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Algae to yeast biorefinery system: Closing the nutrient cycle for a sustainable production of mannosylerythritol lipids (MELs) and organic acids from waste streams

Miguel Figueiredo Nascimento

Supervisor: Doctor Frederico Castelo Alves Ferreira

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Thesis approved in public section to obtain the PhD degree in

Biotechnology and Biosciences.

Jury final classification: **Pass with Distinction**

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2025

Aos meus pais, Ernesto e Cristina, e à minha irmã (e melhor amiga) Leonor, pelo amor e apoio incondicional. Obrigado por nunca me deixarem desistir.

Resumo

Os lípidos de manosileritritol (MELs) são glicolipídicos produzidos por leveduras do género *Moesziomyces*, que se apresentam como uma alternativa promissora aos tensioativos químicos. Os MELs podem ser produzidos partindo de fontes de carbono hidrofílicas (ex. açúcares, glycerol) e hidrofóbicas (ex. óleos vegetais, alcanos). Apresentam propriedades bioquímicas relevantes, como biocompatibilidade ou biodegradabilidade, mas a produção de MELs precisa de ser economicamente viável para que estes sejam competitivos no mercado.

Esta tese visou desenvolver novas soluções inovadoras para otimizar o bioprocesso de produção de MELs, tendo em atenção as emissões de CO_2. Encontra-se dividida em três seções: 1) Produção de MELs a partir de diferenres substratos; ,2) Intensificação e integração de bioprocessos MELs; e 3) Utilização de microalgas e leveduras em co-cultivação para produção de MELs e ácidos orgânicos.

O estudo da combinação de substratos (glucose e óleos vegetais) indicou elevados rendimentos e purezas de MELs, sendo estabelecida como estratégia base de produção. Diferentes resíduos agroindustriais, como soro de queijo e óleo de bagaço de azeite, foram testados. O primeiro foi utilizado como fonte de carbono, permitindo a substituição integral do meio de cultura, sem afetar a produtividade final. O segundo, foi usado em substituição de óleos vegetais ou residuais de fritura, resultando na produtividade mais elevada obtida nesta tese. Foi ainda proposta o uso de nanofiltração para purificat MELs, com perdas mínimas de produto. Finalmente, o bioprocesso MELs foi modelado, permitindo um estudo económico com identificação dos principais pontos críticos.

Na última secção foi investigada a co-cultivação entre microalgas e leveduras para diminuição das emissões de CO_2. A produção de ácido cítrico foi usada como caso de estudo para, identificar os principais parâmetros para obter uma co-cultivação de sucesso. Além disso, foi testado a produção de MELs usando lípidos de microalgas oleaginosas como fonte de carbono.

Palavras chaves: Lípidos de manosileritritol; co-cultivação; microalgas; acidos orgânicos; bioprocesso

Abstract

Mannosylerythritol lipids (MELs) are a class of glycolipid biosurfactants mainly produced by yeasts of the *Moesziomyces* genus,that present a promising alternative to chemically synthetised surfactants. MELs can be produced from a range of different substrates, including n-alkanes, hydrophilic (e.g. sugars) and hydrophobic carbon sources (e.g. vegetable oils). MELs have unique tensioactive properties, biocompatibility, and biodegradability, which make them one of the most promising microbial biosurfactants. However, MELs' market competitiveness requires their cost reduction. This thesis aims to develop new innovative solutions to enhance and optimise MELs bioprocess, while addressing the issue of CO2 emissions. Therefore, this thesis is dived into three main sections: 1,1MELs production from different substrates, 2) Intensification and integrations of MELs bioprocess; and 3) Use of microalgae and yeast in co-cultivation for production of MELs and organic acids.

Firstly, it was discovered that the combination of co-substrates (glucose and vegetable oils), leads to high MELs titres and purities, establishing the main condition for their production. Secondly, different industrial side-streams (Cheese-whey and pomace oil) were tested, revealing that cheese-whey can be used as carbon source and replacement of culture medium, without affecting final productivity. Using pomace oil as a replacement of waste frying oils, led to the highest productivity obtained in this thesis. Furthermore, a novel downstream route was proposed to obtain high MELs purities, with possibility of solvent recycling and minimal product losses. Finally, MELs bioprocess was modelled, performing and economic study and identifying main bottlenecks.

Moreover, it was also accessed the use of co-cultivation between microalgae and yeasts for decreasing of CO_2 emissions. A case study, was developed to produce citric acid to identify the main parameters required for successful co-cultivation, a knowledge transposable to co-cultivation bioprocesses for MELs production. Additionally, MELs were formed using lipids previously produced my oleaginous microalgae.

Keywords: mannosylerythritol lipids (MELs); co-cultivation; microalgae; organic acids; bioprocess

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First of all, I would like to convey my gratitude to my mentors, Professors Frederico Ferreira and Nuno Faria, for their enormous support during the work on this thesis. To Professor Ferreira, one of the most intelligent people I have ever met, for the passion that always bring to the table, and more important for the emotional intelligence and to always have the right words for my worst moments. And to Prof. Nuno Faria, which is not anymore a professor for me, but more a friend who I can rely in a variety of subjects. We have paved a long way, since talking about football, into business, and even mechanic problems in our cars. For me this is just the beginning of an incredible adventure.

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19 pandemic, the beginning in Leipzig was very tough. However, looking back, I couldn't be more proud what I have achieved in my personal development.

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“Nothing behind me, everything ahead of me, as is ever so on the road “

Jack Kerouac, On the Road

Abbreviations

CA – Citric acid

CDW – Cell dry-weight

CW – Cheese-whey

DAG – Diacylglycerides

DHA - Dihydroxyacetone

DO – Dissolved oxygen

DSMZ - Germany collection of microorganisms and cell culture

DV – Diavolumes

EPA - Eicosapentaenoic acid

EtOAc - Ethyl acetate

FAME – Fatty acids methyl esters

FAS – Fatty acid synthase complex

FFA – Free fatty acids

GRAS – Generally recognized as safe

MAG – Monoacylglycerides

ME - mannose-erythritol moiety

MeOH – Methanol

MIT - Massachusetts Institute of Technology

MTBE - tert-butyl methyl ether

MWCO – Molecular weight cut-off

NPV – Net present value

OMW – Olive-mill wastewater

OPNG - O-nitrophenyl-β-D-galactopyranoside

OSN – Organic solvent nanofiltration

PPFD - Photosynthetic Photon Flux Density

RMs – Rhamnolipids

RO – Rapeseed oil

ROI - Return on investment

rpm – Rotation per minute

SBO – Soybean oil

SDG - Sustainable development goals

SLs – Sophorolipids

SPD - SuperPro Designer

TAG - Triacylglyceride

VO – Vegetable oils

vvm – Volume of air per volume of culture medium per minute

WFO – Waste frying oils

YE – Yeast extract

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specific days (0; 2; 5; 7 and 10). After these days, yeast is added (0.01% Vinoculum/Vculture medium) along with NH4Cl (1.5 g/L), thiamine (1 mg/L), $FeSO_4.7H_2O$ (6.3 mg/L), glycerol (50 g/L) and trace elements (1mL). Co-cultivation was carried out for 240 h (10 days), followed by feeds of glycerol (20 g/L), to avoid a concentration below than 10 g/L. The temperature was kept at 26ºC, using an agitation of 130 rpm, and a light regime (light/dark) of 12:12, with an intensity of 60 μmol/(s·m²). pH was manually controlled at 6.5 every day, using 25 and 27% of H_2SO_4 and NaOH, respectively. 314

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initial % of C. vulgaris inoculum, 10% (A, B) and 20% (C, D). The co-cultivation process began by adding 0.01% of yeast (Vinoculum/Vculture medium) along with NH_4Cl (1.5 g/L), thiamine (1 mg/L), $FeSO_4.7H_2O$ (6.3 mg/L) and crude glycerol (50 g/L). Co-cultivation for the 10% inoculum started on the 5th day, while for the 20% inoculum, it started on the 3rd day. The duration of cultivation was 354 hours and 364 hours, respectively. Glycerol feed of 20 g/L was added at 213, 282, and 330 hours for the 10% inoculum, and at 165 and 239 hours for the 20% inoculum. The temperature was maintained at 26ºC with an air-flow rate of 2 vvm and agitation at 120 rpm using a magnet. The pH was automatically controlled at 6.5 with 3% NaOH solution. The light regime was set at 12:12 (light/dark) with an intensity of 500 µmol/(s·m²). Ion NH4+ (squares), ions NO3- (inverted triangles), yeast (hexagons) and algae cell number (hexagons) and (A, C); Glycerol consumption (circles) and citric acid production (triangles) (B, D). Arrow dashed line indicates the beginning of co-cultivation (addition of yeast to the bioreactor). These experiments do not have replicates. 319

Section A: Introduction and state of the art

A.1 Introduction

A.1.1 Motivation and challenges

At the beginning of this year, the world population reached 8 billion people ("World Population Clock: 8.1 Billion People (LIVE, 2024) - Worldometer," 2024). Now more than ever, Earth's resources are disappearing, which can potentially lead to a massive crisis if regulations are not implemented and a shift to green energy and products is not made. Our society heavily depends on fossil-fuel based products, which raises the question: what can we do, as a society, to prevent this?

Last year saw an increase in global catastrophes (including floods in a wide range of places; wildfires; storms, ect), with a global economic cost estimated into USD 290 billion. Moreover, 2023 was considered the hottest year in record ("sigma 1/2024: Natural catastrophes in 2023 | Swiss Re," 2024). These are all consequences of actions taken since the industrial revolution. Time is running out, and each passing day reduces the window for effective action.

Sir David Attenborough stated in his latest book, "*We are all people of Pripyat now. We live our comfortable lives in the shadow of a disaster of our own making. That disaster is being brought about by the very things that allow us to live our comfortable lives.*", a quote that represent the actual reality of our society, showing that we need to learn with mistakes from past, like the one that happened in Pripyat, Chernobyl (Attenborough, 2020). More than ever, it is necessary more actions towards a green and sustainable environment, especially by reducing our use and dependence in petroleum derivatives. This motivation led me to pursue a PhD in biotechnology. Even if my contribution is small, I know I have done something meaningful to our society and future generations.

On of the most widely produced and consumed chemical globally are surfactants, which can be found in a wide range of products. They are part of human's daily life, being present in shampoos and detergents, for example. However, while very effective, these surfactants have significant environmental and health risks due to their non-biodegradable nature and potential toxicity (Ying, 2006). This scenario underscores the urgent need to identify and develop sustainable alternatives.

Biotechnology, by exploiting the use of microorganisms to produce natural products as replacement of their chemical analogues, offers a promising solution. In fact, humans have been

depending on such processes, from bread and alcohol production through fermentation to using plants for medicinal purposes, where many active pharmaceutical ingredients were discovered. Even without understanding chemical properties and biological processes, humans have been relying on nature and microorganisms, like a symbiotic relationship.

In this context, microbial biosurfactants (mBS) emerge as natural alternative to chemical surfactants. They can be produced by various microorganisms (such as yeast, bacteria and fungi), representing a promising green solution. Unlike surfactants, mBS are biodegradable, less toxic and can be applied in a wide range of applications. Additionally, they can be produced by renewable resources and industrial wastes, which contributes to a circular economy.

However, the high production costs of mBS hold back their commercial viability. To be accessible to the global population, the production cost must be reduced to similar prices of chemical surfactants, but, currently these mBS are often categorized as luxury products. As it will be explained in the upcoming sections, this cost is influenced by several factors, including the price of raw materials, efficiency of the strain used (e.g final productivity), and the downstream process to extract the product. Moreover, since mBS are dependent in the performance of a specific strain, the consistency of the product may vary batch to batch, varying final properties, and making it difficult to have a final stable formulation. Finally, the strain used must not pose any risk to human health.

Therefore, despite the challenges associated with the use of microbial biosurfactants (mBS), consumers are increasingly aware of the need for greener products and are more willing to adopt them. This thesis aims to answer this call, by developing sustainable processes that can compete with chemical surfactants, paving the way for a more sustainable future and creating a circular economy.

A.1.2 Concept of the thesis

On this thesis it is explored the microbial production of Mannosylerythritol lipids (MELs[1]), which consist in glycolipid biosurfactants with excellent interfacial properties and other appealing qualities. The molecule consists of a hydrophilic moiety (4-O-β-D-mannopyranosylmeso-erythritol), and fatty acids and acetyl groups as the hydrophobic moiety (see **Figure A*1***). Depending on their level of acetylation at positions C4-C6, MELs molecules can be classified as MEL-A, MEL-B, MEL-C and MEL-D, as it will be explained in detail in **section A.2.**

Therefore, to produce a product based on MELs which would be competitive on the market with other microbial biosurfactants (mBS), and later with surfactants, overall manufacturing costs need to drop significantly. To achieve this, several factors within all segments of MELs production need to be optimized. Namely, 1) finding novel low-cost substrates for MELs production; 2) increasing the efficiency of the bioconversion process itself; and 3) finding cheap and efficient downstream pathways to obtain purified MELs. However, to ensure that these solutions will be viable long term, sustainability must be taken into account. Therefore, the final process must satisfy three key criteria which encompass sustainable development: environmental, economic and social.

The primary goal of this thesis is to develop a sustainable and competitive process that can elevate MELs to an industrial level, by trying to solve some bottlenecks and at the same time pointing out future directions. Consequently, this thesis is structured into four sections, an introductory section A, that provides an introduction and presents the overall state of art for thesis topics, and the three main sections, each one focusing in a distinct area of research.

- **Section B:** This section explores different types of production aiming high MELs titres/productivities, while exploring the use of different side streams (cheese whey, or residues from olive oil industry) that can offer a favourable life cycle impact, but at the same time, that allows to reduce overall costs of the process. The main objective of this section is to create a process based on a circular economy.

[1] In most of literature, mannosylerythritol lipids are often referred as “MEL” (single form), however considering that the main product is a mixture of different congeners of MELs, it was decided to use the plural form “MELs”.

- **Section C:** This section enables the comprehensive integration of MELs bioprocessing, focusing on downstream operations and final economic assessment. At downstream level, the objective was developing a new technology capable of reducing costs, but aligned with sustainable practices, by reducing the use of organic solvents normally used for the extraction of compounds like MELs. Subsequently, the process was mathematically modelled to identify main bottlenecks and determine which areas of research should be prioritized in the future to develop a process even more competitive.

- **Section D:** One of the major challenges facing our society is the release of CO_2 emissions. MELs bioprocess, as every process, will generate CO2 emissions. Therefore, this section explores an innovative approach involving the co-cultivation of microalgae and non-conventional yeasts to produce MELs, while reducing the CO2 emissions. In this context, it was initially studied the use of co-cultivation of microalgae and yeast for production of citric acid, since the production of CA using only yeast it is already a robust and established process. Subsequently, the potential of using lipids produced by microalgae for MELs production. Finally, it was attempted the combination of co-cultivation for MELs bioprocess.

A.1.3 Thesis objectives and research questions

The objective of this thesis is to develop new strategies to produce MELs and other high value products (such as enzymes and organic acids) from waste streams, that explore the features of non-conventional yeasts, alone or synergistically in combination with microalgae, to improve the environmental performance of production processes; therefore, contributing to the creation of a biorefinery that supports circular economy approaches to fight climate changes and its impacts.

This thesis intends to answer the following research questions:

- Can the use of co-substrates with opposite polarities to maximize consumption of the vegetable oil used as substrate, improving MELs yields while maintaining high purities? (**section B.1)**
- Is the use of a carbon source substrate fed-batch process strategy the appropriate to boost MELs yields? (**section B.1)**
- Are *Moesziomyces* spp. able to utilize lactose and consequently produce β-galactosidase? (**section B.2**)
- Can cheese-whey (side stream of cheese industry) be used simultaneously as carbon source and replacement of mineral medium and yeast extract, usually used on culture medium formulation? (**section B.2**)
- Can olive pomace residues be used as a carbon source for MELs production? What is the impact on MELs titres of using a carbon source richer on free fatty acids? (**section B.3)**
- Can an efficient downstream process be developed for the treatment of MELs, i.e. MELs harvested from fermentation broth, to improve MELs purity using sustainable solvents and simple unit operations? Can this technology be used to others microbial biosurfactants? (**section C.1**)
- What are the main bottlenecks in MELs bioprocess to decrease product production costs? Specifically: i) How does the scale-up impacts the unit cost of production? ii) How to overcome equipment downtime? iii) Which categories contribute the most to the total process cost? iv) Titre vs productivity. Which the metric to optimize aiming at a higher impact on profitability? (**section C.2)**

- Can co-cultivation of microalgae and yeast be used to optimize a bioprocess, while reducing CO_2 emissions? Specifically: i) What's the optimal culture medium that supports both microorganism? ii) What's the appropriate ratio of yeast to microalgae? iii) In which stage should the yeast be introduced into the culture? **(section D.1).**
- Can lipids from oleaginous microalgae be used as carbon source to produce MELs? (**section D.2)**
- Can co-cultivation between microalgae and yeast used for MELs production? Can decrease CO_2 emissions? (**section D.3**)

A.1.4 Thesis outline

Section A includes the state of the art of both MELs bioprocess development and the co-cultivation of microalgae and yeasts. It is provided an overview of MELs structure, properties and characterization, and the progress made so far in the three main areas of a bioprocess (upstream, and the use of different side streams; fermentation, including different studies aiming the achievement of higher titres; and downstream processing which includes all the technologies tried to purify MELs). Additionally, it also offers an overview in the studies reporting the use of microalgae and yeast in co-cultivation to produce a specific product or whole biomass.

Overall, **section B** focus on the development of MELs production, including bioreactor studies, using different feeding strategies and waste materials, to create competitive and sustainable process. In detail, **section B.1** focus on the development of a baseline condition that aims the production of high titres of MELs, while maintaining a high purity. **Section B.2** studies the production of β-galactosidase by *Moesziomyces* spp. and the potential use of cheese-whey (side stream of cheese industry) as a carbon source and culture medium replacer. **Section B.3** studies the use of different residues generated during olive oil production, and their use in bioreactors.

Section C.1 focus on the application of a new technology, organic solvent nanofiltration (OSN) and in-house development of specific membranes, aiming to have pure MELs with a minimal environmental footprint. **Section C.2** provides an economic analysis of the whole MELs bioprocess, pointing out main bottlenecks.

