in all calculated yields. On the other hand, AMS-grown bacteria showcased significantly better yields (up to 7.4 times), resulting in a biomass yield of 0.20 g CDW/g COD consumed. This value is close to the range of 0.20-0.23 g CDW/g COD (0.30-035 g CDW/ g MeOH) reported in literature (Bélanger et al., 2004; Peyraud et al., 2012). Regarding *K. phaffii*, the biomass yield of 0.18 g CDW/g COD consumed was similar to previous reported values belonging to genetically engineered strains growing on MeOH at 30ºC, between 0.10-0.28 g CDW/g COD consumed (0.15-0.42 g CDW/ g MeOH) (Looser et al., 2015). In general, methylotrophic monocultures have showed biomass yields up to 0.29 g CDW/g COD

consumed (0.44 g CDW/ g MeOH) (Abou-Zeid & Baghlaf, 1983; Vigentini et al., 2005), meaning there is space for improvement.

Regarding the co-culture's yields, although it had a similar protein yield to *K. phaffii* regarding consumed substrate, the biomass yield was better for the co-culture (1.4 times). This means that the conversion of the substrate into CDW was more efficient, potentially due to the cooperation between species mentioned previously. Furthermore, since *M. extorquens* demonstrated signs of growing poorly in this medium, using other strains or optimizing the medium composition could be the answer for improved yields.

In previous research, synthetic microbial communities showed increased biomass and protein yields, as well as improved stability, preventing contamination better than pure culture (Goldberg, 1985; Harmand et al., 2019; Rokem et al., 1980), which is particularly important in a continuous process. Furthermore, not only improved yields are important, but also the facilitating of the down streaming process at the fermentation level. If more aggregates are present, the easier it is to extract the biomass form the culture medium. The co-culture followed by the monoculture of *K. phaffii* exhibited lower frequency of singlets regarding total events when compared to AMS- and MMMV-grown bacteria, with less than half of the population (Figure 14). This further confirms the importance of selecting yeast strains for cost-effectiveness of the process.

Although these batch experiments provided a better understanding of the effect of culture and medium composition on the various parameters of SCP quality, this is far from what is possible to achieve in continuous industrial production, which generally supports higher productivities (Goldberg, 1985). For example, a continuous process of SCP production from *K. phaffii* can achieve values of 130 g CDW/L and productivities of 10 g $L^{-1}$ $h^{-1}$, 100 and 1000 times higher, respectively, than achieved in this study (G. H. Wegner, 1990). Therefore, moving to a continuously operated process would prove beneficial for increased yields.

## 5.5 Acid precipitation for biomass harvesting

The acid precipitation assay performed on *K. phaffii* over the course of 3 hours lead to significant biomass recovery in the pellet using hydrochloric acid and formic acid with a maximum value of 92 $\%_{CDW}$, almost twice of the amount achieved by natural precipitation (54$\%_{CDW}$). The type of acid did not significantly influence the biomass precipitation, except at pH=3.5 in which hydrochloric acid performed 9% better. It would be then plausible to assume that other acids could work just as efficiently but further tests should be performed to test this theory. When choosing an acid, its strength and origin are important. For example, hydrochloric acid is a stronger acid (pKa = -6.3) than formic

acid (pKa = 3.75) which meant more formic acid was added to obtain the same pH. The more acid is needed, the greater the costs, which could favor the use of hydrochloric acid. However, using a pure inorganic chemical is not as sustainable as using an organic acid recovered from other bioprocesses (e.g. organic acids produced during anaerobic digestion).

If the type of acid did not contribute significantly to the difference in biomass harvesting, it would be expected that the pH did. However, it was only possible to observe that the highest pH (4.5) did not perform well compared to all the other conditions (2, 2.5, 3, 3.5 and 4). Although this made it difficult to conclude whether higher pH or lower pH work better, it is possible to infer that it should be in the range of 2.5-4. Besides performance itself, the aim of reducing production costs, the potential negative effects of acid in biomass quality and corrosion of the equipment (Salgar-Chaparro et al., 2020), further favors the use of the higher pH values in this range, since less acid is added.