Overall, **section D** focus in the design of system to couple phototrophic microalgae and non-conventional yeasts culture. **Section D.1** focus on the development of a co-cultivation system using microalgae and yeast for production of citric acid, which is a more mature process, and thus more stable and robust, than the MELs bioprocess, and thus selected as a first case study to answer research questions related with bioprocess development based on co-cultivation of microalgae and yeast. This section was important to define key parameters for a successful co-cultivation system, which was used later in the development of a co-cultivation system aiming MELs production. **Section D.2** focus on the development of a sequential process, that starts with the production of lipids from oleaginous microalgae, that will be used in the next stage as carbon source for MELs production. **Section D.3** focus on the development of a co-cultivation for MELs

production, by redesigning new bioreactors and using the parameters previous defined in **section D.1.**

This thesis is structured as compilation of scientific papers, with each section interconnected for the reader's benefit. Ideally, each section aims to result in a scientific paper published in a peer-review journal. Therefore, the reader is asked to bear in mind that some redundancies in the information provided will be present, especially in material and methods sections.

A.1.5 Research contribution in publications

Parts of **section A**, especially **section A.2** it is already prepared for publication as the review article manuscript *"Bioprocessing strategies for sustainable large-scale production of Mannosylerythritol lipids: Bottlenecks and future perspectives*", developed with co-authorship of Dr Petar Keković (former PhD student of the group). Furthermore, also part of **section A.2**, was published (https://www.mdpi.com/2311-5637/10/5/246) as a mini-review *"Unlocking the Potential of Mannosylerythritol Lipids: Properties and Industrial Applications"* in the journal *Fermentation* (part of special issue: Production of Added-Value Products from Renewable Resources and Engineered Cell Factories) with Joana Almeida as first author and myself as co-author.

Part of the research presented in **section B.1** it is published in the journal *Applied Biochemistry and Biotechnology* with the title ''*Substrates of Opposite Polarities and Downstream Processing for Efficient Production of the Biosurfactant Mannosylerythritol Lipids from Moesziomyces spp."* (https://link.springer.com/article/10.1007/s12010-023-04317-z) with Dr Nuno Faria as first author and myself as co-author.

Research presented in **section B.2** it is published (https://link.springer.com/article/10.1007/s13399-022-02837-y) in the Journal *Biomass Conversion and Biorefinery*, with the title ''*Moesziomyces spp. cultivation using cheese whey: new yeast extract-free media, β-galactosidase biosynthesis and mannosylerythritol lipids production*''). Moreover, these results were the topic of an oral presentation given at the 13th European Congress of Chemical Engineering and 6th European Congress of Applied Biotechnology (ECCE/ECAB 2021, virtual conference).

Research presented in **section B.3** it is already prepared for submission as the manuscript ''*Development of mannosylerythritol lipids sustainable biorefinery: Assessment the use of olive oil as carbon source towards a circular bioeconomy and economic evaluation of the process"*.

Research presented in **section C.1** it is published (https://www.mdpi.com/2077-0375/13/1/81) in the journal *Membranes* (part of special issue: Membrane Science towards Sustainable Development Goals (SDGs)), with the title "*Novel downstream processing setup for biosurfactants produced from lipid-based substrates*". Work co-developed with Dr Petar Keković. Moreover,

these results were the topic of an oral presentation given at Congress of Microbiology and Biotechnology (MICROBIOTEC 19), at University of Coimbra, Portugal, in 2019.

Research presented in **section C.2** is prepared for submission as the manuscript ‘’*Techno-economic analysis of Mannosylerytritol Lipids bioprocess: Main Bottlenecks and future perspectives”*.

Research presented in **section D.1** is already prepared for submission as the article manuscript ‘‘*Development of a co-cultivation system of Yarrowia lipolytica and Chlorella vulgaris: Citric acid bioprocess as a case stud* ‘’. This work was developed at Helmholtz Centre for Environmental Research – UFZ, Leipzig.

Research presented in **section D.2** is published (https://www.mdpi.com/2076-2607/10/12/2390) in the journal *microorganisms* with the title ‘’ *Production of Mannosylerythritol Lipids Using Oils from Oleaginous Microalgae: Two Sequential Microorganism Culture Approach”*.

Some results achieved in **sections B.1**, **B.3**, **C.1** , can also be found in an international patent application, with the title ‘’ *Device, system and process for the enhanced production of mannosylerythritol lipids (MELs) integrating fermentation and product separation from fermentation broth by non-invasive methods” (*PT 118115 / Portuguese patent application - PCT application submitted) (Petar; Keković et al., 2022)

Outside the main scope of this thesis, but still on the field of sustainable bioprocess development, I was the first author of a review article published during my PhD, (https://doi.org/10.1016/j.procbio.2022.03.020) in the journal *Process biochemistry* with the title ‘’*Integrated perspective on microbe-based production of itaconic acid: From metabolic and strain engineering to upstream and downstream strategies*’’.

A.2 State of the art in Mannosylerythritol lipids and their potential impact in society

A.2.1 Overview of surfactants and microbial biosurfactants

Our planet is currently facing drastic climatic changes, resulting of decades of intensive use of fossil fuels, mainly for energy and chemical production. This has caused an imbalance in the global ecosystem and led to rising average temperatures, due to CO_2 emissions (Dai, 2011). Now more than ever, solutions are needed to counteract this phenomenon, or future generations may not have a sustainable place to live.

One of the most globally and widely used chemicals are surfactants, with a market value estimated at US$ 45.72 billion in 2023 and projected to reach US$ 69.3 billion by 2032, with a compound annual growth rate (CAGR) of 4.7% ("Surfactants Market Size, Growth, Trends | Report 2023-2032," 2023)(Dixit et al., 2020). Surfactants are amphiphilic molecules (ionic or non-ionic) that can interact with polar and apolar compounds due to their's structure, which consists of a polar moiety and apolar head (Kronberg et al., 2014). Even though their primary structure might seem simple, these molecules can form different supramolecular structures when above their critical aggregation concentration (CAC), depending on the nature of the solvent. The most common structures formed are micelles, which happens when surfactants are placed in a hydrophilic medium, leading to the assembly of hydrophobic tails to avoid contact with water, creating a "cage-lie" structure. Other types and complexes structures can also be formed, such as: cylindrical and spherical bilayers, which are formed by uni- or multilamellar structures; helical ribbon and tubules, mostly formed by chiral surfactants; and bicelles or disk aggregates, which can be made mixing different surfactants in the same solution (Ghosh et al., 2020).

Driven by these unique characteristics, surfactants can adsorb at different interfaces, decreasing surface tension and promoting different self-structures; thus they can be useful for wide range of applications, such as, cleaning agents, cosmetics, pharmaceuticals, leather, paper textiles chemicals and food processing (Rodrigues et al., 2006). These molecules are so unique, that they can also be found in humans, such as pulmonary surfactants, which are essential to lowering surface tension, preventing their collapse during breathing.

Nevertheless, surfactants used in the different industries are produced from petrochemicals (non-renewable products), contributing to green gas houses emissions. Furthermore, it is estimated that 60% (w/w) of the total surfactant produced ends up in the aquatic environment

(Pradhan and Bhattacharyya, 2017), due to direct product discharge, leakage, and inefficient removal from water in wastewater treatment stations. Additionally, synthetic surfactants can persist and accumulate in the environment due to their slow biodegradability, which are toxic to microorganisms (Ying, 2006). Additionally, several surfactants are reported to cause irritation for human skin and eyes.

Now, more than ever, consumers are becoming increasingly aware of the impact of chemicals on the environment and human health. In response to climate changes, in 2015 the United Nations established sustainable development goals (SDG), aiming to address a broad range of global challenges, including climate change and environmental degradation (SDG 3, 12, 13) (United Nations, 2015). Specifically, SDG 4 proposes a substantial reduction of number of deaths and illness from hazardous chemicals population by 2023. The European union has also introduced additional regulation, setting milestones, such as the reduction of net greenhouse gas emissions by at least 55% until 2030, when compared to 1990 levels.

In this regard, microbial biosurfactants (mBS) started to be envisioned as an alternative to chemical surfactants, given their higher biodegradability and lower toxicity (Marchant and Banat, 2012). They are categorized into different classes based on their structure: glycolipids, lipopeptides, fatty acids, polymeric and particulate mBS. Currently, the market of mBS is dominated by glycolipids, such as sophorolipids (SLs), rhamnolipids (RMs), and it is expected to reach $USD 24.3 million by 2032 (Fact.MR, 2022).

Also, major multinational companies are intensifying their investments in research and scale-up production of mBS. For instance, Evonik and Unilever have announced a partnership in 2022 for the construction of RMs production facility in Slovakia. Similarly, BASF and Holiferm also announced a partnership for SLs production, with an investment of 21.4 M€. This clearly shows the growing market demand for such products.

Mannosylerythritol lipids (MELs), the focus of this thesis, constitutes a third emerging mBS. The actual MELs process have a technology readiness level (TRL) of 4, a value lower than the one for SLs/RLs, with TRLs at 8/9, yet they exhibit significant potential. Therefore, in the following sections, an overview will be provided of current processing technologies and practises across all

stages of MELs bioprocess, highlighting why this molecule stands out as one of the most promising mBS.

A.2.2 Why MELs?

In 1956, Boothroyd et al., isolated MELs for the first time (Boothroyd et al., 1956). Produced by the fungus *Ustilago* spp (PRL 627), they were described as “extracellular oil” and were heavier than typical vegetable oils. Interestingly, because it contained mannose-erythritol molecules in its composition, at the time it drew attention in scientific community (Fluhartyt et al., 1969). As the molecule was studied further, new microorganisms were discovered to produce MELs (see **Table A1**). Currently they are mainly produced by *Moesziomyces* sp. yeasts.

MELs structure contains a 4-O-β-D-mannopyranosyl-meso-erythritol as the hydrophilic group and fatty acids short-chains as the hydrophobic group (**Figure A*1***). According to the number and position of the acetyl group MELs are classified as: MEL-A, di-acylated congener; MEL-B, mono-acylated congener in C6; MEL-C, mono-acylated congener in C4 and MEL-D, deacylated congener (Arutchelvi et al., 2008). Other factors influence the structure of MEL, such as the amount of acylation in mannose, the fatty acids length, and their saturation.

Depending on the type and the final concentration, MELs can self-assemble in diverse structures. Imura et al. (Imura et al., 2006), explored into this phenomenon, determined the CAC and described the many architectures that different kinds of MEL could form. They came to the conclusion that, at CACs of 4 and 4.5 μM, respectively, MEL-A and MEL-B assemble in huge unilamellar vesicles. Nonetheless, they form sponge structures (L3 phase) when the concentration of MEL-A rises to more than 20 μM. Above the CAC, MEL-B forms characteristic multilamellar vesicles. It's interesting to note that two whole different structures result from a slight variation in both MELs (the presence or not of one acetyl group). Considering these different structures, Yamamoto et al., (Yamamoto et al., 2012) have tested both MELs (MEL-A and MEL-B), where they have observed that MEL-B has high hydrophilicity (capacity of water retention), when compared to others. Regarding MEL-C and MEL-D, both form lamellar phases, with CACs of 4 μM and 12 μM, reducing the surface tension of water to 24.4 mN.m-1 and 24.6 mN.m-1, respectively (Fukuoka et al., 2011; Morita et al., 2008c). Later it will be show in detail how different MELs structures have impact in a final application.

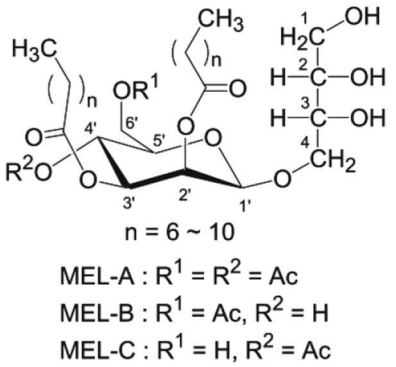


Figure A1: Chemical structure of MELs and their types. MEL-A: di-acylated; MEL-B: mono-acylated in C6; MEL-C: mono-acylated in C4.

Due to their structural diversity, environmental compatibility, low toxicity and versatile biochemical functions, MELs seem to be a promising sustainable alternative to many of the modern chemical detergents, cosmetic agents and pharmaceuticals currently used in our society (Arutchelvi et al., 2008). Namely, because of the low critical micelle concentration (CMC), which is around 0.0027 mM, a value 10-fold lower, when compared with SLs and RMs, with values ranging from 0.12-0.30 mM) (Imura et al., 2006; Kitamoto et al., 2002; Konoshi et al., 2008; Lang and Wullbrandt, 1999). This factor implies that for the same application, lower concentration of MELs can be used, and having the same effects as SLs or RMs. Furthermore, Keković et al. (Petar Keković et al., 2022), recently assessed the ecotoxicity of surfactants (e.g tween-80) as well mBS (MELs, SLs and RMs), using the model marine *Artemia franciscana*. The authors have proved that MELs (IC value of 999.95 $mg.L^{-1}$) are even less toxic than RMs and SLs, with an IC value 45.4 and 27.7% higher, respectively.

Table A1: Summary of the microorganism used to produce MELs. For each microorganism it is indicated the genus, specie and the strain. The ones used in bioreactor (V ≥ 1 L) are marked with *.

Genus	Species	Strain	Reference
Moesziomyces (Pseudozyma; Candida; Vanrija)	*antarcticus (antarctica)*	T-34	(Kitamoto et al., 2001a, 1990b; Morita et al., 2007a)
		JCM 10317*	(Madihalli et al., 2020; Morita et al., 2007b)
		ATCC 20509*	(M. Adamczak and Bednarski, 2000; Marek Adamczak and Bednarski, 2000; Dzi and Adamczak, 2013)
		KCTC 7804*	(Kim et al., 2006)
		PYCC 5048	(N. Faria et al., 2014; Petar Keković et al., 2022; Nascimento et al., 2022a, 2022b)
		ATCC 32657	(Bhangale et al., 2013)
		ATCC 28323	(Dzięgielewska and Adamczak, 2013)
		MTCC 2706*	(Mawani et al., 2021)
	bullatus (aphidis; rugulosus)	DSM 70725*	(Beck et al., 2022; Beck and Zibek, 2020; Dzięgielewska and Adamczak, 2013; Rau et al., 2005b; Yang et al., 2023)
		DSM 14930*	(Rau et al., 2005b)
		PYCC 5535	(N. Faria et al., 2014; Nascimento et al., 2022a, 2022b)
		MUCL 27852*	(Goossens et al., 2016)
		XM01*	(Yu et al., 2022)
	hubeiensis	CBS 10077	(Beck and Zibek, 2020; Konishi et al., 2008)
		JCM 16987*	(Andrade et al., 2017)
	tsukubaensis	JCM 10324T	(Morita et al., 2007b)
		JCM 16987	(Morita et al., 2007d)
		CBS 422.96	(Beck and Zibek, 2020)
	parantarcticus (parantarctica)	JCM 11752^{T}	(Andrade et al., 2017)

Genus	Species	Strain	Reference
Moesziomyces (Pseudozyma; Candida; Vanrija)	*shaxiensis*	CBS 10075	(Beck and Zibek, 2020; Fukuoka et al., 2007)
	fusiformata	JCM 3931^T	(Morita et al., 2007d)
	flocculosa	JCM 10321^T	
	thailandica	JCM 11753^T	
	graminicola	CBS 10092	(Morita et al., 2008c)
	siamensis	CBS 9960	(Beck and Zibek, 2020; Morita et al., 2008a)
Ustilago	*maydis*	L9*	(Liu et al., 2011)
	scitaminea	NBRC 32730*	(Morita et al., 2009a; T. Morita et al., 2011)
	nuda	PRL 627	(Bhattacharjee et al., 1970)
Kurtzmanomyces	*Sp.*	I-11	(Kakugawa et al., 2002)

T, type strain; JCM, Japan Collection of Microorganisms; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; PYCC, Portuguese Yeast Culture Collection; DSM, German Collection of Microorganisms and Cell Cultures; MUCL, Collection of filamentous fungi, yeasts and arbuscular mycorrhizal fungi of the Université catholique de Louvain; CBS, Centraal bureauvoor Schimmel culures; NBRC, NITE (National Institute of Technology and Evaluation) Biological Research Center

A.2.3 Disparity of analytical methods and data representation

As previously seen, MELs molecular structure are complex, and have different congeners, which even increase the level of complexity. Therefore, in order to enable a good comparison of data obtained from various literature sources, it is important to note the discrepancies between sources in terms of analytical methods used for quantitative MELs characterization, as well as to carefully report the common omission of data critical to estimation of process efficiency. The methods used so far, for MELs quantification are represented in **Table A2**. Additionally, it was created a classification system, where “A” and “C”, corresponds to the best (most accurate and precise) and worst (non-reliable), respectively. Through all the text and in the following tables (**Table A3** and **Table A4),** it is represented the final MELs titre and the method used, by using the classification system herein created. This will allow the reader to assess the reliability of the reported MELs titre.

In this regard, the one more precise and accurate method (classification A1) corresponds to high pressure liquid chromatography, accoupled with evaporative light scattering detectors (HPLC-ELSD), allowing to quantify MELs directly, and distinguish between different MELs types (Beck et al., 2022; Goossens et al., 2016; Morita et al., 2009a, 2008b). This method gives the most complete information on achieved MELs concentrations and provides data on residual lipids, such as: free fatty acids (FFA), monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG).

Alternatively, the fatty acids composing the hydrophobic moiety of the molecule can be esterified with methanol and analysed with gas chromatography (GC). This method (classification A2) relies on the fact that MELs molecules contain short fatty acid chains (C8-C12), while most hydrophobic carbon sources contain longer fatty acid chains (C14-C22) (N. Faria et al., 2014; N. T. Faria et al., 2014). Still as, while rare, some working strains produce MELs with longer fatty acid chains, along with the possibility, even with low probability, that certain fatty acids can be present, but not incorporated in the product, makes this method slightly less reliable.

The Anthrone method (classification B) is a well-studied spectrophotometric method sometimes used for determining glycolipid concentrations, including MELs (Hodge et al., 2008). This method relies on the reaction between the Anthrone reagent and the carbohydrate moiety of the

biosurfactant, resulting in a measurable coloration (620 nm). While this method is used in scientific reports for determining MELs levels in the broth (Kim et al., 2006; Madihalli et al., 2020), it is flawed due to its imprecision. Specifically, it relies on the assumption that not only other carbohydrates present in the broth (originating from the substrate or of metabolic origin) , but also other glycolipids and poly-carbohydrates (even from the cell membrane) do not react with Anthrone reagent, which might lead to an incorrect result (Loewus, 1952), affecting the ability of constructing a reliable mass balance of the bioprocess.

Despite the availability of the methodologies for MEL characterisation, numerous literature sources present data derived by unreliable, indirect measurements of MELs concentration. Gravimetrical methods (classification: C) rely on performing a liquid-liquid extraction of an aliquot of the fermentation broth (2x times at least), evaporating the solvents and measuring the final mass. In these reports, the assumption often made is that the total collected mass consists solely of MELs (Marek Adamczak and Bednarski, 2000; Dzięgielewska and Adamczak, 2013), which is not true, since most of the times, crude MELs are significantly contaminated with residual lipids. Obviously, the result is an unconvincingly high yield, and, again, the inability to construct a mass balance of the process.