Another important factor tested was incubation time, which should be minimized at an industrial level to maximize productivity. Acid precipitation seemed to stabilize after 75 to 90 min for the suggested optimal range. Between the last time point (180 min) and the previous (105 min), 4 to 24%$_{CDW}$ of the biomass recovery happened in the acid precipitation samples whereas up to 70%$_{CDW}$ was observed in the MQ controls. This suggests that natural sedimentation occurred between this period and the effect of the acid took place until stabilization first occurred. Therefore, when thinking of scaling-up this process, it would be important to see what is more cost-effective, conjugating simultaneously time and recovered biomass. If the effect of acid is the only interest, then 90 min is enough time for the precipitation to take place.

Thus, this study proved not only that acid precipitation is faster than natural sedimentation, but it is also more efficient, which is a very promising conclusion, related to the fact that this process has not been done to precipitate biomass for SCP production. Base or acid precipitation is a method usually applied in the precipitation and purification of proteins derived from plant and animal raw materials. It has also been performed to purify proteins from commercially available microalgae *Nannochloropsis oculata* (Cavonius et al., 2015). On the other hand, purification and extraction of biomass for SCP is usually done through solid-liquid separation (centrifugation, microfiltration) (Sillman et al., 2019) followed typically by other treatments such as freeze- or spray-drying (Morgan et al., 2006). Acid pretreatment could prove a valuable alternative to other solid-liquid separation methods since it does not require energy or suffer from membrane fouling. However, since biomass recovery in these conventional processes is typically between 95-99%, which is higher than shown through acid precipitation, a combination between these methods would likely aid in improving the

cost-efficiency. For example, other processes with lower biomass recovery values, such as chemical flocculation (80-90%), were previously shown to be economically competitive when combined with more efficient processes (e.g., microfiltration) (Ma et al., 2016).

Further tests with different acids (recovered or otherwise) and full assessments comparison between methods should be performed to understand the potential of this process.

# Chapter 6

# Conclusions and future work

Lab-scale production of SCP on methanol was successfully performed through mono and co-cultivation of *M. extorquens* and *K. phaffi* in batch mode. Initial tests were performed to assess the optimal cultivation parameters (temperature, MeOH concentration, and medium composition) using 96-well plates but were mostly inefficient due to the constant evaporation, condensation and mixing problems shown at this scale. Regarding medium composition, a new modified medium (MMMV) showed up to 8 times more final CDW than other reported media (DSMZ1629, MMM, AMS) at small scale, but the same was not observed at the bioreactor scale, where AMS resulted in 3 times higher final CDW. Furthermore, AMS-grown bacteria showed signs of stress, with up to 50% in aggregates, and the potential accumulation of PHB. On the other hand, MMMV-grown bacteria demonstrated poor growth throughout time, with increasing damaged cells population (up to 60% of the population). Both AMS-grown and MMMV-grown *M. extorquens* performed poorly in terms of protein content (up to 25%$_{CDW}$) compared to the yeast monoculture (up to 44%$_{CDW}$), with the latter comparing favorably with literature. Similar behavior between the co-culture and the yeast co-culture was observed in terms of growth (up to 3.2 times higher than bacteria) and protein content. However, the co-culture exhibited significantly higher biomass yield (1.4 times). Through flow cytometry, bacterial and yeast population were distinguished in the co-culture, and it was possible to observe that the presence of yeast likely aided in bacterial growth.

To reduce production costs, a novel acid precipitation assay for biomass harvesting was performed on a *K. phaffii* culture. Biomass precipitation was independent of the type of acid (formic and hydrochloric acid) and of the pH used (optimal range between 2.5-4), leading to a maximum biomass recovery value of 92% (CDW), almost twice of the amount achieved by spontaneous precipitation (54%$_{CDW}$).

This thesis provided a detailed analysis of the behavior mono- and co-cultures on methanol and evaluated innovative ways to decrease the production costs. Although this route is promising for SCP production to be competitive with traditional protein sources, there is tremendous space for further improvement of yields and protein content. For example, one of the strains used displayed an inconsistent behavior and only batch experiments were performed, which is industrially unpromising. Further work should focus on the following steps:

1. Analysis to check for possible contaminations (validate the presented data)

2. Analysis of the amino acid profile (assess nutritional quality)
3. Analysis of the nucleic acid content (assess the need for costly RNA removal strategies)
4. Analysis of added-value compounds such as PHB and carotenoids (compare the cost-effectiveness of other biomass end-products)
5. Selection of other strains and co-culture (improve yields and nutritional quality)
6. Evaluation of the co-feeding of other substrates along methanol (e.g., formate and hydrogen to improve yields and nutritional quality)
7. Optimization through continuous mode (assess impact on co-culture dynamics and yields)
8. Medium optimization through scanning of inhibitory compounds/concentrations (improve yields and reduce the media supplementation costs)
9. Replace dosage of external chemicals through integrated pH strategy (e.g. using $NH_4OH$ as both nitrogen source and base)
10. Combine acid precipitation with other downstream processes (assess cost-effectiveness in a bigger picture)
11. Develop a product with favorable organoleptic properties (assess marketability)
12. Increase knowledge on the effects of SCP in food and feed regarding long-term consumption (assess health concerns)

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# Appendix

## A.1 Media Composition

### A.1.1 Nutrient Broth & Agar + 1% MeOH

**Table A1:** Composition of Nutrient Broth and Agar containing 1% MeOH.

| Component | Concentration (g/L) |
|---|---|
| **Peptone** | 5.0 |
| **Meat extract** | 3.0 |
| **Methanol** | 0.1 |
| **Agar** | 15 |

Adjust pH to 7.0 using 5M NaOH.

### A.1.2 Sabouraud Dextrose Agar

**Table A2:** Sabouraud Dextrose Agar composition.

| Component | Concentration (g/L) |
|---|---|
| **Dextrose (glucose)** | 20 |
| **Peptone** | 10 |
| **Agar** | 50 |

### A.1.3 AMS

Prepare the following stock solutions:

**Table A3:** Composition of the stock solutions used for AMS medium.

| Stock solution | Component | Concentration (g/L) |
|---|---|---|
| **Salts (A)** | $MgSO_4.7H_2O$ | 10 |
| | $NH_4Cl$ | 5 |
| | $CaCl_2.6H_2O$ | 1.5 |
| **FeNaEDTA (B)** | FeNaEDTA | 5 |
| **Basic Buffer (C)** | $Na_2HPO_4.2H_2O$ | 35.63 |
| **Acid Buffer (D)** | $K_2HPO_4$ | 27.2 |
| **Trace Solution (E)** | $Na_2EDTA.2H_2O$ | 0.5 |
| | $FeSO_4.7H_2O$ | 0.2 |
| | $H_3BO_3$ | 0.03 |
| | $CoCl_2.6H_2O$ | 0.02 |
| | $ZnSO_4.7H_2O$ | 0.01 |
| | $MnCl_2.4H_2O$ | 0.003 |
| | $NaMoO_4.2H_2O$ | 0.003 |
| | $NiCl_2.6H_2O$ | 0.002 |
| | $CuSO_4.5H_2O$ | 2.5 |
| **Vitamin solution** | Riboflavin | 0.1 |
| | Thiamine-HCl x $2H_2O$ | 0.5 |
| | Nicotinic Acid | 0.5 |
| | Pyridoxine-HCl | 0.5 |
| | Ca-pantothenate | 0.5 |
| | Biotin | 0.001 |
| | Folic Acid | 0.002 |
| | Vitamin B12 | 0.01 |

The stock solutions should be autoclaved separately to avoid precipitation except for vitamins, which are filter-sterilized. To prepare a twice concentrated AMS media, it is necessary to add 200 mL of solution A, 2 mL of solution B, 20 mL of solution C, 20 mL of solution D, 2 mL of solution E and 10 mL of vitamin solution to 750 mL of distilled water.

### A.1.4 MMM & MMMV

Prepare the following stock solutions:

**Table A4:** Composition of MMM medium.

| Stock solution | Component | Concentration (g/L) |
|---|---|---|
| **P solution** | $NaH_2PO_4.H_2O$ | 25.9 |
| | $K_2HPO_4.3H_2O$ | 33.1 |
| **$MgCl_2$** | $MgCl_2.6H_2O$ | 406.6 |
| **$(NH_4)_2SO_4$** | $(NH_4)_2SO_4$ | 2464.28 |
| **$CaCl_2$** | $CaCl_2.2H_2O$ | 294.04 |
| **C7 metals** | Sodium citrate | 13.41 |
| | $ZnSO_4.7H_2O$ | 0.345 |
| | $MnCl_2.4H_2O$ | 0.198 |
| | $FeSO_4.7H_2O$ | 0.500 |
| | $(NH_4)Mo_2O_{24}.4H_2O$ | 2.471 |
| | $CuSO_4.5H_2O$ | 0.250 |
| | $CoCl_2.6H_2O$ | 0.476 |
| | $Na_2WO_4.2H_2O$ | 0.109 |

Autoclave stock solutions separately before adding to 1 L of 2x concentrated media. For MMM, add 100 mL of P solution, 0.500 mL of MgCl2 solution, 8 mL of (NH4)2SO4 solution, 0.002 mL of $CaCl_2$ solution and 2 mL of C7 metals solution to 970 mL of distilled water. For MMMV, also add 10 mL of the vitamin solution described above to 960 mL of distilled water.

### A.1.5 DSMZ1629

DSMZ1629 media was prepared 2x concentrated by adding the following components to 1L of distilled water:

**Table A5:** Composition of DSMZ1629 medium.

| Component | Concentration (g/L) |
|---|---|
| **$NH_4Cl$** | 3.24 |
| **$MgSO_4.7H_2O$** | 0.40 |
| **$K_2HPO_4$** | 4.80 |
| **$NaH_2PO_4.2H_2O$** | 2.20 |
| **$Na_2EDTA$** | 0.03 |
| **$FeSO_4.6H_2O$** | 0.006 |
| **$ZnSO_4.7H_2O$** | 0.009 |
| **$CoCl_2.6H_2O$** | 0.006 |
| **$MnCl_2.4H_2O$** | 0.00128 |
| **$H_3BO_3$** | 0.002 |
| **$Na_2MoO_4.2H_2O$** | 0.0008 |
| **$CuSO_4.2H_2O$** | 0.0006 |
| **$CaCl_2.6H_2O$** | 0.006 |

Although the recipe suggests adjusting pH to 7.1, this was not done. The 1L bottle was autoclaved and after cooling, used for the media experiments.