Finally, complete information regarding the fermentation efficiency is rarely presented. This should include detailed information regarding the amounts of substrate used, their composition, profiles of product concentration over the fermentation duration, productivity, yields, and residual substrate levels in the end of the fermentation. Even when reliable analytical techniques are used, the omission of a part of the information gives unreliable results which impairs a critical comparison with studies using different process approaches and carbon sources.

These issues are especially prominent in literature sources reporting production of MELs in the bioreactor scale. Out of the literature sources presented in **Table A3** and **Table A4**, only 25% report the levels of residual lipids at the end of the fermentation (Goossens et al., 2016; Kim et al., 2006). This impairs the scientific community to have an insight into final product purity, but also, it avoids the comparison and transfer of knowledge from article to article. The joint effort to develop a sustainable industrial process, transparent, reliable and comprehensive data representation should be imperative.

Apart from all the methods described above to quantify MELs, there is also complementary techniques used to detect and give a full characterization of the MELs, such as thin-layer chromatography (TLC) complemented with nuclear magnetic resonance (NMR). Although the method HPLC-ELSD gives the overview of all types of MEL congeners, these methods (β1 and β2) are of faster applications and are broadly accessible, and they can give information about the whole molecule. While TLC allows to distinguish between MELs congeners, NMR permits the characterization of molecules (acetylation, type of fatty acids chain). Recently, Beck et al (Beck et al., 2019), developed a new type of combined methodologies (β2), using high-performance thin-layer chromatography (HPTLC) with MALDI-TOF mass spectrometry (MS). This method allows to identify a variety of MELs congeners, varying on acetylation and on the type of fatty acid chain, and even giving information on molecular weight (MW) and hydrophobicity. Furthermore, using this new combined methodology, the authors were able to identify a new molecule within MELs, where erythritol is replaced by mannitol, forming mannosylmannitol lipids (MMLs). Mostly it is mainly produced in *M. parantarticus,* but it was also found to be produced in *M. bulattus.*

Table A2: Summary of methods used for MELs quantification, detection and characterization. For MELs quantification, a classification system was created, where "A" corresponds to the most accurate and precise method, and "C" to the most unreliable method, with a high variation in the final concentration. β1 and β2 correspond to methods only used for characterization of different MELs congeners in the final mixture.

Type of analysis	Classification	Method	Brief description of the method	Article
Quantification of MELs	A1	HPLC-ELSD	MELs were quantified using HPLC equipped with a silica gel column and a nevaporative light scattering detector (ELSD).	(Goossens et al., 2016; Konishi et al., 2008; Liu et al., 2011; Morita et al., 2009a, 2007a, 2007b; T. Morita et al., 2011; Rau et al., 2005b)
	A2	GC-FID	Fatty acids of samples were determined by methanolysis and GC analysis of methyl esters, where MELs were quantified by the amount of C8, C10 and C12.	(Bednarski et al., 2006; N. Faria et al., 2014; N. T. Faria et al., 2014; Petar Keković et al., 2022; Nascimento et al., 2022b, 2022a; Santos et al., 2019; Yu et al., 2022)
	B	Phenol sulphuric acid (Anthrone method)	Initially, MELs were extracted with ethyl acetate followed by hydrolyzation with anthrone reagent and sulphuric acid. The OD was measured at 620 nm.	(Bhangale et al., 2013; Kim et al., 2006; Kitamoto et al., 2001a, 1990b; Yang et al., 2023)
			Crude MEL was extracted with ethyl acetate, concentrated, and dissolved in chloroform. The amount of MELs were measured through mannose (result of MELs hydrolysation)	(Madihalli et al., 2020)
			For each sample taken from fermentation broth, the authors washed the supernatant with ethyl acetate, which was then evaporated. Then, the obtained residue was dissolved in water, and then using Anthrone method for MELs quantification.	(Mawani et al., 2021)
	C	Gravimetrically	MELs samples were extracted with ethyl acetate and washed with hexane (to remove lipids). The sample was concentrated, and the total content of MELs were measured gravimetrically.	(Marek Adamczak and Bednarski, 2000; Dzi and Adamczak, 2013; Dzięgielewska and Adamczak, 2013; Niu et al., 2019)

Type of analysis	Classification	Method	Brief description of the method	Article
Characterisation of MELs	β1	TLC with NMR and LC-MS	MELs formation were analysed by TLC (which allows to distinguish between types of MELs), followed by NMR and LC-MS, allowing to have a detailed characterization of the structures of MELs produced.	(Mawani et al., 2021; Yu et al., 2022)
	β2	HPTLC–MALDI-TOF–MS	Initially MELs variants are separated using HPTLC. At the same time, MALDI-TOF-MS is conducted, which will allow the connection of each peak from MS to every band observed on HPTLC. This novel method allows the identification of MEL congeners, with different degrees of acetylation and fatty acid chain lengths.	(Beck et al., 2019; Beck and Zibek, 2020)

A.2.4 Integrated perspective on MELs bioprocess: from upstream to downstream

A review of technological techniques available in the literature for developing various areas of MELs manufacturing will be provided. Rather of dividing the entire process from beginning to end into two halves, as is standard practice, the procedure is divided differently here. The fermentation (fermentation in shaking flask or bioreactor) is at the core of the process, where the majority of MELs are produced, with all preceding phases (substrate preparation and pre-treatment, for example) occurring upstream. Similarly, all unit activities after MEL bioconversion (MEL recovery, purification, and so on) are labelled as downstream steps in the process.

A.2.4.1 Upstream processing

The first step in constructing the blueprint for a MELs production process includes the substrate selection. Although it is well established that MELs can be produced from a variety of substrates (mainly hydrophobic substrates, such as vegetable oils), not all satisfy the various criteria needed to be included in a sustainable process. Besides the most obvious issues, such as substrate-to-product conversion efficiency and interaction with the working microorganism, other matters, affect the decision-making regarding the selection of an adequate substrate. At this point, and as it will be discussed in **section C.2,** it is necessary to perform and early economic assessment (using software like SuperPro or AspenPlus), which will allow to determine bottlenecks, and which unit operations to study in detail. For example, even if good yields are obtained from leftover substrates that require pre-treatment (acid/basis or enzymatic treatment), the total cost of treating the residue might prevent the process from being scaled up. As a result, in the future, it is critical to apply modulation tools and conduct economic/environmental analysis of the entire process.

A.2.4.1.1 Metabolic pathways and carbon sources used for MELs production

MELs can be produced from a variety of substrates, as reported in literature (**Table A3**). However, the selection of the substrate which would be used in industrial-scale production is a complex question, with several different parameters which should be taken into consideration. An ideal substrate for sustainable large-scale production should be metabolically compatible and

easily consumed by the microorganism, with the application of simple pre-treatment processes, while entering the metabolic pathway in a way which prompts MELs production and cell growth/maintenance in an optimal ratio. Furthermore, it should be cheap, neither used for food or feed production, and ubiquitous - widely available, with possibility of production on various soils and in different climates. Finally, an ideal substrate should be fully renewable, with a minimal carbon footprint in terms of substrate production and pre-treatment.

The metabolic pathway of MELs production, from substrate to product, is already well explored. As reported by Morita et al., (Morita et al., 2014), the complete MELs molecules can be produced by both sugar- and oil-based substrates. However, losses occur when only one type of carbon source is used. Namely, the hydrophobic moiety of the molecule can be synthesised from glucose through the process of de-novo fatty acids synthesis (Kitamoto et al., 1998), while the process is energetically more efficient when a lipid substrate is used and the fatty acids (derived from the lipid hydrolysed by lipases), which can be integrated directly through β-oxidation chain shortening, as shown by Kitamoto et al. Similarly, when a sole lipid-based carbon source is fed to the cells, the glycerol released during lipid hydrolysis has to first be converted to glucose through the process of gluconeogenesis, before it is further converted to mannose and erythritol which represents the hydrophilic moiety of MELs. All these efficiency-lowering sidesteps are avoided when an optimal balance of both carbon source types are used in an adequate ratio, and with a well-defined feeding strategy.

Recently, Wada et al. (Wada et al., 2022) have used two types of substrates (olive oil and glucose), measuring the metabolites produced during MELs synthesis. Interestingly, while 94% of olive oil was converted to biomass and MELs, only 59% of glucose was used in the production of these two compounds. Furthermore, the authors report that the levels of acetyl-coA, adenosine triphosphate (ATP), adenosine diphosphate (ADP) were 95, 45 and 93% lower when using olive oil, compared with glucose, respectively. The whole metabolic pathway representing the use of hydrophilic or hydrophobic carbon sources is represent in **Figure A2**.

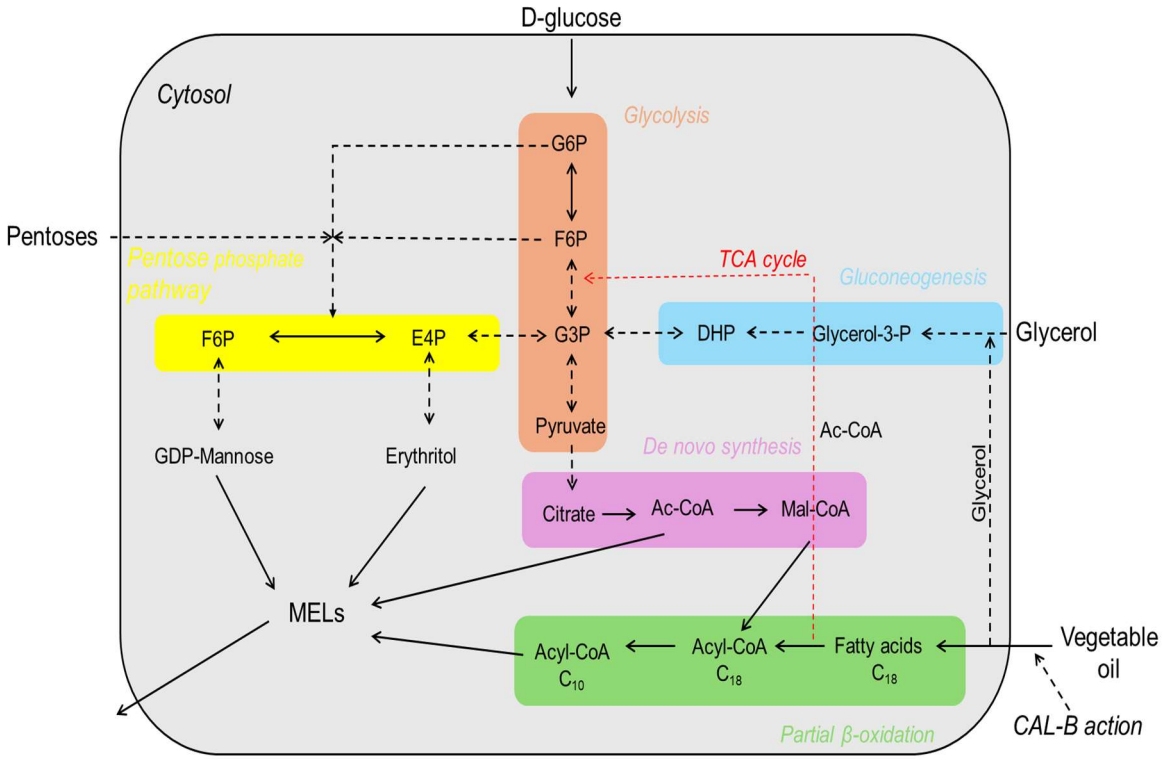


Figure A2: Metabolic pathways for the production of MELs using D-glucose, glycerol and vegetable oils as carbon source. G6P – glucose-6-phosphate; F6P – fructose-6-phosphate; G3P – glyceraldehyde-3- phosphate; E4P – erythrose-4-phosphate; DHP – Dihydroxyacetone-P ; Ac-CoA – Acetyl-CoA; Mal-CoA – Malonyl-CoA.

A.2.4.1.2 Substrate and feed strategy impact on MELs production

In many existing well developed biotechnological processes, recently there was an active effort to shift towards alternative substrates, in an effort to achieve higher overall process sustainability. Usage of low value feedstock can reduce upstream costs and avoid unwanted competition with the food supply market. These usually include biomass-based residues (agricultural waste, including lignocellulosic residues), or unwanted wastes from various industries (crude glycerol, cheese whey, paper pulp, or sugarcane bagasse and molasses).

Many literature sources report the use of various carbon sources for MELs production, ranging from sugars (glucose, xylose and others) and glycerol, alkanes, to vegetable oils and industrial residues. As seen in **Table A3**, the high productivities are achieved when using hydrophobic substrates (e g. Vegetable oil), although most associated with a low final purity of MELs.

Unfortunately, as it will be discussed in the following sections and also shown in **Table A3** and **Table A4**, most of the studies do not report the impurities (residual lipids) presented in crudes MELs mixture.

Kitamoto et al., (Kitamoto et al., 1990b), were the first ones to use vegetable oils (SBO, Ro, coconut oil, ect) as carbon source (80 g/L), reporting the highest titre of MELs, when using SBO (34 g/L) as carbon source. Interestingly, Konishi et al., (Konishi et al., 2008), using glucose (20 g/L) and soybean oil (40 g/L) in a fed-batch fermentation (every 4 days, a feed of equal concentrations of glucose and soybean oil was fed, until day 16 of fermentation) led to 76.3 g/L, increasing in 47.5% the final titre, when compared with same conditions, but using only SBO (40 g/L). These results go accordingly with some results shown in this thesis (**section B.1**), where the combination of both carbon sources improve final MELs titre. Feed strategies where glucose (Morita et al., 2007d) and glycerol (Morita et al., 2007c) are the main substrates show lower productivity and a 10-fold lower yield of MELs compared to feed strategies including vegetable oils.

Sugar-based renewable substrates performed relatively poorly compared to other renewable substrates. Wheat straw (2.5 g/L) (N. Faria et al., 2014), residual honey (5.4 g/L) (Bhangale et al., 2013), and sugarcane juice (12.0 g/L) (T. Morita et al., 2011) all resulted in low MELs titres and productivity, despite mostly good yields and low levels of residual substrates. On the other hand, lipid-based residues performed better, with reports of waste frying oil (WFO) (55.0 g/L) (Niu et al., 2019) and soap stock (107 g/L) (Dzięgielewska and Adamczak, 2013), reaching some of the highest MELs titres, with good productivity. Note that this last value of MELs titre was obtained with method “C” (gravimetrically).

As previously mentioned, residual lipids present in the final product reduce its value and efficiency for most applications. However, many literature sources reporting MELs production using lipid-based substrates often overlook the significance of this value, and experimental results for these fermentations are most often given without data on residual lipids present in the broth at the end of the fermentation, or in the collected product. This information is of crucial importance, not only in terms of denoting the quality and applicability of the final product, as well as any

potential needs for further purification steps, but also for constructing a more comprehensive mass balance of the process, enabling further improvement of the feeding strategy.

Even so, in some cases MELs determination was performed using method C, gravimetrically (the studies previously reported, using soap stock or WFO as carbon sources), disregarding the possibility of lipids presence in the final mixture (even after some crude purification steps), preventing the estimation of the efficiency of the bioconversion step of the process, and obviously, obtaining overestimated titres.

A.2.4.1.3 Substrate pre-treatment

As stated previously, MELs producing microorganisms, especially the most used *Moesziomyces* strains, can uptake a variety of carbon sources as substrate for MELs production: ranging from sugars (including pentoses and hexoses) and alcohols to lipids and alkanes. However, waste substrates are often of more complex nature, and require some pre-treatment before use in fermentation.

Although these strains are known for secreting a plethora of extracellular enzymes, not all macromolecular structures present in the renewable substrates can be fully hydrolysed by the yeasts autochthonous enzymes. Morita et al. (Morita et al., 2009a), report that sucrose can be successfully used for MELs production using the *Ustilago scitaminea* fungus. Faria et al. (Faria et al., 2019, 2015), have shown that *Moesziomyces* strains (*M. antarcticus* and *M. bulattus*) are efficient xylanase producers, and are able to directly uptake xylan derived from lignocellulosic residues for MELs production. Similarly, Mhetras and Gokhale (Gokhale, 2018), have reported *M. hubeinsis* as a cellulose-free xylanase producer. Still, as there are no reports of MELs-producing species that have cellulolytic properties, there are limited possibilities of the use of biomass without prior hydrolysis. As these hydrolysis steps either require large amounts of chemicals and energy (chemical hydrolysis), or are expensive and time-consuming (enzymatic hydrolysis), which in both cases increase the final costs of the process (possible bottlenecks of the process). Alternatively focus should be shifted towards the use of industrial intermediates and by-products, which are already processed and rich in readily available sugars.

Most importantly, the lipases produced by the strains, especially CAL-B (Candida antarctica lipase B), for which production some of these strains are already used in an industrial setting, are

of crucial importance for breaking down the lipids and using the resulting free fatty acids (usually after chain shortening) into the newly synthesised MELs molecule. The pre-treatment of lipid-based wastes usually includes particle removal or pH neutralization. Rancid raw materials can also be used, as the free fatty acids released during auto-oxidation can be directly used by the cells, if peroxide levels are non-inhibitory. During his PhD studies, Petar Keković (Keković, 2022), have explored the pre-treatment of vegetable oils with CAL-B, produced in separated fermentation by using *M. antarcticus*, instead of a commercial enzyme. It was used free fatty acids (FFA; product result from hydrolysis) and fatty acid methyl esters (FAME; resulted from transesterification, here used immobilized CAL-B) as hydrophobic carbon source. The resulting MELs titres were 12.68 and 12.26 g/L, respectively, which are 40% higher than those obtained with SBO (7.45 g/L).

Nevertheless, even the residues that don't require a pre-treatment, all studies use substrates in combination with enriched medium (containing yeast extract, which is expensive). The use of an enriched medium in different nutrients is a constraint for the total price of the process (around 21% of total OPEX, operational expenditure) (Dhanarajan and Ramkrishna, 2014). In **section B.2**, this problem was addressed and overcome by using cheese-whey (by-product of cheese industry) as it will be described.

Table A3: Summary of the results obtained in studies reporting the production of MELs in shake flasks with different microorganisms, using synthetic substrates (S), industrial residues (R), or substrates produced by other microorganisms (M). It is indicated for each substrate the yield and the productivity (directly retrieved or calculated using data available therein). “n.a”, No information available to determine the parameter; “ – “values obtained by design of experiments (DoE); “*”, Report of lipids composition (free fatty acids) in the end of the fermentation. Furthermore, for each study it is represent the method used to quantify MELs, by using the classification system created (see table 2). At grey, it is represented the studies performed within the research group I belong.