## A.2 Calibration curves for CDW (g/L) calculation

### A.2.1 *M. extorquens* and *K. phaffii*

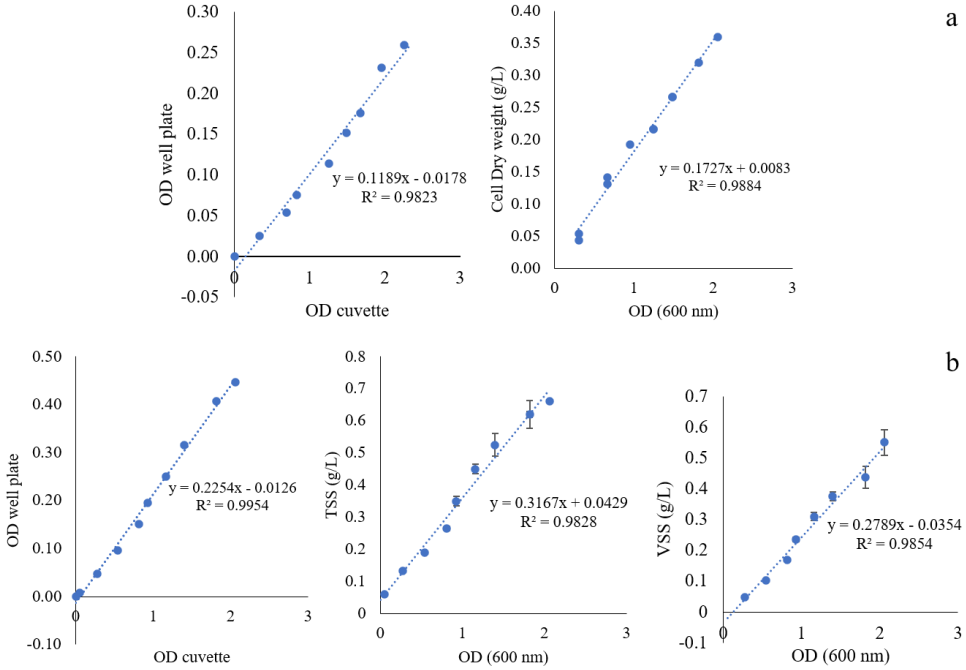


**Figure A1:** Calibration curves used for *K. phaffii* (a) and *M. extorquens* (b).

### A.2.2 Co-culture of *M. extorquens* and *K. phaffii*

CDW (g/L) for the culture was calculated based on the mass ratio between bacteria and yeast in the bioreactor experiments. It was necessary to obtain the cell counts for each strain through flow cytometry. First, primary (**Figure A2**a) and secondary gating (**Figure A2**b) was performed in the yeast and bacteria monocultures. Lastly, distinction between intact and damaged cells was also done ((**Figure A2**c). Through these three consecutive filters, it was possible to calculate the average ratio between the two strains in the co-culture samples.

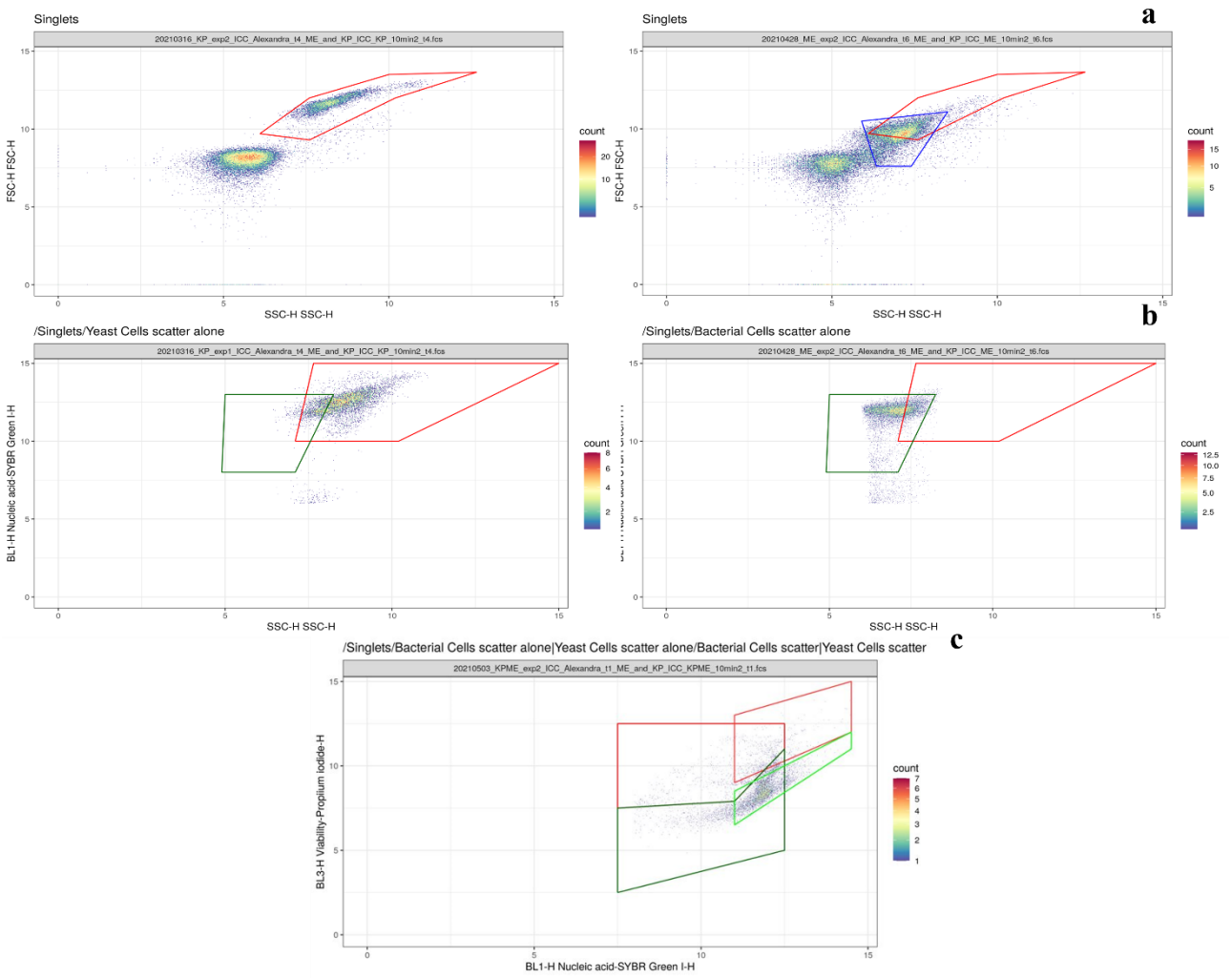


**Figure A2**: Examples of primary gating (a) and secondary gating (b) for *K. phaffii* (left) and *M. extorquens* (right) samples from the monocultures. The blue or green gating refers to bacteria and the red to yeast. Finally, an example of tertiary gating is shown for a co-culture sample taken after 14.5h. The red gating refers to damaged cells and the green to intact cells.

This ratio of size was correct to mass ratio (m/m) by following the assumptions.

1. *M. extorquens* has an average reported size of 3.2 µm(T. J. Strovas et al., 2007)
2. *K. phaffii* has an average reported size of 5 µm (Gmeiner et al., 2015)
3. Both cells have a sphere shape
4. The volume ratio is the same as the mass ratio

This resulted in a co-culture containing 19% of *M. extorquens* and 81% of *K. phaffii*. CDW (g/L) was then calculated using the OD-TSS (g/L) calibration curves of each having in mind this ratio.

## A.3. Preliminary tests

### A.3.1 pH measurements on 96-well plates

The pH was measured throughout the preliminary tests in 96 well-plates to confirm significant or insignificant growth of the cultures.

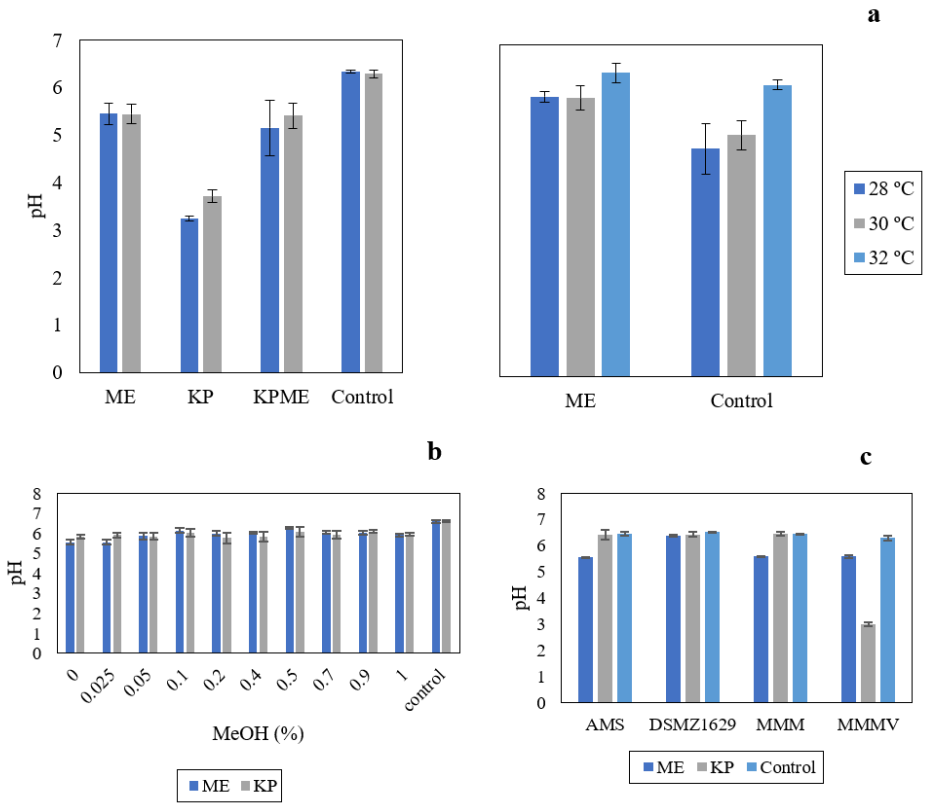


**Figure A3:** Final pH for K. phaffii (KP), the co-culture (KPME) and M. extorquens (ME) grown on AMS (a, left) and MMMV (a, right) at different temperatures (28, 30 and 32 ºC), at different MeOH (%) concentrations and on different media (c) in 96-well plates.