Substrate used	Brief description of the process	MELs (g/L)	Productivity (g/L/h)	Yield (g/g)	Article
SBO^{S}	The authors have tested different types of oil, and different nitrogen sources, including wastes. The best results was attained using SBO.	34.0$_{(B*)}$	0.18	0.43	(Kitamoto et al., 1990b)
Glucose^{S} and SBO^{S}	Fed-batch fermentation with feed of 40 g.L^{-1} of SBO and 2 g.L^{-1} of YE every 4 days, during 16 days of fermentation.	74.3$_{(A1*)}$	0.19	0.46	(Konishi et al., 2008)
n-alkanes (10 to C18)^{S}	Fed-batch fermentation with feeds of 6% of octa-decane every 7 days, during 30 days of fermentation.	140.0$_{(B)}$	0.19	0.76	(Kitamoto et al., 2001a)
Sucrose^{S}	Fed-batch fermentation with feeds of 150 g.L^{-1} of sucrose every 7 days, until 21 days of fermentation.	12.8$_{(A1)}$	0.03	0.03	(Morita et al., 2009a)
Glucose^{S}	Fed-batch fermentation with feed of 120 g.L^{-1} of glucose every 7 days, until 21 days of fermentation.	12.8$_{(A1)}$	0.03	0.04	(Morita et al., 2007a)
Glycerol^{S} and 2% of mannose^{S}	Fed-batch fermentation with feed of 100 g.L^{-1} of glycerol and 20 g.L^{-1} of mannose, every 7 days, for 21 days.	16.3$_{(A1)}$	0.03	0.04	(Morita et al., 2007b)
Crude glycerol^{R}	The authors studied different nitrogen sources, ratios of MeOH and scaled-up to bioreactors.	6.7$_{(A1)}$	0.06	0.13	(Liu et al., 2011)
Xylose^{S}	The authors explored the capacity of three different *Moesziomyces spp* to assimilate pentoses and studying the effect of ratio C/N in MELs production.	4.8$_{(A2*)}$	0.02	0.14	(N. T. Faria et al., 2014)
Glucose^{S}		5.4$_{(A2*)}$	0.02	0.13	
Xylose^{S} + glucose^{S}		4.9$_{(A2*)}$	0.02	0.11	
Glucose^{S} + different inhibitors^{S}	The authors have studied the effect of different inhibitors (acetate, furfural and formate), on MELs production. Furfural revealed as the one with higher inhibitory effect on MELs production. This study	-	-	-	(Santos et al., 2019)

Substrate used	Brief description of the process	MELs (g/L)	Productivity (g/L/h)	Yield (g/g)	Article
	provides insight to the best conditions to use, when using lignocellulosic residues as main substrate.				
Cocunut water^{R}	MEL production only from a residue, without any supplementation of any medium component.	$3.9_{(C)}$	0.02	0.78	(Madihalli et al., 2020)
Sugarcane Juice^{R}	Apart from the industrial residue tested as a carbon source, the authors also evaluated different nitrogen sources and temperatures.	$12.0_{(A1)}$	0.06	0.54	(T. Morita et al., 2011)
Soap stock^{R}	The authors evaluated different wastes (glycerol, post-FFA, WFO, post refining waste, whey permeate), using different microorganisms.	$107.2_{(C)}$	0.45	0.54	(Dzięgielewska and Adamczak, 2013)
Residual honey ^{R}	They have started with 14% of residual honey, and in the end, only 1.5% of residual lipids were left	$5.4_{(B)}$	0.39	*n.a*	(Bhangale et al., 2013)
Wheat straw^{R}	Fed-batch SSF with pre-hydrolysis of wheat straw	$2.5_{(A2*)}$	0.01	0.042	(N. Faria et al., 2014)
WFO^{R}	Comparison of MEL production using waste cooking oil (home-prepared) versus SBO.	$55.0_{(C)}$	0.68	0.23	(Niu et al., 2019)
Whey permeate^{R} + Waste fish oil^{R}	The authors used 8% (w/v) of whey permeate, treated with commercial β-galactosidase, and 8% (w/v) of waste fish oil. In this study the authors also tested other waste fats (pork, post-refining fatty acids)	$26.7_{(A2)}$	0.19	0.148	(Bednarski et al., 2006)
SBO^{S} + sweetwater^{R}	The authors have used 7% of SBO as hydrophobic carbon source with different % of sweetwater (16-24) as hydrophilic carbon source. Best result was obtained using 22% of sweetwater.	$7.5_{(B)}$	0.04	*n.a*	(Mawani et al., 2021)
SBO^{S}	The authors have tested different nitrogens sources (where the best was $NaNO_3$) and also tested different concentrations of $NaNO_3$ (2 $g.L^{-1}$) and SBO (70 $g.L^{-1}$). Later, the authors produced nanomicelles of MELs for antimicrobial assays and drug release (clarithromycin).	$64.5_{(A2)}$	0.38	0.92	(Yu et al., 2022)
Glucose^{S}	The authors have used different species and tested different culture medium. The idea was to replace the culture normally used, having yeast extract, and replace by trace elements and vitamins, without affecting the growth of the fungi.	-	-	-	(Beck and Zibek, 2020)
FFA^{S}/FAME^{S}	Native lipases produced by M. antarcticus were used to partially hydrolyze vegetable oil, forming free fatty acids	12.6/12.3	0.37/0.54	0.075/0.11	(Keković, 2022)

Substrate used	Brief description of the process	MELs (g/L)	Productivity (g/L/h)	Yield (g/g)	Article
	(FFA). While methyl alcohol esters were produced using immobilized CAL-B. Both were used as hydrophobic carbon source (addition at 4th day, in a fermentation started with 40 g/L of glucose).				
Cheese whey^{R} + WFO^{R}	The authors have used only 40 g/L of lactose (cheese whey) with 20 g/L of WFO without any supplementation of culture medium. For the first time, the authors observed the production of β-galactosidase in *Moesyzomycess* spp.	13.9$_{(A2^*)}$	0.06	0.23	(Nascimento et al., 2022a) – **Section B.2**
Crude oil^{S} + NaCl^{S}	The authors have studied the effect of NaCl (0, 3.5, 5 and 10%), where they found that 3.5% of NaCl is enough to produce MELs in unsterile conditions. Using	3.0$_{(A2^*)}$	0.009	0.07	(Petar Keković et al., 2022)

A.2.4.2 Bioprocesses development for MELs production

There is a multitude of literature sources providing details on MELs production in shake flasks, however the transition to bioreactors is not quite often, and only a few numbers of publication report the production of MELs in bioreactors (**Table A4**).

Most fermentations are performed at similar temperatures, ranging from 25 to 28 °C. As for agitation and aeration, they were usually reported to be set up in a cascade, in order to maintain the dissolved oxygen (DO) in the broth at a fixed value. This ranges from 20 up to 50% (Marek Adamczak and Bednarski, 2000; Kim et al., 2006). Agitation speeds range from 150 to 750 rpm, which should not present a problem for yeast cultures. Considering the critical role of oxygen in MELs production (one of the bottlenecks of the bioprocess; for more information see **section B.1**), it is expected that these studies (**Table A4**) to present kLa (oxygen mass transference) under the best conditions for maximum MELs titre. This would allow a better comparison between studies, as different bioreactors and different impellers can be used, influencing the uptake of oxygen. However, so far, none of the studies have reported this information, which makes it hard to assess the eventual oxygen limitations. Additionally, this omission complicates the scale-up process while attempting to replicate other conditions.

A.2.4.2.1 Working microorganisms

Several species of yeasts and a few filamentous fungi are reported to be efficient MELs producer strains within scientific reports spanning the last several decades. Most reported producer strains are presented in **Table A1**. However, performance of microorganisms varies not only in terms of their productivity, but also of the substrates that can be used efficiently for MELs production, and their performance in laboratory scale bioreactors, which is linked to their morphology.

Most successful fermentations, in terms of MELs titres and yields, from literature sources use yeasts from the *Moesziomyces* genus. Kitamoto et al. (Kitamoto et al., 1990a) report relative high yields of 0.425 $g_{MELs}/g_{substrate}$ achieved by *M. antarcticus* with the use of SBO. The same species was reported to achieve high MELs titers of 107 and 140 g/L on soapstock (Dzięgielewska and Adamczak, 2013) and n-alkanes (Kitamoto et al., 2001a), respectively. while performing portly on a substrate consisting solely of glucose, with maximum MELs concentration reaching only 12.8 g/L (Morita et al., 2007c). Other species of the same genus also achieve good results, with *M.*

bullatus being used in fermentations with WFO (Niu et al., 2019) and soap stock, achieving titres of 55 and 77.7 g/L, respectively. Konishi et al. (Konishi et al., 2008), reported that *M. hubeiensis* was able to reach titres of 74.3 g/L, with a relative decent yield of 0.464 $g_{MELs}/g_{substrate}$.

On the other hand, production of MELs by fungi of the genus *Ustilago* rarely reports competitive results in terms of MELs productivity and yields. Morita et al. (Morita et al., 2009a), reached only 12.8 g/L with *U. scitaminea*, using sucrose as carbon source. Similarly, Liu et al. (Liu et al., 2011) report that the fungus *U. maydis* produced only 6.7 g/L of MELs with crude glycerol as the carbon source. As for production in laboratory-scale bioreactors, most reported literature states that successful MELs production fermentations were performed with *Moesziomyces* yeasts as working microorganisms. Finally, in the effort towards developing sustainable large-scale MELs production, it is important highlight the various challenges when using filamentous fungi in bioreactors. Their use leads to technological challenges resulting from their morphological properties (Gibbs et al., 2000). With this in mind, and with the overwhelming reports of yeast fermentations achieving better results in terms of MELs production, it seems that the way forward is by using *Moesziomyces* yeasts in industrial bioprocesses. Continuous efforts to improve the working microorganism should be maintained, as they are crucial for developing an efficient process. This is particularly important for developing genetic engineered strains, capable of resist to several factors (e.g inhibitors present in industrial side streams, such as acetic acid, or polyphenols).

A.2.4.2.2 Feeding strategy Yield, productivity and titre

In most of the studies reported in **Table A4**, the type of fermentation more used is fed-batch, allowing to achieve high MELs titres. In fact, the best titre (165 g/L) reported in literature for MELs was obtained from a long fed-batch fermentation that used large amounts of SBO (around 186 g/L), glucose (around 50 g/L) and mineral medium (14 g/L of yeast extract (YE) and sodium nitrate) (Rau et al., 2005b). In this study and in many others reported in **Table A4**, SBO is used as both a carbon source and an anti-foam agent. Often, the amount of SBO used to control the foam in bioreactor is not specified. For example, Rau et al. (Rau et al., 2005b), reported a MELs yield of 0.93 $g_{MELs}/g_{substrate}$,, indicating that 92% of carbon in substrate is direct towards MELs production, with only 8% used for other products (e.g biomass, proteins, ect). This value is clearly

unreliable and nearly impossible, and it seems the authors have not considered in the calculations the amount of oil added as an anti-foam agent.

Other sources report that high titres were achieved using multiple feeds of vegetable (Marek Adamczak and Bednarski, 2000; Goossens et al., 2016; Kim et al., 2006). Recently, for the first time, Yang et al. (Yang et al., 2023), reported the production of MELs in a pilot scale (1 m^3). In this study, the authors used a similar strategy performed by Rau et al. (Rau et al., 2005b), using fed-batch strategy (feeds of 3,1 and 1% (v/v) were fed at days 3, 4 and 6, respectively) during 10 days, resulting in a MELs concentration of 76.7 g/L. This study is particularly motivating as it validates MELs bioprocess on a 1m^3 scale, demonstrating this bioprocess can indeed make into industry scales. However, the use of SBO or RO as a main substrate increases the issues of process scale-up, since it is a threat for food availability and prices, requiring a large arable land area for production (Anto et al., 2020).

Only a few papers give information on MELs produced from waste materials in bioreactors. Dzegliewska and Adamczak (Dzięgielewska and Adamczak, 2013), achieved very high titres of MELs (120.5 g/L) using RO, previously broken down into FFA using commercial lipases and supplemented with fresh biomass, however yields and residual substrate were not reported, and the quantification method used belongs to “C” category (see **Table A2**). Morita et al. (T. Morita et al., 2011), successfully used sugarcane juice supplemented with urea to produce MEL-B (25.1 g/L).

It can be observed, based on information presented in **Table A4**, that full transparency in terms of substrate to MELs conversion efficiency is a rare occurrence in most of the sources. Mostly omitted information is regarding the level of residual lipids, as well as the purity of the collected MELs. Although MELs titre, yield and productivity, which are usually reported, give important information about the fermentation efficacy, they are not sufficient to construct a more thorough mass balance needed for up-scaling the process and planning production in an industrial scale.

A.2.4.2.3 Issues with upscaling MELs production in bioreactors

Due to a high need for oxygen in the bioconversion step of the process, bioreactors require thorough mixing and intense aeration to achieve sufficient yields of MELs. However, due to the surface activity of the molecule, as well as the presence of extracellular protein in the broth,

foaming is a problem that needs to be dealt with during upscaling MELs production to an industrial scale. Some sources state that vegetable oil addition was used in the first days of fermentation (Kim et al., 2006; Rau et al., 2005b). In some cases, these feeds are overlooked, and not considered when calculating the mass balance and yields of MELs. As the foam fraction contains significant amounts of MELs, it can present an opportunity to efficiently recover MELs from the system. In this regard, Andrade et al. (Andrade et al., 2017), report that foam created during fermentation was collected and processed, and proved to be an efficient way to collect MELs. Moreover, Yang et al. (Yang et al., 2023), reported the collection of foam to an external tank, within an industrial context, solving one of the problems caused during MELs fermentation.

Although other strategies of controlling excessive foam formation are also efficient, such as addition of synthetic foam-controlling agents or the use of oxygen-enriched air, they tend to decrease the overall sustainability of the process, by using petrochemical-based compounds which remain in the effluents or product, or by driving up the process costs. Focus should be put on feed strategies that include lipid-based substrates in key days when foam formation is expected, as well as alternative mixing and aeration methods and innovative bioreactor setups.

Table A4: Summary of the results obtained in studies reporting the production of MELs in bioreactors, with different microorganisms. It is indicated for each study, the final titre of MELs, purity, yield and productivity (directly retrieved from the study or calculated using data available therein). n.a. – not available. Additionally, for each study it is represent the method used to quantify MELs, by using the classification system created (see **Table A2**).

Type of each operation/reactor	Set-up of each reactor	MELs (g/L)	Residual lipids (g/L)	MELs purity (%)	Productivity (g/L/h)	Yield ($g_{MELs}/g_{substrate}$)	Brief description of the process	Authors
Fed-batch (30 L of working volume in a 72 L bioreactor)	Initial pH of 6.2 (and not monitored afterwards). The agitation and aeration rate were 300 rpm and 3.3 vvm (540 $l.h^{-1}$), respectively.	165(A1)	*n.a*	*n.a*	0.58	0.93	The authors started with 17 $g.L^{-1}$ of SBO and 23 $g.L^{-1}$. From day 1 until day 2.8 day of fermentation a concentrated solution (glucose 285 $g.L^{-1}$, sodium nitrate 16 $g.L^{-1}$, yeast extract 14 $g.L^{-1}$) was fed at 125 $ml.h^{-1}$. To prevent foaming, was fed 6.1 l of SBO (from 0.75 to 3.75 day)	(Rau et al., 2005b)
Batch (2 L of working volume in a 5 L bioreactor)	DO was kept at 50 %, using 1 vvm as aeration rate and varying the agitation from 150 to 500 rpm.	45(C)	*n.a*	*n.a*	0.31	0.5625	The authors started with 80$g.L^{-1}$, studying different substrates (the best one was SBO). They also have studied different vvm (1 and 2) with different % of DO (25,50 and 75 %) in the medium	(Marek Adamczak and Bednarski, 2000)
Fed-batch (10 L o working volume in a 15 L bioreactor)	Agitation was 216 rpm, imposing a cascade of aeration rate (ranging from 0.8 to 0.36vvm).	69(A1)	17% (w/w)	51	0.16	0.51	They started with 73.4$g.L^{-1}$ of rapeseed oil (RO) and after 8.9 days they feed with 55.4$g.L^{-1}$ of RO.	(Goossens et al., 2016)
Fed-batch (1.5 L of working volume in a 2 L bioreactor)	DO was maintained at 30% using 1 vvm as aeration rate.	32(A1)	*n.a*	*n.a*	0.17	0.25	They started with 50 $g.L^{-1}$of crude glycerol, and only after dropped until 15 $g.L^{-1}$the fermentation was initiated. After that, a mixture was fed (460 $g.L^{-1}$of crude glycerol and 132.2 $mg.L^{-1}$of ammonium citrate) at 3 $ml.h^{-1}$. They also studied the	(Liu et al., 2011)

Type of each operation/reactor	Set-up of each reactor	MELs (g/L)	Residual lipids (g/L)	MELs purity (%)	Productivity (g/L/h)	Yield ($g_{MELs}/g_{substrate}$)	Brief description of the process	Authors
							effect of methanol (2,5 and 10%).	
Fed-batch (2 L of working volume in a 5 L bioreactor)	DO was kept at 20 %, using a range of vvm (0.2-2vvm) as aeration rate, and an agitation varying from 500 to 750 rpm. Initial pH of 8 and kept at 4, after 24 hours of fermentation. It was used two feeds of SO at 44 and 104 h.	95(B)	20 (SO)	82	0.48	0.475	They started with 15g.$L^{-1}$of SBO and glucose. To prevent foam appearance, they used two feeds of SBO at 44h and 104 h. They also studied the effect of controlling pH (3,4 and 5).	(Kim et al., 2006)
Batch (3 L of working volume in a 5 L bioreactor)	In the first 24 h, they imposed an agitation of 100 rpm and 0.4 vvm as aeration rate, after that, they changed to 150 rpm and 0.8 vvm until 84 h of fermentation.	1.26	*n.a*	*n.a*	*n.a*	*n.a*	The authors created and integrated bioprocess. By using cassava wastewater (composed of sugars) as a carbon source, the foam created was recovered, freeze dried and passed in an ultrafiltration membrane to purify MELs.	(Andrade et al., 2017)
Fed-batch (3 L of working volume in a 5 L bioreactor)	Initial pH was 6.2 and there is no information about other parameters, such as agitation, vvm or % of DO.	120.5(C)	*n.a*	*n.a*	0.63	*n.a*	They used commercial lipases to break RO and tested the effect of starting with different concentrations of fresh biomass (5,50,100 and 500 g.L^{-1}). They started with 20 % of post-FFA, 2% of glucose, and with 500 g.L^{-1} of fresh biomass.	(Dzi and Adamczak , 2013)
Fed-batch (2 L of working volume)	DO was set up at 50%, using a cascade of agitation, ranging from 100-500 rpm, with an aeration rate of 1 vvm.	45.5(C)	*n.a*	*n.a*	0.32	*n.a*	The authors have started with 40 g.L^{-1} of glucose, with feeds of 80 g.L^{-1} of SBO at day 2 and 4, during 6 days of fermentation.	(M. Adamczak and Bednarski, 2000)

Type of each operation/reactor	Set-up of each reactor	MELs (g/L)	Residual lipids (g/L)	MELs purity (%)	Productivity (g/L/h)	Yield ($g_{MELs}/g_{substrate}$)	Brief description of the process	Authors
Batch (1 L of working volume in a 5 L bioreactor)	The agitation was fixed at 600 rpm with an aeration rate of 0.5 vvm.	25.1(A1)	*n.a*	*n.a*	0.15	0.13	The authors used 19.3 % of sugarcane juice supplemented with 1 $g.L^{-1}$ of urea, producing only MEL-B.	(T. Morita et al., 2011)
Batch (3 L of working volume in a 5 L bioreactor)	The agitation was fixed at 600 rpm, with an aeration rate of 1 vvm. pH was kept at 6.0 and the temperature at 30ºC	21.5(B)	*n.a*	*n.a*	0.13	*n.a*	The authors have used 7% of SBO with 22% of sweetwater.	(Mawani et al., 2021)
Fed-batch (7 L of working volume in a 10 L bioreactor)	The agitation was fixed and 250 rpm, with an aeration rate of 1 vvm.	113.6(A2)	*n.a*	*n.a*	0.59	94.6	Fermentation started with 20 $g.L^{-1}$ of glucose and SBO. A feed of 30 20 $g.L^{-1}$ was added at day 2 and 5.	(Yu et al., 2022)
Pilot scale; Fed-batch (500 L of working volume in a 1000 L bioreactor)	Agitation was kept at 200 rpm and aeration rate was no less than 0.3 vvm.	76.7(B)	*n.a*	*n.a*	0.40	0.69	First study reporting the production of MELs in a pilot scale. The authors have shown that the addition of 0.2 mM of Fe^{2+} and 0.1 mM of Fe^{3+} improved MELs titre. Fermentation started with 80 $g.L^{-1}$ of SBO as carbon source. A feed of 3,1 and 1% (v/v) was fed at days 3,4 and 6, respectively, being used as carbon source and anti-foam (which was collected to a tank).	(Yang et al., 2023)
Fed-batch (3 L of working volume in a 7 L bioreactor)	pH was kept at 6. DO was maintained at 10% using a cascade of agitation, ranging from 400 to 1200 rpm. Aeration rate was set up at 0.7 vvm.	50.5(A1)	16.2	68	0.19	0.208	The process started with glucose as carbon source, and after consumption, RO was added. At this stage, two scenarios were tested, varying the amount of RO added (16-20 and 9-10 $goil.gbiomass^{-1}$). The authors also developed a simulator, using kinetic equations,	(Beck et al., 2022)

Type of each operation/reactor	Set-up of each reactor	MELs (g/L)	Residual lipids (g/L)	MELs purity (%)	Productivity (g/L/h)	Yield ($g_{MELs}/g_{substrate}$)	Brief description of the process	Authors
							which predicted the actual values of the process.	

A.2.4.3 Downstream processing

Product collection (extraction), separation and purification within the downstream processing section of the process are associated with about 60% of overall production costs (Najmi et al., 2018). Levels of residual substrate in the fermentation broth at the end of the process are an important factor in terms of process sustainability. Unused substrate is an indicator of an inefficient substrate-to-product conversion and affect negatively the economy of the process. Furthermore, presence of substrate in waste streams can cause serious harm to the environment, and so increases waste processing costs.

Finally, residual substrate present in the final product is especially problematic for biosurfactants produced from vegetable oils. This drops the quality of the product drastically and imposes the need for complex downstream processing. The previously mentioned issues with transparent reporting of residual substrate in scientific communication cause confusion when comparing data from different authors.

A.2.4.3.1 Techniques used to purify MELs

Through all the literature (**Table C5**), the initial method used for harvesting MELs fraction from the culture broth are based on an organic solvent extraction (e.g ethyl acetate) (Rau et al., 2005a), or by sedimentation/decantation, often coupled with heating/boiling, (Worakitkanchanakul et al., 2009). After obtaining final crude mixture of MELs (contaminated with residual lipids), several techniques were explored. Example of chromatography column based processes for MELs purifications are able to achieve high product purities (> 97%), however the amount of MELs recovered was limited to only 4 % (Kim et al., 1999) or 50 % (Morita et al., 2007d), using as eluents chloroform mixtures with methanol (MeOH) or acetone, respectively. Both studies reported the use and disposal of large volumes of solvents, being toxic to environment and human health (Smyth et al., 2010), but also its recyclability is problematic, namely due to the formation of stable azeotropes (Shephard et al., 2016). An example of an organic solvent extraction method for MELs extractions is reported in the study by Rau et al. (Rau et al., 2005a), which uses sequential liquid-liquid extraction employing 1:2 of n-hexane, 1.6:1 of MeOH, 3:1 of MTBE and 3:1 of cyclohexane (v/v of fermentation broth), but able to recover 8% of the MELs produced. The MTBE used in the initial extraction has the potential to be recycled by distillation,

but the other solvents would require challenging separation and should be considered waste. In another study, Shen et al. (Shen et al., 2019), achieved 80% of MELs recovery employing 2.5:1 of MeOH and 3:1 n-hexane (v/v of fermentation broth) using solvent shifts (e.g avoiding the need of solvents to be completed evaporated prior to addition of new ones). Still, again solvents recyclability is hampered by formation of complex mixtures and stable azeotropes.

In this regard, these issues were tried to be solved during my PhD studies, where it was developed a new technology, using organic nanofiltration (OSN), to remove residual lipids, as it will be described in **section C.1**.

A.2.5 Development of novel applications

In order to have a more sustainable process, novel applications should be developed, as they give value to the product itself, and encourage the strive for continuous development of the process. Bioproducts can often directly substitute already used chemical products, due to similar properties. However, additional possibilities for applications of these products exist, primarily due to their uncommon properties, such as low toxicity, biocompatibility, etc.

Based on the properties of MELs, scientists have in the previous decades suggested, and tested, their use for various applications. These include applications in the fields ranging from medicine and cosmetics to agriculture and bioremediation, where MELs would be used as both a specialty and a bulk chemical. These proposed applications of MELs are presented in **Table A5**.

A.2.5.1 Biomedical/Pharmaceutical industry

Due to MELs lack of toxicity and their positive interaction with the human body, many cosmetic and medical applications has been proposed. These applications are based on MEL's beneficial interactions with various cell types, antimicrobial properties, as well as their nanostructure formation capabilities (e.g. liposomes structures to transport more effectively drugs to their site of action).

One of the first function to be explored was the antimicrobial activity. Kitamoto et al. (Kitamoto et al., 1993) tested the activity of MEL-A and MEL-B on gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Mycobacterium rhodochrous*, *Staphylococcus aureus*), gram-negative bacteria (*Pseudomonas aeruginosa*, *Pseudomonas rivoflavina*, *Escherichia coli*), and in fungi (*Candida albicans*, *Aspergillus niger*). They concluded that MELs exhibit a strong inhibitory effect on gram-positive bacteria, and some sensitivity to *Pseudomonas* strains. Since most food pathogens belong to gram positive bacteria, more studies were performed using MELs against these pathogens (Ceresa et al., 2020; Lipids-a et al., 2020; Shu et al., 2020; Yamauchi et al., 2022). Studies have shown that MELs can damage the integrity of cell membranes and interfere with the adhesive capacity of bacteria, avoiding biofilm formation. This is particularly significant for pharmaceutical and biomedical applications in equipment treatment and medical implants, but also in the food and feed industry, where it can be used as food preservative and in the treatment of farms. For example, Shu et al. (Shu et al., 2022), have seen that when using MEL-A (1.5%),not

only improve the loaf volume and gas retentention; but also achieved a killing rate of 99.7% for *Bacillus* cereus vegetative cells and 75.54% for its spores.

In the field of medicine, MELs proposed applications are based on beneficial interactions with various cell types, antimicrobial properties, as well as their nanostructure formation capabilities. Several studies have stated the use of MELs for anticancerogenic applications, based on their ability to damage cancer cells and cause their differentiation. Isoda et al. (Isoda et al., 1999), reported that MELs induce neurite outgrowth, opening the possibility of applications for neural damage repair. Morita et al. (Morita et al., 2011) observed MELs anti-inflammatory capabilities by affecting exocytotic release in cell lines, while Ueno et al. (Ueno et al., 2007), and Inoh et al. (Inoh et al., 2001) proposed possible applications for gene delivery due to MELs transfection capabilities and successful incorporation into the host cells in liposome form. Similarly, MELs can be possibly used for drug delivery, in the form of nanoparticles formed with metals (Bakur et al., 2019).

Recently, Wu et al. (Wu et al., 2022), designed a drug delivery complex liposome for antibiotic delivery, using MEL-B, soybean lecithin (SL) and cholesterol (LipoSC-MELB). These liposomes loaded with amoxicillin were tested against *Helicobacter pyrol*i, which is responsible for gastritis and peptic ulcer disease in humans. Remarkably, the authors have shown these liposomes can be used for treatment of *H. pyroli* infection, a condition affecting a significant portion of the global population. Additionally, MELs were observed to prevent biofilm formation of Staphylococcus aureus, with possible use for medical implant, equipment treatment, or even as food preservatives (Ceresa et al., 2020).

A.2.5.2 Personal care and cosmetics

Currently there is already a company (Toyobo©) merchandising MEL-B as a cosmetic. On this field, Yamamoto et al. (Yamamoto et al., 2012), tested MELs on participant's skin, and found it had great moisturizing effects, while Choi et al. (정관영, 2016) patented a ceramide-based skin care product containing MELs in its formulation indented for reducing skin wrinkling. Bae et al. (Bae et al., 2019a) observed that MELs inhibit melanogenesis in human melanocytes and a skin-equivalent, opening the possibility of the development of a skin-whitening product, while the same authors in a different study (Bae et al., 2019b), reported that MELs can potential be used for

treating UVA irradiation damage to skin, based on in-vitro tests. In a different context, two other reports from groups of scientists lead by Tomotake Morita tested MELs interaction with hair and hair-growth cells, reporting in-vivo results of MELs potential use for hair damage repair, as well as stimulation of fibroblasts and papilla cells, critical elements of hair growth (Morita et al., 2010a, 2010b) .

Due to the complexity and pre-requirements needed for in-vivo tests for medical applications, in this area only one of the reports has results based on tests performed in realistic conditions. These relate to MELs antimicrobial properties, where they were observed to prevent gram-positive bacteria proliferation.

A.2.5.3 Agricultural applications

Agricultural applications of MELs are mostly based on its tensioactivity and bioactivity. Fukoka et al. (Fukuoka et al., 2015) tested MELs applicability as an agrospreading agent, due to its beneficial interaction with hydrophobic plant surfaces. Similarly, MEL-A applied on leaf surfaces was shown to prevent conidial germination of the pathogenic fungus *Blumeria graminis* for some plants with hydrophobic leaf surfaces (Yoshida et al., 2015).

Moreover, MELs toxicity against mosquito larvae and pupae was tested, a LC50 between 30-60 µg/mL was obtained, depending on the stage of the larvae, which is a moderate toxicity. On the other hand, MELs-synthesised silver nanoparticles, shown to be highly toxic, with a LC50 of approximately 1 µg/mL. The authors propose that nanoparticles with silver increase the bioactivity of MELs against mosquito larvae and pupae (Ga'al et al., 2021a, 2021b). A recent study by Matosinhos *et al*. (Matosinhos et al., 2023), studied the effect of MELs in lettuce seed germination, plant growth and root development, concluding that MELs can have both a biostimulant and a phytotoxic effect, depending on their concentration.

A.2.5.4 Other applications

Other applications were proposed based on some of the many specific properties MELs have. In this regard, knowing the capacity of MELs to reduce surface tension and create emulsions, it was tested its detergent activity. It was found that MELs are stable at high temperatures and pHs, and, in a 1:1 portion with a commercial detergent, they improve the efficiency of stain removal (Sajna et al., 2013).

Furthermore, MELs interact positively with n-alkanes, making them more bioavailable and enhancing their biodegradation, which opens the possibility of their use in bioremediation (Yu et al., 2015). This, coupled with the fact that most MELs-producing microorganisms can consume n-alkanes, as was mentioned earlier, makes this hypothesis even more compelling ((Kitamoto et al., 2001a). It was found that MELs have the capacity of maintaining a good stability and activity under extreme temperatures, pH and salt concentration values (Andrade et al., 2015; Jose de Andrade and Maria Pastore, 2016). Additionally, MEL-A improves the fluidity of biodiesel and hydrocarbon fuels at low temperatures, opening the possibility for MELs to be applied as fuel additives (Madihalli et al., 2016). This property was used to show that MELs were able to prevent ice particle growth, making MELs a promising ice agglomeration control agent (Kitamoto et al., 2001b).

The diversity of applications in which MELs excels over others mBS, really indicate that there are many more undiscovered opportunities for this molecule to enter the market of mBS and compete with SLs. Focus should be put on MELs exceptional properties which give them an advantage over chemical agents and other mBS. This includes not only replacing existing agents but also innovating novel applications that leverage the multifunctional nature of this molecule.

Therefore, development of the process and new applications should progress simultaneously. The following sections will detail the advancement made within this thesis to create a more competitive and sustainable process.

Table A5: Summary table of MELs potential applications reported in the literature. *n.a* – Not available.

Application area	Specification	Brief description of the results
Biomedical/ Pharmaceutics	Anti-microbial activity	Both MELs were strongly active against gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, *Mycobacterium rhodoochrous*, *Staphylococcus aureus*), killing more than 99%
		MELs had antimicrobial activity against *S. aureus* and biofilm disruption activity.
		MEL-A (80% of purity) inhibited the germination of *Bacillus cereus* spores.
		MEL-A (80% of purity) inhibited planktonic cells and biofilm of *S. aureus.*
		MEL-B inhibited the growth of bovine mastitis causative *S. aureus.*
		The combination of MEL-A (80% of purity) with high hydrostatic pressure led to a higher bactericidal effect against *Listeria monocytogenes* (than the hydrostatic pressure alone).
		MELs inhibited the growth of *E. coli* and *P. aeruginosa*. The combination of MELs and antibiotics potentiated antibiotics' efficiency.
Biomedical/ Pharmaceutics (continuation)	Anti-tumour	MELs induced the differentiation of Human Promyelocytic Leukemia cells HL60 and inhibited Protein Kinase C activity.
		MELs inhibited Tyrosine Kinase activity, inhibiting proliferation and inducing the differentiation of Human Myelogenous Leukemia cells K562.
		MEL-B (90% of purity) reduced cell viability and induced death by apoptosis of B16F10 Mouse Melanoma cells.

Application area	Specification	Brief description of the results	MELs used	References
	Anti-inflammatory	MELs inhibit the secretion of inflammatory mediators by Rat Basophilic Leukemia RBL-2H3 cells (a mast cell line).	MEL-A MEL-B	(Y. Morita et al., 2011)
	Neural repair	MELs induce the outgrowth of neurites from and enhance the activity of acetylcholinesterase in PC12 pheochromocytoma cells.	MEL-A	(Isoda et al., 1999; Shibahara et al., 2000)
	Genetic material transfection or drug-carrying	MEL-A increased the efficiency of gene transfection by cationic liposomes with a cholesterol derivative or DC-Chol.	MEL-A	(Inoh et al., 2004)
		MEL-A-containing cationic liposome was able to deliver siRNA rapidly and directly.	MEL-A	(Inoh et al., 2011)
		MELs were used as stabilizing agents for silver and zinc oxide nanocomposites, gold nanoparticles and for silver and magnetic iron oxide nanocomposites synthesis, to be used in human liver cancer cells inhibition (HepG2).	*n.a*	(Bakur et al., 2022, 2019a, 2019b)
		Nanoliposomes made of soybean lecithin and cholesterol, when incorporated with MEL-B, have enhanced stability to pH 3-7 and deliver amoxicillin for *Helicobacter pylori* infection treatment in vivo.	MEL-B (Toyobo©)	(Wu et al., 2022)
		MEL-B nanomicelles successfully carried berberine for *H. pylori* biofilm disintegration and infection eradication.	MEL-B (Toyobo©)	(Cheng et al., 2023)
	Drug Delivery	Preparation of MELs nanomiceles for drug delivery (clarithromycin). It was shown that, by varying the pH, it is possible to control clarithromycin delivery (pH 1.2, in 2 h, 37.1% of drug was delivery, while, at pH 7.4, only 9.7% was released).	MELs mixture	(Yu et al., 2022)
	Immunoglobulin purification	MEL-A shows high binding affinity towards HIgG, HIgA and HIgM.	MEL-A	(Im et al., 2001; Ito et al., 2007)
Cosmetics and personal care	Formulation stabilization	Emulsification of pseudo-ceramide is stabilized by molecular association with MELs.	Damy chemicals	(Kim et al., 2014)
		MELs stabilize the foaming, emulsification, and wetting properties of Sodium Lauryl Sulphate.	MELs mixture	(Zanotto et al., 2023)
		Coating cosmetics (lip primer, foundation and sunscreen) pigments with MELs, enhance their skin adhesion.	*n.a*	(Kitagawa et al., 2010)