## A.3.2 Effect of medium composition on *M. extorquens* culture consistency

**Figure A4:** Erlenmeyer inoculated with *M. extorquens* in AMS (left) or MMMV (right).

# A.4. Bioreactor experiments

## A.4.1 AMS replicates

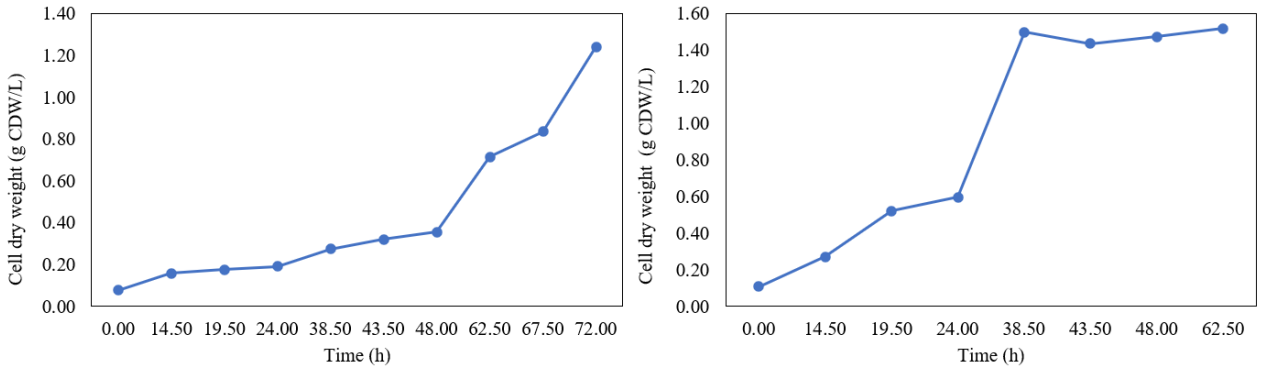


**Figure A5:** CDW (g CDW/L) *M. extorquens*' replicated experiments grown on AMS medium.

## A.4.2 Protein productivities

The protein productivities were calculated from $t_1$ until $t_4$ based on the difference in protein content (mg/L) between the timepoint in question and the one before for each tested culture: *K. phaffii* (KP), *M. extorquens* (ME) and the co-culture (KPME). Negative productivities after $t_5$ were neglected for statistical analysis since the cultures were not growing exponentially anymore and thus were not active.

**Table A6:** Protein productivities of *K. phaffi* (KP), *M. extorquens* (E) and their co-culture (KPME) at different timepoints throughout the batch experiments.

| | | Productivity (mg/L/H) | | | |
|---|---|---|---|---|---|
| **Time point** | Time(h) | KP | KPME | ME in MMMV | ME in AMS |
| **$t_1$** | 14.5 | 4.8±0.2 | 3.8±1.1 | 1.02±0.7 | 1.6±1.5 |
| **$t_2$** | 19.5 | 2.6±0.7 | 2.5±0.4 | 0.82±0.3 | 0.8±1.0 |
| **$t_3$** | 24.0 | 12.1±0.5 | 3.8±2.9 | 0.1±0.0 | 1.6±1.8 |
| **$t_4$** | 38.5 | 3.0±2.5 | 10.5±3.4 | 0.2±0.3 | 1.8±1.5 |
| **$t_5$** | 43.5 | 0.5±0.7 | -1.9±4.7 | 0.0±0.7 | 1.7±2.1 |
| **$t_6$** | 48.0 | | -1.5±2.9 | -0.1±0.6 | -0.2±0.6 |
| **$t_7$** | 62.5 | | | | 0.4±1.1 |
| **$t_8$** | 67.5 | | | | 1.1 |
| **$t_9$** | 72 | | | | 0.7 |