Application area	Specification	Brief description of the results	MELs used	References
	Skin whitening	MELs (85% of purity) inhibit melanogenesis via suppressing ERK-CREB-MiTF-tyrosinase signalling in human melanocytes and a three-dimensional human skin equivalent.	MELs from DKBIO, MEL-B	(Bae et al., 2019a)
	Hair growth promotion	MEL-A (80.1% of purity) produced from soybean oil increases cultured Fibroblast cells and 3D Human Skin model cells viability and activates Human Papilla cells.	MEL-A	(Morita et al., 2010b)
	Damaged hair repair	MEL-A (99% of purity) and MEL-B (90% of purity) shown similar activity as ceramides for hair damage repair, and increase of hair flexibility.	MEL-A MEL-B	(Morita et al., 2010a)
	Skin repair and moisturization	MELs ameliorate UVA-induced aquaporin-3 downregulation by suppressing c-Jun N-terminal kinase phosphorylation in cultured human keratinocytes.	MELs from DKBIO	(Bae et al., 2019b)
		MEL-A had a recovery effect on SDS damaged skin cells	MEL-A	(Morita et al., 2009b)
		MEL-A and MEL-B produced with olive oil show activities similar to natural ceramides on the cell viability and SDS-induced damage repair of cultured human skin cells; MEL-B increased the water content in the stratum corneum and reduced water loss by perspiration.	MEL-A MEL-B	(Yamamoto et al., 2012)
		MELs with carbon chains with 10 or more carbons exhibit better cell damage repair than a natural C18 ceramide, particularly MEL-D C10 (MELs purified by acetylation level and carbon chain size, see original paper)	MELs purified	(Kondo et al., 2022)
		MEL-B protected both HaCaT and 3D skin cell models from UVB- and SDS-induced damage by up-regulating the expression of the skin barrier damage-associated key mRNA genes and proteins LOR, FLG, and TGM1 (MELs mixture 34.94% MEL-A, 28.46% MEL-B and 11.32% MEL-C).	MELs mixture.	(Jing et al., 2022)
		▪ MEL-B liposomes increase skin permeability to water-soluble compounds (calcein) in mice.	MEL-B (Toyobo©)	(Tokudome and Tsukiji, 2020)
	Antioxidant	▪ MEL-C (purity of 80.7-92.5%) has antioxidant activity through DPPH radical and superoxide anion scavenging and protection of cultured human fibroblast cells against H_2O_2-induced oxidative stress	MEL-C	(Takahashi et al., 2012)
	Anti microbial	▪ MELs have antimicrobial activity against Malassezia furfur, the yeast that causes dandruff. A shampoo formulated with MELs and SLS had increased anti-dandruff activity	*n.a*	(Mawani et al., 2023)

Application area	Specification	Brief description of the results	MELs used	References
Bioremediation	Oil spills	▪ MELs increase the bioavailability and biodegradation rate of n-alkanes, diesel, kerosene and crude oil (MELs mixture: 68% MEL-A, 28% MEL-B and -C and 4% MEL-D).	*n.a*	(Hua et al., 2004; Kitamoto et al., 2001a)
			MELs mixture	(Farooq et al., 2024)
	Food preservation	▪ MEL-A enhances the rheological properties and water holding capacity of frozen dough, minimizing the freezable water content, while killing *B. cereus* cells and spores	MEL-A	(S. Liu et al., 2024; Shu et al., 2022, 2019)
		▪ Emulsification of essential oils with MEL-B (*Thymus vulgaris*, *Lippia sidoides* and *Cymbopogon citratus*), leads to an enhance of essential oils' antioxidant activity and preservation of antimicrobial activity.	MEL-B	(Zanotto et al., 2023)
Agriculture	Agro-spreader	▪ MEL used as agrochemical spreader for biopesticides for hydrophobic plant surfaces (MELs mixture: 58% MEL-A, 25% MEL-B and 10% MEL-D).	MEL mixture	(Fukuoka et al., 2015)
	Wetting agent	▪ MEL solutions showed good wetting ability on poorly wettable Gramineae plant surfaces.	MEL-A, MEL-B, MEL-C	(Fukuoka et al., 2015)
	Biocide	▪ MEL-Ag nanoparticles; activity against mosquito larvae and pupae	MELs mixture	(Ga'al et al., 2021)
		Powdery mildew was suppressed on MEL-treated leaves.	MEL-A	(Yoshida et al., 2015)
		▪ MELs, combined with other ingredients, are used for nematodes control.	NA	(Farmer et al., 2018)
		▪ MEL-B, biostimulant and phytotoxic effect on lettuce plant germination and growth for given concentrations.	MEL-B (Toyobo©)	(Matosinhos et al., 2023).
Others	Fuels additive	▪ MEL-A enhances the fluidity of fuels at low temperatures.	MEL-A	(Madihalli et al., 2016)
	Jet biofuel	▪ MELs are used as precursors for fuel with lipid chains comprising 6 to 14 carbons production.	*n.a*	(Faria, 2014)
	Enhanced oil recovery	▪ MEL-B can create emulsions with heavy oils.	MEL-B	(Jose de Andrade and Maria Pastore, 2016)

Application area	Specification	Brief description of the results	MELs used	References
	Detergent	▪ MELs had stability over wide pH and temperature ranges and improved detergent efficiency in removing stains from fabric in a proportion of 1:1 (w detergent/w MELs)	MELs mixture	(Hellmuth et al., 2014; Sajna et al., 2013)
	Ice prevention	Suppression of agglomeration and growth of ice particles	MEL-A	(Kitamoto et al., 2001b)

A.2.6 Approaches towards a sustainable large-scale production of MELs

To produce a market-competitive MELs-based product, the overall manufacturing costs must be significantly reduced. This requires optimization across all segments of MELs production, such as: 1) finding novel low-cost substrates for MELs production; 2) increasing the efficiency of the bioconversion process itself; and 3) finding cheap and efficient downstream processing pathways to obtain purified MELs. However, to ensure that these solutions will be viable long term, sustainability must be considered. Therefore, the final process must satisfy three key criteria which encompass sustainable development: environmental, economic and social.

Firstly, renewable substrates with a beneficial life cycle impact should be favoured. The production process should have a low need for toxic and non-sustainable chemicals, such as solvents, non-recyclable or compostable materials, and should not generate polluting waste. Also, the product formulation itself should have low ecotoxicity and the affinity to biodegrade into compounds not presenting a danger to the environment. However, for a process to be accepted by the profit-driven industry, it has to be economically profitable. Costs for equipment, materials and energy, and workforce expenditures should be as low as possible. Modifications within the bioconversion process itself can contribute to facing this challenge, by increasing titres, yields and productivity. To satisfy the social aspect of sustainable manufacturing, the production process must be developed in a socially responsible way. This practically means that downstream treatment of the product should be in line with specific requirements for various applications depending on societal needs; substrate selection should take into consideration resource security and avoid unethical competition with the food sector; and strive to achieving a final product which is safe for human health.

An important concept in sustainable development is circular economy. This is especially important when developing a cheap, bulk material, which would require large quantities of raw material. This can be achieved by incorporating waste streams from other industries into media formulation.

Finally, the key parameter defining the economy of the process in all its segments is the intended application of MELs, which gives value to the product. For high-end applications

(medical, cosmetic), where small amounts of MELs of great purity are needed, production costs and downstream expenses can justifiably go up. The priorities for process development in this scenario shift from reaching high titres and using cheap substrates, towards avoiding residual substrates and achieving highly efficient MELs purification. In the other scenario, for applications where MELs are used as a bulk chemical, such as agricultural, petrochemical, and others, high MELs titres should be favoured, and some residual substrates can be tolerated. Overall process costs should be low, by constructing a process setup where productivity is as high as possible, with minimal energy expenses. In this case, MELs mixtures of lower purity are often acceptable, so in selecting the adequate downstream treatment strategies which consume less chemicals and require low energy should be considered.

A.2.7 The potential of using microalgae and yeast in co-cultivation

As has been mentioned several times throughout this thesis, most countries, in line with Paris agreement signed in 2016, have implemented laws to reduce CO_2 emissions. The International Energy Agency (IEA) reports that the increased deployment of clean energy will be the primary driver of the 0.1% increase in worldwide CO_2 emissions between 2022 and 2023. Indeed, emissions increased at a 0.5% annual pace from 2010 to 2023—the slowest growth rate since the Great Depression—demonstrating the promise of better energy sources once more. In this sense, reducing emissions and developing innovative solutions that can replace products based on fossil fuels are the goals (Z. Liu et al., 2024; “Major growth of clean energy limited the rise in global emissions in 2023 - News - IEA,” 2023).

Nevertheless, MELs, one of the most promising mBS, was presented in the previous section along with the work that has been done thus far to further this bioprocess. But every bioprocess that uses heterotrophic digestion as its primary energy source also produces a significant amount of CO_2. Thus, the primary objective is to develop a bioprocess that can substitute products derived from fossil fuels with little to no CO_2 emissions.

About 2.8–2.9 billion years ago, the first photosynthetic microorganisms called cyanobacteria, or blue-green algae, first evolved on Earth. By turning CO_2 into O_2, they completely changed the planet as we know it today, by promoting suitable conditions for the appearance of other species (Patel et al., 2017). Microalgae in this category of microorganisms began to be thought of as a possible addition of other microorganisms (yeasts/bacteria) to be used in bioprocess, because of their ability to convert CO_2 into valuable molecules (e.g., biofuels, bioethanol) by using wastewaters/nutrient rich effluents (Ferreira et al., 2017). Furthermore, this kind of cultivation does not compete with agricultural systems for arable areas (Lopes et al., 2023).

This sub-section will discuss the potential applications of microalgae in co-cultivation with yeasts and their use in the synthesis of important building blocks capable of replacing fossil fuel-based products.

A.2.7.1 Microalgae properties and products

Algae are photosynthetic creatures that can withstand a broad range of temperatures, salinities, pH levels, and light intensities. They may grow in a variety of aquatic environments, including lakes and wastewater. Algae may grow alone or in harmony with other living things. Rhodophyta (red algae), Phaeophyta (brown algae), and Chlorophyta (green algae) are the general classifications for them. They can be divided into two categories based on size: microalgae, which are tiny single cells, and macroalgae, which are multicellular, large-sized, and visible to the human eye (Khan et al., 2018).

Microalgae are special microorganisms, since they have the capacity to produce a wide range of valuable compounds, such as: 1) Lipids (Song et al., 2023); 2) Proteins (Abreu et al., 2012); 3) Vitamin, where commercial microalgae powders are already known to be a source of Riboflavin, vitamin B12, folate (Edelmann et al., 2019); 4) Bioactive compounds as lutein, that protect eyes from oxidative stress (Jeon et al., 2014); and 5) Antioxidants, such as astaxanthin, known to be anti-inflammatory, anti-diabetic (Oslan et al., 2021). In fact, a company Algalif is already industrially producing astaxanthin, by using the microalga *Haematococcus pluvialis.*

Additionally, these microalgae can be used as a source of vital compounds used for human nutrition, such as omega-3 fatty acids (eicosapentaenoic acid (EPA)) and docosahexaenoic acid (DHA). Omega-3 fatty acids can be found primarily in fish, however, it is estimated that the actual production of EPA and DHA can only meet 30% of the actual demand for human consumption (1.3–1.4 Mt/year) (Hamilton et al., 2020). In this regard, some studies started to study and optimise the process for the production of omega-3 fatty acids using microalgae, as well reviewed by Perdana et al. (Perdana et al., 2021). For example, Gu et al. (Gu et al., 2022) have evaluated the production of EPA and DHA in 11 different microalgae strain, where they observed that the most promising one is *Phaeodactylum tricornutum* when grown in bioreactors (5-L photobioreactor). Interestingly, the authors have found that EPA content (5% of dried biomass) it is approximately constant in a wide-range o temperature (13-27ºC) and salinities (35-50 g/L).

A.2.7.2 Co-cultivation between microalgae and yeast

Even though the topic of co-cultivation is relatively recent, such cooperation between different microorganism has been occurring since ever. One of the most ancient and well-known relationship are lichens, which are symbiotic associations between a heterotrophic mycobiont (ie. Fungus) and one or more autotrophic photobionts (green algae and or/cyanobacteria) (Asplund and Wardle, 2017). In this type of symbiosis, the fungus provides a shelter for the algae, and this one provides food for the fungus (WA et al., 2022). This relationship has allowed algae to colonise a wide range of environments across the planet, significantly contributing to Earth's ecosystem.

Most of microalgae can have two types of growth, heterotrophic (relying in external carbon sources for obtaining energy) and autotrophic (using sunlight and CO_2). However, some essential molecules (lipids, especially omega-3 fatty acids as previously described) are mainly produced via autotrophic route. For instance, the oleaginous microalgae, such as *Neochloris oleabundans,* can accumulate until 60% of lipids in biomass (Gouveia et al., 2009) as it will be discussed in **section D.2**. Still, the maximum biomass achieved in these autotrophic processes can go up until 2-5 g/L, being not enough and to profitable, when compared with processes using oleaginous yeasts.

In this regard, knowing the capacity of cooperation between fungus and yeast, co-cultivation started to be envisaged as a tool to overcome these challenges, especially for the production of biodiesel. This process relies in the fact that photosynthetic algae consume CO_2 and produce O2. This caption can be coupled to heterotrophic system to optimize CO_2/O_2 exchanges, benefiting from a symbiotic effect and improved biofuel production (Wrede et al., 2014). Moreover, co-cultivation process has the advantage of reducing costs, since only limited quantity of sugar will be added for yeast growth and there is no need for continuous aeration since oxygen is generated by microalgae and carbon dioxide is released by the yeast cells (Chioke, 2021). In **Table A*6*** it is representing a summary of the studies reporting the use of co-cultivation between microalgae and yeast, mainly from improvement of biomass and lipids production.

Specifically, Yen et al. (Yen et al., 2015) have co-cultivated oleaginous yeast *Rhodotorula glutinis* and the microalgae *Scenedesmus obliquus*, in a 5-L photobioreactor. Their results show that co-

culture experiments resulted in biomass increases of around 40-50% and total lipids increases about 60-70%, when compared with mono-cultivations.0020

Different from the studies reported in **Table A*6***, Santos et al. (Santos et al., 2014), developed a different type of process, by connection two reactors for gas exchange. In this process, *Rhodosporidium toruloides,* was grown in stirred-tank reactor for lipids production, and the CO_2 generated was used to stimulate the autotrophic growth of *Chlorella protothecoides* in a vertical photobioreactor. The CO_2 bio-fixed by the microalgal biomass reached an estimated value of 29 mg/L/h in the photobioreactor receiving gas stream from the fermenter, a value 1.9 folder higher when compared to control photobioreactor.

Overall, this sub-section provides an overview of the importance of co-cultivating yeasts and microalgae, summarising studied that reports this process. The information described here has shaped the decisions, guiding the research into-cultivations, which will be further explained in **section D.3.**

Table A6: Summary of most of the results obtained in studies reporting the use of different microalgae and yeast co-culture systems. It is indicated for each study the species used, a brief description of the system, including the scale (i.e., Shake flask or bioreactor), the key parameters, and the biomass and lipid (mg/L/day). Values were directly retrieved from the study or calculated using data available therein. Y- yeast; M - microalgae

Microalgae strain	Yeast strain	Description	Key parameters	Biomass (mg/L/day)	Lipids (mg/L/day)	Ref
Spirulina platensis	*Rhodotorula glutinis*	Monosodium glutamate was used as a promising way to synthesize lipids using wastewater as medium in shake flask fermentation.	**T:**30ºC **Agitation:**140 rpm **Illuminance:** 4,000 lx **Cultivation time:** 5 days	320	44	(Xue et al., 2010)
C. protothecoides	*R. toruloides*	A yeast and a microalgae were, for the first time, grown in two separate reactors connected by their gas-phases, taking advantage of their complementary nutritional metabolisms, i.e., respiration and photosynthesis.	**T:**30 °C (Y), 28 °C (M) **Aeration:**1 VVM (Y), 0.5 VVM (M) **Illuminance:** 4 klux (M) **Cultivation time:** 15 hours	0.22 g/L (M), 6.15 g/L (Y)	2.20 (M), 120 (Y)	(Santos et al., 2014)
Scenedesmus obliquus	*Rhodotorula glutinis*	The co-culture conditions, including the carbon source concentration, temperature and dissolved oxygen level, were first optimized in flask trials, before the scale up to a 5L photo-bioreactor (PBR).	**Agitation:** 150 rpm **Aeration:**1 VVM (2% CO_2) **Light intensity:** 100 PPFD μmol/m/s **Cultivation time:** 7 days	250	600	(Yen et al., 2015)

Microalgae strain	Yeast strain	Description	Key parameters	Biomass (mg/L/day)	Lipids (mg/L/day)	Ref
Mainly *Scenedesmus obliquus*	Mainly *Candida pimensis*	A microorganism co-cultureformed by microalgae and yeast isolated from municipal wastewater plant was grown in an optimized medium (by selecting micronutrients' distribution) in 1L PBR.	**T:** 24 ºC **Light intensity:** 400 PPFD *µ*mol/m/s **Light regime (light/dark):** 12:12 h **Cultivation time:** 12 days	40	242	(Suastes-Rivas et al., 2020)
Chlorella vulgaris	*Rhodotorula glutinis*	The co-culture was performed in shake flasks using glycerol and crude glycerol as carbon sources enhanced biomass and lipid production.	**T:** 30 ºC **Agitation:** 140 rpm **Light intensity:** 3.0 klux **Light regime (light/dark):** 16:8 h **Cultivation time:** 5 days	7.25	920	(Cheirsilp et al., 2012)
Chlorella vulgaris	*Rhodotorula glutinis*	Starch processing effluent was used as afermentation substrate for wastewater treatment and biofuel feedstock production. The authors tested different culture conditions (e.g. different inoculations times) in 200 mL shake flasks to maximize conversion of nutrients to single-cell oil.	**T:**15°C **Agitation:** 50 rpm **Light intensity:** 150 PPFD *µ*mol/m^2/s^1 **Cultivation time:** 6 days **Inoculation:** both species at the same time	563	302	(Lu et al., 2023)

Microalgae strain	Yeast strain	Description	Key parameters	Biomass (mg/L/day)	Lipids (mg/L/day)	Ref
Chlorella sp.	*S. cerevisiae*	The influence of light intensity on the biomass growth, oil accumulation and its property in terms of fatty acid composition for biodiesel was evaluated together with role of yeast in carbon dioxide biofixation in a mixed culture using 2.5 L bubble column reactor.	**T:**28 ºC **Light intensity:** 115 PPFD μmol/m^2/s^1 (for cell growth) and 184 PPFD μmol/m^2/s^1 lux (for product formation) **Aeration:** 0.1 vvm **Cultivation time:** 3 days	660 613	119.34 129.34	(Shu et al., 2013)

Section B: Intensive production of Mannosylerythritol Lipids (MELs) using different agro-industrial residues

B.1 MELs production intensification: The use of co-substrates and development of a fed-batch fermentation

B.1.1 Outline

Microbial biosurfactants can replace fossil driven surfactants with positive environmental impacts, owing to their low eco-toxicity and high biodegradability. However, as previously described in **section A.2**, their large-scale production and application are restricted by high production costs, mainly driven by the use of large quantities of vegetable oils. Such costs can be reduced by utilizing renewable raw materials and simplifying downstream processing. However, it is essential to first identify conditions that meet the predefined requirements before altering the substrates and scale-up the production.