## A.4.3 Concentration of anions and cations

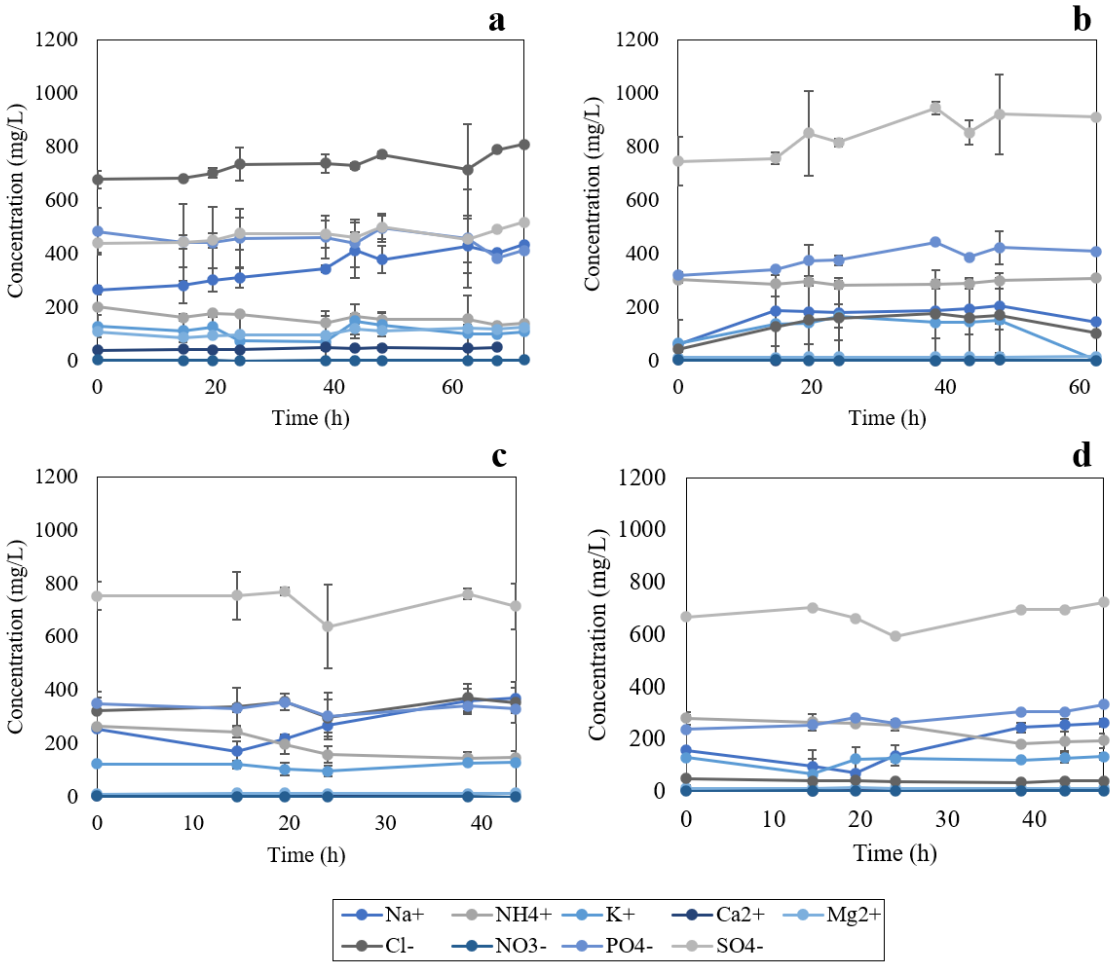


**Figure A6:** Concentration of cations and anions throughout time for *M. extorquens* grown on AMS (a), *M. extorquens* grown on MMMV (b), *K. phaffii* (c) and their co-culture (d).

## A.4.5 Co-culture behavior on MMMV-grown medium

**Figure A7:** Co-culture "petal-like" pellet in a sample taken at the end of the experiment.

## A.6 Acid precipitation assay

**Table A7:** P values obtained through Tuckey test when comparing biomass recovered in the pellet in formic acid (FA), hydrochloric acid (HCl) treatment and control samples (MQ) across multiple pH values.

| | pH | p value |
|---|---|---|
| 2.5 | FA-HCL | 0.82 |
| | HCL-MQ | <0.01 |
| | FA-MQ | <0.01 |
| 3 | FA-HCL | 0.27 |
| 3.5 | FA-HCL | <0.01 |
| | HCL-MQ | <0.01 |
| | FA-MQ | <0.01 |
| 4 | FA-HCL | 0.9 |
| | HCL-MQ | <0.01 |
| | FA-MQ | <0.01 |
| 4.5 | FA-HCL | 0.99 |
| | HCL-MQ | 0.01 |
| | FA-MQ | 0.01 |