In this regard, here a novel strategy for MELs production explores the combination of hydrophilic and hydrophobic carbon sources sideways. Co-substrate MELs production by *Moesziomyces antarcticus* was 3-fold higher than using D-glucose with low levels of residual lipids. The use of waste frying oil instead of soybean oil (SBO) in co-substrate strategy (starting with glucose and feeding SBO at 4^{th} day) resulted in similar MELs production. *Moesziomyces antarcticus* cultivations, using 3.9 M of total carbon in substrates, yields 7.3, 18.1 and 20.1 g/L of MEL, and 2.1, 10.0 and 5.1 g/L of residual lipids, for D-glucose, SBO, and a combination of D-Glucose and SBO, respectively. To boost productivity, the same strategy was applied, but starting with the use of glucose and WFO (with low impact in titre, when replacing SBO) from day 0. This led to an increase in productivity from 0.84 g/L/day to 2.0 and 2.4 g/L/day, for *M. bullatus* and *M. antarcticus*, respectively.

As a case study, only *M. bullatus* was used for bioreactor studies, considering the problems caused by *M. antarcticus.* Therefore, co-substrate strategy was applied, investigating different feed regimes: 1) two large feeds of 20 g/L of WFO at day 3 and 6; and 2) daily feeds of 6.66 g/L until day 6 of fermentation. After 9 days, a final productivity of 6.1 $\pm$ 0.4 and 5.4 $\pm$ 0.1 g/L/day, respectively. This approach effectively resolved issues like foaming, without requiring large amounts of vegetable oil. Remarkably, when using 2 large feeds, the yield obtained is 0.6 $\pm$ 0.1 mol of C_{MELs}/mol of $C_{substrate}$, corresponding to one of the highest yields reported in the literature.

This chapter established a foundation for MELs bioprocess, which will be further explored in the following sections.

B.1.2 Introduction

As previously described in **section A.2**, MELs bioprocess is not yet fully established. As represented in **Table A4**, the highest productivity reported (12 g/L/day) (Rau et al., 2005b), was achieved from a long fed-batch fermentation that used large amounts of soybean oil (around 50 g/L), glucose (around 186 g/L) and mineral medium (14 g/L of yeast extract (YE) and sodium nitrate. However, these values still fall significantly short on the reported for SLs (88.8 g/L/day) (Gao et al., 2013). The discrepancy in values explains why MELs currently face challenges in competing at commercial level with the other mBS.

Besides the work performed by Rau et al, only a few works, regarding the development and process intensification of MEL production, were performed. In all of these studies, one of the major problems related with MELs production was the foam and its appearance during the fermentation, therefore, most of them have relied in the intensive use of soybean oil (SO) or rapeseed oil (RO), since they can be used as an anti-foam and carbon source at the same time, as it can been **Table A4**. Furthermore, the use of SO and RO as main substrates poses challenges on process scale-up, as it threatens food availability and prices by requiring a large arable land area for production (Anto et al., 2020). Therefore, sustainable processes involving renewable residues are required for an economically feasible large-scale MELs production. Some studies were attempted in bioreactor, using crude glycerol (Liu et al., 2011), cassava wastewater (Andrade et al., 2017) and sugarcane juice (T. Morita et al., 2011), although, as it possible to observe in **Table A4**., the yields and productivities are lower, when compared with fermentations using SO or RO.

The aim of this chapter was to intensify the process of sustainable MELs production, by using an co-substrate strategy that allows the achievement of high titres and MELs purities, without requiring the use of large feeds of vegetable oil or expensive downstream. Later the strategy was scaled-up for bioreactors, where it was tested different operation condition (different kLa) and feed strategy.

B.1.3 Materials and methods

B.1.3.1 Microorganisms and maintenance

Moesziomyces antarcticus PYCC 5048^T and *M. bullatus* PYCC 5535^T were obtained from the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal, and plated in YM Agar (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, D-glucose 10 g/Land agar 20 g/L) and incubated for 3 days at 25 °C. Stock cultures were prepared by propagation of yeast cells in liquid media described above for the inoculum (2.2) and stored, in 20% (v/v) glycerol aliquots, at -80 °C.

B.1.3.2 Media and cultivation conditions

An inoculum was prepared by transferring the stocks cultures of *M. bullatus* into an Erlenmeyer flask with 1/5 working volume (50 mL) of medium containing 0.3 g/L MgSO4, 3 g/L of NaNO3, 0.3 g/L KH2PO4, 1 g/L yeast extract, and 40 g/L of glucose and incubating at 27 °C, 250 rpm, for 48 h. Fermentation was initiated with 10% (v/v) of inoculum added into an Erlenmeyer flask with 1/5 working volume (50 mL) containing 0.3 g/L MgSO4, 0.3 g/L KH2PO4, 1 g/L yeast extract, 3 g/L NaNO3 and carbon source, either hydrophilic (D-glucose), or hydrophobic (SBO or waste frying oil, WFO) with different concentrations and proportions (described in the sections below), for 14/ or 10 days at 250 rpm and 27°C.

Initially three different conditions were tested in *M. antarcticus* and *M. bullatus* cultivations: i) The use of soybean oil (SBO; OliSoja, Portugal) (from 20 to 80 g/L) sole carbon source; ii) The use of D-glucose as sole carbon source, starting the culture with D-glucose (40 g/L) and followed by of D-glucose (40 or 80 g/L); and iii) Co-substrate strategy using, starting the culture with D-glucose (40 g/L) and with further supplementation at day 4 of cultivation of SBO waste fried oil, in different proportions, to a total carbon added in cultivation of 2.6 and 3.9 M. Following these results, it was decided to replace SBO by waste frying oil (WFO), initiating the cultivation with 40 g/L of glucose (1.3 M of carbon) and feeding at day 4 with 20 g/L of WFO (1.3 M of carbon). All cultures were carried out in biological duplicates and incubated at 27ºC, 250 rpm, for 14 days.

To enhance productivity, the co-cultivation strategy previously described was adopted to start the fermentation with 40 g/L of glucose (1.3 M) and 20 g/L of WFO (1.32) as carbon source, followed

by the rest of mineral medium. This approach was compared to only using 40 g/L of WFO in the beginning (2.6 M). All cultures were carried out in biological duplicates and incubated at 27ºC, 250 rpm, for 7 days.

Additionally with the objective to access the role of oxygen in MELs production (as detailed in sub-section B1.4.3), different ratios of volume of headspace/medium volume ($v_{headspace}/v_{culture\ medium}$, 0.25, 1.5, 4 and 9) were investigated exclusively for *M. bullatus* cultures. The fermentation started with 40 g/L of glucose (1.3 M of carbon) and 20 g/L of WFO (1.3 M of carbon), followed by the rest of mineral medium components. All cultures were carried out in biological duplicates and incubated at 27ºC, 250 rpm, for 7 days.

B.1.3.3 Oxygen volumetric mass transference coefficient (kLa) determination

To determine kLa of the bioreactors, 1-L of working volume was used with all the mineral medium and glucose present (as previously described), varying the presence or absence of WFO, in order to study the effect of WFO. kLa was measured at different aeration rates (0.5, 1 and 2 vmm) and agitation speeds (200, 400, 600, 800 rpm). The method used to determine the coefficient was the dynamic method, as described by Linek et al (Linek et al., 1993), where nitrogen is sparged into the bioreactor to remove the oxygen, and then, the stream with air was connected and the dissolved oxygen determined, until reaching the steady state. kLa was determined using **Equation *1***.

$$\frac{dCAL}{dt} = kLa * (C_{Al}^{*} - C_{AL})$$

Equation 1 : Determination of kLa. C_{AL} corresponds to dissolved oxygen concentration in liquid phase; C_{AL}* corresponds to dissolved oxygen concentration in steady state (when it reaches a plateau).

B1.3.4 Bioreactors conditions and parameters

The experiments were performed in a 2-L bioreactor (New Brunswick™ Bioflo®/CelliGen® 115), using 1 L of working volume, with the medium as previously described in section 2.2, using 40 g/L of glucose and 20 g/L of WFO as carbon sources, varying the feed strategy and the agitation/vvm. The temperature was set up at 27ºC and pH control was implemented only if the value dropped below 4, by using 1 M solution of NaOH.

Initially, two different strategies were attempted using fed-batch fermentation (with two pulses of 20 g/L WFO) to maintain DO at 15% using a cascade mode by varying operational parameters such as agitation and vvm. The first strategy, based on existing literature (see **Table A4**), used a cascade system mode for agitation and vvm (ranging from 150-500 rpm and 0.5-1 vvm, respectively). The fermentation process took 12 days and feeds of WFO were added at day 4 and 8. The second strategy expanded the range of agitation and air-flow (150-800 rpm and 0.5-2vvm, respectively). In this scenario, considering the high consumption of WFO, it was necessary to WFO feeds to days 3 and 6 to prevent foam formation, as it will be explained later. The fermentation process took 9 days

In another experiment, using the best operational parameters from the second strategy (agitation and air-flow ranging from 150-800 rpm and 0.5-2 vvm, respectively), a semi-continuous feeding approach was attempted. The pump was adjusted to give a feed of 4.17 mL of WFO every 12 hours during 6 days, performing a total of 40 g/L (matching the the total amount supplied in previous strategies). The fermentation process took 9 days.

B.1.3.5 CO_2 production rate

The volumetric production rate of CO_2 (rCO_2) was calculated using **Equation *2***, as previously described by Santos et al (Santos et al., 2014). In this equation, C is the number of moles of gas per volume unit, calculated from the ideal gas law for the bioreactor conditions (T=27ºC, P=1 atm); Qv is the air flow rate (L/h); V, volume of culture medium; $CO_{2\ out}$, the molar fraction of CO_2 in outlet gas stream, measured by a sensor (Vernier).

$$\mathrm{rCO2} = \frac{(\mathrm{C} * \mathrm{Qv} * \mathrm{CO2out})}{\mathrm{V}}$$

Equation 2 : Determination of CO_2 production.

B.1.3.6 Viscosity determination

From samples taken from each bioreactor performed, it was evaluated the viscosity of fermentation broth by using a viscometer (Brookfield viscometer DV-II+ pro).

B.1.3.7 Growth and biomass determination

Yeast growth was determined by measuring the cell dry weight (CDW), periodically, during fermentation time. CDW was determined from 1 ml of culture broth by centrifugation at 10 000 x g for 6 min, followed by cell pellet washing with deionized water (twice) and drying at 60 ºC for 48 h.

B.1.3.8 MEL quantification and extraction

During the fermentations, 1 mL of culture broth were periodically taken and freeze-dried. The fatty acid content of the biological samples was determined by methanolysis and GC analysis of methyl esters, as already reported in previous studies (N. T. Faria et al., 2014; Welz et al., 1990). MEL were quantified through the amount of C8, C10 and C12 methyl esters, considering a molecular weight between 574 and 676 g.mol-1 depending on the length of the two acyl chains (C8–C12) and the degree of acetylation. The residual lipids fraction was quantified through the amount of C14, C16 and C18 methyl esters.

At final day of fermentation, for bioreactor and some shake flasks, there were beads enriched in MEL (see figure A1), and so the point taken was not representative of the MEL and residual lipids present. Therefore, the extraction was carried out, adding an equal volume of ethyl acetate to the fermentation broth and mixing, allowing the creation of two phases. Then the organic phase retrieved and evaporated in a rotary evaporator, recovering ethyl acetate and obtaining a crude fraction of MEL and residual lipids. A sample of crude MEL was weighted, following the same procedure described above (methanolysis and GC analysis).

B.1.3.9 High Performance liquid chromatography (HPLC) analysis

The quantification of D-glucose was performed using HPLC. Culture broth samples were centrifuged at 10000 rpm for 6 min, the supernatants filtered through a 0.22 μm-pore size-filter and injected into HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany) and an Rezex ROA Organic Acid H+ column (300 mm× 7.8 mm, Phenomenex, Torrance, CA, USA), at 65°C. Sulfuric acid (5 mM) was used as mobile phase at 0.5 ml/min.

The consumption of oil (triglycerides - TAG) in the culture samples was also analysed by HPLC, as described by Badenes et al (Sara M. Badenes et al., 2010). 500 μL of each sample was retrieved and mixed with 1 μL of acetic acid 58.5 Mm and 499 μL n-hexane. Then it was centrifuged at 10000 rpm for 2 minutes, and the organic phase was extracted and injected into the HPLC system, equipped with a Chromolith Performance RP-18 endcapped (100mm x 4.6mm x 2μm) column, an auto sampler (Hitachi LaChrom Elite L-2200), a pump (Hitachi LaChrom Elite L-2130) and a UV detector (Hitachi LaChrom Elite L-2400) set up at 205 nm. The flow rate was set up at 1 ml/min and the injection volume was 20 μL. Three mobile phases were employed: phase A consisted of 100% acetonitrile, phase B consisted of water 100% and phase C comprising a mixture of n-hexane and 2-propanol (4:5, v/v). Quantification was carried out using calibration curves of Glyceryl trioleate and Glyceryl trilinoleate (>98 %, Sigma-Aldrich GmbH) for TAG.

B.1.3.10 Statistical analysis

Statistics were performed, using Graph-pad, by analysis of variance (two-way ANOVA) and p-values of the differences between groups were corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p < 0.05$

B.1.4 Results and discussion

As previously discussed, *Moesziomyces* spp. have the ability to use different carbon sources for the production of MELs, ranging from vegetable oils (yielding high titres but low purity MELs) to hydrophilic carbon sources (resulting in low titres but with high purities MELs). However, there is no established condition in the literature, that simultaneously can achieve high yields of MELs but also with high purity, which is crucial for the downstream process (less steps of purification, as elaborated in **section C.1**). In this regard, the work here presented in this section aims to: 1) To identify an optimal condition that combines hydrophobic/hydrophilic carbon sources to enhance MELs yield, while reducing the ratio residual lipids/MELs, thereby facilitating downstream; and 2) Using the condition previously assessed, the second objective consists into studying different feed regimes in bioreactor to enhance MELs productivities and establish a robust bioprocess that can be further used. These two parameters will serve as the foundation for the subsequent sections.

B.1.4.1 MELs production using hydrophobic (SBO) or hydrophilic (glucose) carbon sources

The initially idea was to access how MELs production is affected when using only SBO as sole carbon source, even though if it was a condition already tested in different studies. Yields and purity, as well as substrate utilization and product/residues formation, were assessed (**Table B1** and **Figure B1**). Although purity definition often considers all form of contaminants, here purity is presented as a ratio of MELs to the sum of MELs and total residual lipids, major contaminants after MELs liquid-liquid extraction. As is it possible to observe in **Figure B1**, when increasing the initial concentration of SBO (from 20 to 80 g/L), MELs production increased non-linearly for both strains, rising from 8/9 to 20 g/L. However, the residual lipids also exhibited a non-linear increase (from 1 to 28 g/L, approximately). Consequently, as predicted, the higher the amount of SBO used, the lower the purity of MELs: 1) in M. antarcticus cultivation decreased from 92% (w/w) to 40% using 20 and 80 g/L, respectively, of SBO; 2) in M. bullatus purity decreased from 88% (w/w) to 45% using 20 and 80 g/L, respectively. These results goes accordingly to what Kitamoto et al. (Kitamoto et al., 1990c), observed for different types of oil (with an yield of 34 g/L of MELs when

using M. antarcticus T-34), however the yield is 1.7-fold lower than the obtained in this study, which can be explained by the different strain, or by the quantification method used to determine MELs concentration (method B, as represented in **Table A2)**.

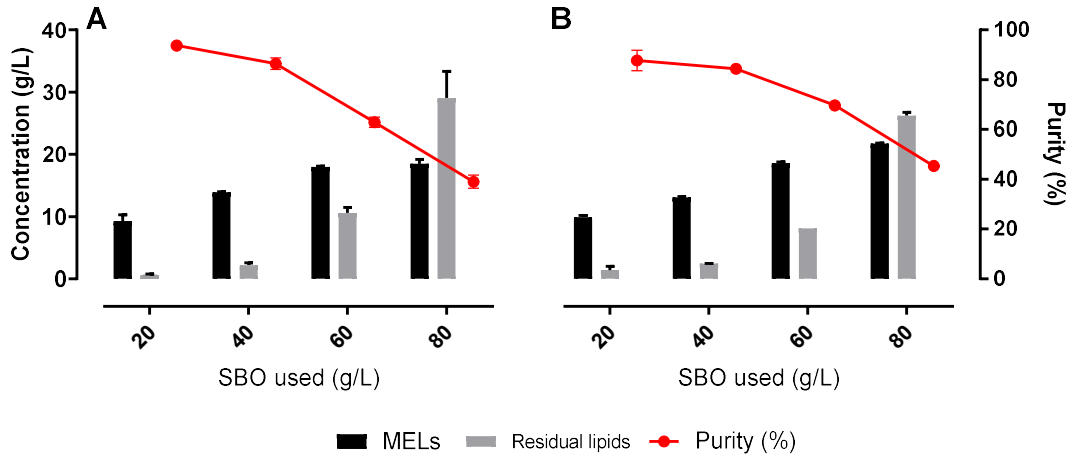


Figure B1: MELs (black bars) and residual lipids (gray bars) obtained after 14 days of cultivation of *M. antarcticus* PYCC 5048^T (A) and *M. bullatus* PYCC 5535^T (B) in SBO at different concentration (20, 40, 60 and 80 g/L). Red line corresponds to the final purity of MELs (Ratio of g of MELs to the sum of g of MELs and residual lipids)

Table B1*:* Maximum MELs obtained (g/L), Yields of MELs produced ($g_{MELs/}g_{Substrate}$) and residual lipids not consumed ($g_{residual\ lipids/}g_{Substrate}$) for *M. antarcticus* PYCC 5048^T and *M. bullatus* PYCC 5535^T using SBO as solo carbon source (20, 40 60 and 80 g/L) with the respective concentration of carbon (1.3, 2.6, 3.9 and 5.2 M).

Strain	**Condition (SBO g/L)**	**C substrate (M)**	**MELs (g/L)**	**Productivity (g/L/day)**	**Y $_{MELs/Substrate}$ (g/g)**	**Residual lipids (g/L)**	**Y of Residual lipids/Substrate (g/g)**	MELs purity (g/g)
***M. antarcticus* PYCC 5048^T**	**80**	5.2	19.5	1.39	0.24	29.1	0.40	0.37
	60	3.9	18.1	1.29	0.30	10.0	0.13	0.64
	40	2.6	14.0	1.00	0.35	1.9	0.02	0.88
	20	1.3	9.3	0.66	0.50	0.8	0.01	0.92
	80	5.2	21.8	1.56	0.27	26.6	0.33	0.45

Strain	Condition (SBO g/L)	C substrate (M)	MELs (g/L)	Productivity (g/L/day)	$Y_{MELs/Substrate}$ (g/g)	Residual lipids (g/L)	Y of Residual lipids/Substrate (g/g)	MELs purity (g/g)
***M. bullatus* PYCC 5535^T**	**60**	3.9	18.7	1.34	0.231	8.1	0.10	0.70
	40	2.6	13.2	0.94	0.33	2.4	0.03	0.85
	20	1.3	9.9	0.71	0.50	1.4	0.02	0.88

$Y_{MELs/Substrate}$ – maximum MELs yield (g/g); MELs purity (g/g) at the end of the fermentation- Ratio of g of MELs to the sum of g of MELs and residual lipids

Then, it was tested also the use sole D-glucose carbon sources. In **Table B2**, it is possible to observe that MELs titres were lower when compared with SBO, in equimolar amounts of carbon. The low level of residual lipids observed may be attributed with cellular synthesis rather than external addition (part of metabolic pathway for energy uptake by yeasts). Furthermore in *M. antarcticus* cultures, the increase of D-glucose from 80 g/L to 120 g/L slightly increased MEL titres: from 5 to 7 g/L. In *M. bullatus* cultures no differences on MELs titres were observed.

Table B2: Maximum MELs obtained (g/L), Yields of MELs produced ($g_{MELs/g_{Substrate}}$) and residual lipids not consumed ($g_{residual\ lipids/g_{Substrate}}$) for *M. antarcticus* PYCC 5048^T and *M. bullatus* PYCC 5535^T using co-substrate strategy (marked at bold orange) as alternative to D-glucose or SBO as sole carbon source, at 2.6 and 3.9 M of total carbon. Marked with an *, represents the condition where SBO was replaced with WFO.

Strain	Condition (SBO feeding, g/L)	C (M Glu + SBO)	MELs (g/L)	Productivity (g/L/day)	Residual lipids (g/L)	MELs purity (g/g)
***M. antarcticus* PYCC 5048^T**	0	2.6	5.1 ± 0.6	0.36 ± 0.04	1.9 ± 0.2	0.73 ± 0
	20 (WFO)*		**11.8 ± 0.3**	**0.84 ± 0.02**	**3.43 ± 0.3**	**0.76 ± 0.01**
	20		**14.4 ± 0.6**	1.03 ± 0.04	**0.9 ± 0.3**	**0.94 ± 0.02**
	40 (WFO)		20.71			
	40		14.0 ± 2.8	1 ± 0.2	1.8 ± 0.4	0.89 ± 0
	0	3.9	7.3 ± 0.2	0.52 ± 0.01	2.1 ± 0.0	0.78 ± 0
	20		**16.9 ± 2.7**	1.21 ± 0.19	**3.9 ± 1.2**	**0.82 ± 0.02**
	30		**20.0 ± 2.1**	1.43 ± 0.15	**5.1 ± 1.7**	**0.8 ± 0.04**
	40		**22.9 ± 2.5**	1.64 ± 0.18	**7.3 ± 1.0**	**0.76 ± 0.01**
	60		18.1 ± 1.7	1.29 ± 0.12	10.0 ± 0.1	0.64 ± 0.02
***M. bullatus* PYCC 5535^T**	0	2.6	3.0 ± 0.2	0.21 ± 0.01	1.7 ± 0.1	0.64 ± 0
	20 (WFO)*		**9.9 ± 0.05**	**0.71 ± 0**	**3.4 ± 1.9**	**0.76 ± 0.11**
	20		**11.5 ± 0.2**	0.82 ± 0.01	**2.7 ± 0.4**	**0.81 ± 0.02**
	40		13.2 ± 2.2	0.94 ± 0.16	2.4 ± 0.6	0.85 ± 0.01
	0	3.9	2.6 ± 0.5	0.19 ± 0.04	4.1 ± 0.5	0.39 ± 0.02
	20		**12.5 ± 1.5**	0.89 ± 0.11	**6.3 ± 1.8**	**0.67 ± 0.04**
	30		**14.1 ± 2.3**	1.01 ± 0.16	**8.9 ± 0.4**	**0.61 ± 0.03**
	40		**16.5 ± 1.6**	1.18 ± 0.11	**9.4 ± 0.2**	**0.64 ± 0.02**
	60		18.7 ± 0.1	1.34 ± 0.01	8.1 ± 0.2	0.7 ± 0

$Y_{MELs/Substrate}$ – maximum MELs yield (g/g); MELs purity (g/g) at the end of the fermentation- Ratio of g of MELs to the sum of g of MELs and residual lipids

B.1.4.2 Co-substrate strategy in MELs production from *Moesziomyces* spp.

The co-substrate strategy included two set of conditions totalizing 2.3 and 3.9 M of carbon. An initial 1.3 M of carbon, corresponding to 40 g/L of D-glucose, was fed to *M. antarcticus* and *M. bullatus* cultivations, with further supplementation, after 4 days: 1.3 M from 40 g/L of D-glucose or 20 g/L of SBO, or 2.6 M from mixtures of D-glucose and SBO, up to 80 g/L and 40 g/L, respectively. The cultivation was carried out for 14 days.

Throughout the cultivation process, in the majority of conditions investigated, orange to reddish oil colouring beads were observed at around day 7 (**Figure F1**). These beads are MELs-enriched with the presence of residual lipids (mostly monoglycerides), and their appearance is observed in different cultivation conditions, as it will be observed in the subsequent chapters. Interestingly, while for *M. antarcticus* the beads disappeared over the next days of cultivation, for *M. bullatus* it did not happen. The formation of these beads its still not clear, and might be related with a MELs titre threshold, but also with the interaction with residual lipids, since the disappearance of beads in *M. antarcticus* cultivation did not result in a decrease in MELs titre in the following days. In this regard, quantification of MELs produced over time was challenged, due to the heterogeneity of fermentation broth. Therefore, the values of MELs titre (14^{th} day) were obtained through total extraction of the culture broth with EtOAc (as described in section **B.1.3.8**), and are represented in **Figure B*2*** and **Table B2**.

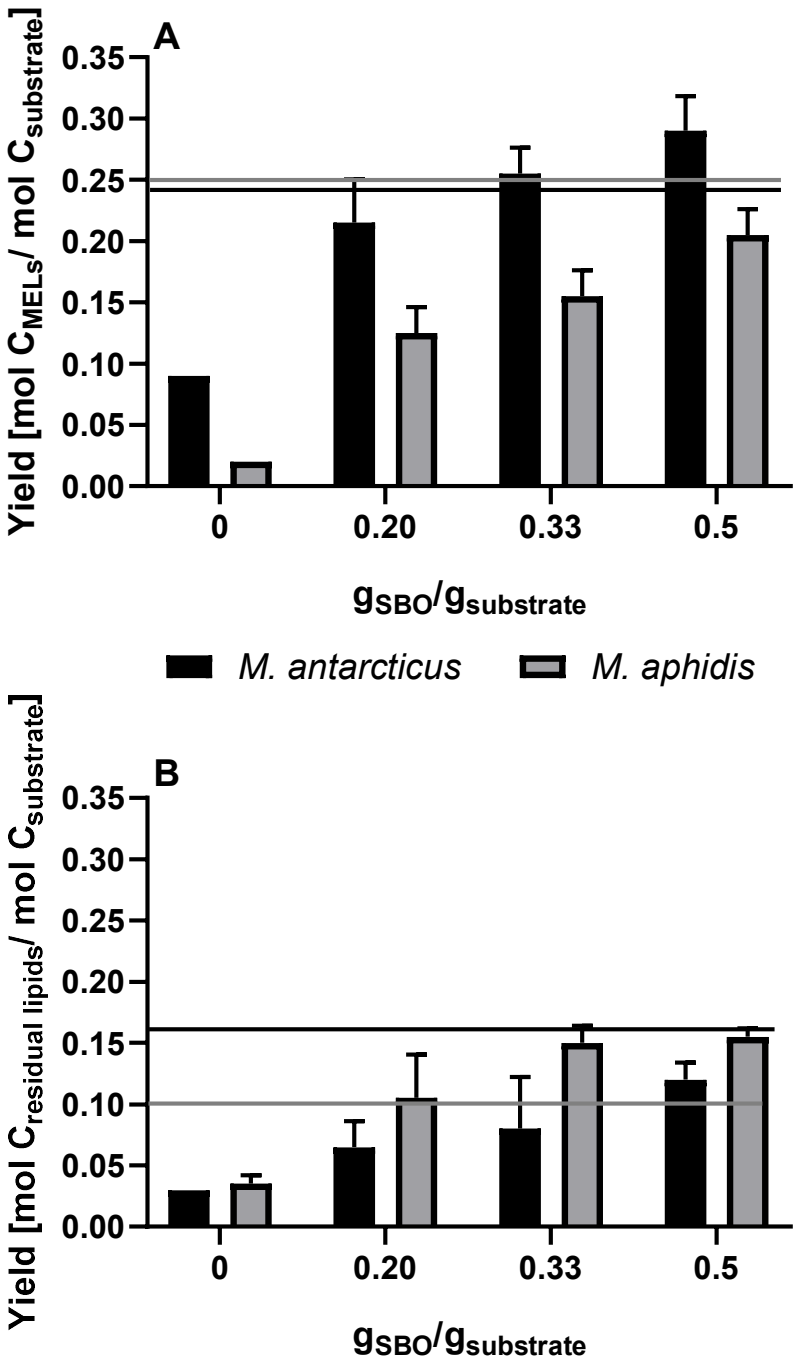


Figure B2: Carbon yields of MELs (A) and residual lipids (B) for *M. antarcticus* PYCC 5048^T and *M. bullatus* PYCC 5535^T, after 14 days of cultivation using a total 4 M of carbon source: Sarting with 40 g/L of glucose (1.33 M in carbon) and at day 4 a feed with D-glucose and/or SBO. Lines represent yield obtained in *M. antarcticus* (black line) and *M. bullatus* (grey line) when cultivated with SBO as sole carbon source (4 M in carbon, corresponding to 60 g/L of SBO).

Moesziomyces antarcticus cultivation using D-glucose as sole carbon source, at 2.6 M of carbon, yielded 5.1 g/L of MEL. Interestingly, a similar titre of around 14 g/L was obtained when the same 2.6 M of total carbon was fed under the co-substrate strategy, with 20 g/L of SBO, or using SBO as sole source, at 40 g/L. However, the cultivations following co-substrate strategy led to significantly lower values of residual lipids than the ones using SBO as sole carbon source, 0.9 g/L and 1.8 g/L, respectively, increasing the MEL purity up to 94% (**Table B2**).

In case of *M. bullatus* cultivation, MELs titres obtained under the co-substrate strategy, with a total carbon concentration of 2.6 M, were slightly lower when compared with cultivation using SBO as sole carbon source (11.5 and 13.2 g/L, respectively). Furthermore, no improvement in MELs purity was observed when employing the co-substrate strategy compared to using only SBO (**Table B2**). These results may suggest that there no synergistic effects when using substrates with opposite polarities in *M. bullatus* cultivations.

Increasing the total carbon to 3.9 M resulted in the same trend as described before for 2.6 M. Similar or higher MEL titres, but lower residual lipids, were observed in *M. antarcticus* cultivations when the co-substrate strategy was followed (**Table B2**). The maximum MELs titres (22.9 g/L) was obtained for a *M. antarcticus* cultivation, when feeding at 4th day with 40 g/L of SBO, corresponding to a yield of 0.30 $mol_{product}/mol_{carbon}$ (**Figure B3 A**) and 0.29 $g_{MEL}/g_{substrate}$. As expected, the carbon conversion rate decreases to 0.26 when only feeding with 30 g/L of SBO. Both co-substrate conditions (30 and 40 g/L of SBO feeding) led to an improvement in the conversion rate and titre, compared when using 60 g/L of SBO was sole carbon source (0.24 and 17.1 g/L respectively). Additionally, the residual lipids yield decreased from 0.16 $mol_{residual\ lipids}/mol_{carbon}$, when using SBO as sole carbon source at 60 g/L, to 0.08 and 0.12 when using 30 and 40 g/L of SBO (at 4th day), respectively, in co-cultivation strategy (**Figure B3 B**).

As previously described in **section A.2**, depending on the type of substrate used (either hydrophilic or hydrophobic), the metabolic pathways differs. The production of MELs from D-glucose include the activation of the fatty acids biosynthesis (*de novo* synthesis), after glycolysis, to produce medium to long fatty acyl-chains. Then, the acyl groups will, undergo through partial beta-oxidation in the peroxisome (Freitag et al., 2014), the so-called chain shortening pathway, to yield the shorter lipidic chains that are incorporated into mannose-erythritol moiety (Kitamoto et

al., 1998; Wada et al., 2020). From the results obtained in this chapter (**Table B2**), *M. antarcticus* can produce higher MELs titres (5.1 ± 0.6 g/L) than *M. bullatus* (3.0 ± 0.2 g/L) when using D-glucose a sole carbon source, which might be related with a higher peroxisomal activity. This theory is supported with the observation of higher lipids accumulation by *M. bullatus*, but lower MELs titres, indicating that although *M. bullatus* can undergo through fatty acid biosynthesis, these medium to long acyl-chains are accumulated in other carbon storage molecules than MELs. Oppositely, the behaviour on MELs production seems to be similar between those strains when vegetable oil was used as sole carbon source.

Generally, in cell cultivation, the addition of a new substrate might lead to adaptation periods between substrates with consequences on product formation and productivity of the whole process. In this regard, the use of D-glucose followed by the feeding of vegetable oil, will lead to the production of extracellular lipases, as previously reported (N. T. Faria et al., 2014), which will be important to shorten the adaptation period on cultivations with feeding of SBO, allowing a quick incorporation of residual lipids into the cells. In this study, lipase activity was also determined in cultivations using 40 g/L of glucose as sole carbon source (**Figure F5**), where it is possible to observe that for *M. antarcticus* lipase activity increases through cultivation time (with a value of 2 IU/mL at day 4), and for *M. bullatus* it is more or less constant (0.5 IU/mL).

In establishing the co-substrate strategy, D-glucose was used to cultivate and stabilize microbial cultures, potentially producing hydrophilic MELs building blocks and/or promoting MELs-genes induction and producing lipases. Nevertheless, further addition of D-glucose is not expected to favour the production of a secondary product such as MELs. Further D-glucose addition increases C/N ratio, known as an important factor for secondary metabolite production, such as reserve lipids or glycolipids. However, MELs production from D-glucose requires de novo MEL-acyl groups building-up, through acetyl Co-A accumulation in the cytosol, and more importantly, fatty acid biosynthesis trough fatty acid synthase complex (FAS), which requires two NADPH molecules per each step of elongation (see **Figure A2**). Then, to be incorporated in mannose-erythritol moiety (ME), these acyl molecules synthesised should undergo partial β-oxidation in the peroxisome. The described metabolism results in a low maximum theoretical MELs production capacity in *Moesziomyces* spp. when using D-glucose. Further supplementation of optimized amounts of vegetable oil, instead of D-glucose, may boost MEL production. Prior lipase

production induced by D-glucose, will promote the hydrolysis of oil into glycerol, which enters in glycolytic pathway, yielding mannose and erythritol, along acyl groups, which are incorporated into ME moieties (after partial β-oxidation) to produce MELs.

The results obtained illustrate the potential of this promising cultivation strategy attaining high MEL titres while maintaining lower residual lipids, and thus facilitating further downstream processing.

B.1.4.3 Improving MELs production sustainability by replacing hydrophobic carbon sources by waste frying oil on co-substrate strategy

So far SBO has been treated as preferential substrate for MELs production, as case study, however it is use is not sustainable, as previously described. In this regard, the use of waste frying oils (WFO) as hydrophobic carbon source to produce MELs in a co-substrate strategy from *M. antarcticus* and *M. bullatus*, have the potential to increase the sustainability of the process. Therefore, it was decided to use co-substrate, by starting the fermentation with 40 g/L of glucose, following by the addition of 20 g/L of WFO at 4th day of cultivation (**Figure B3** and **Table B2**). The parameters analysis of WFO was already analysed and described by Arévalo Robles (Robles Arévalo, 2015), showing that it contains high peroxide value (58 mEq/kg) than the refined SBO (<10 mEq/kg), along with a different fatty acid chain composition (WFO major fatty acid is oleic acid, 18:1, while SBO is linoleic acid 18:2).

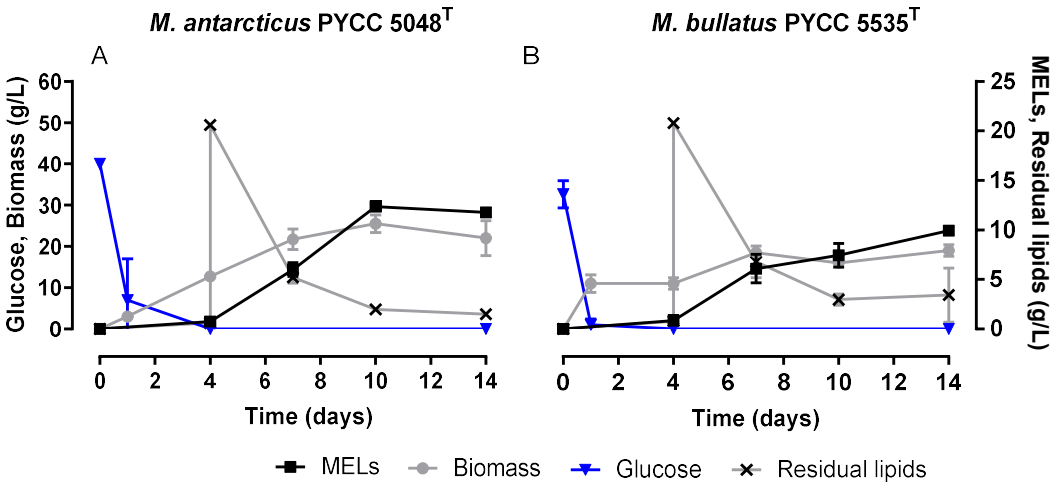


Figure B3: Cultivation of M. antarcticus PYCC 5048T (A) and M. bullatus PYCC 5535T (B) using D-glucose (40 g/L) as carbon source in the beginning and supplemented with 20 g/L of WFO at day 4. Fermentation was carried during 14 days at 27 ºC, and with an agitation of 250 rpm. For both graphics it is represented yeast biomass (circles); MELs production (squares); residual lipids (crosses) and glucose (inverted triangles). Standard deviation lower than 1 g/L are not represented.

MELs maximum titres of 12.6 and 10.0 g/L were observed at day 10 and 14, for *M. antarcticus* and *M. bullatus*, respectively. Residual lipids determined were relatively low after 14 days, around 1.5 g/L and 3.4 g/L in *M. antarcticus* and *M. bullatus*, respectively. The utilization of WFO in co-substrate strategy resulted in MEL titres of around 15% lower than the ones obtained when using SBO under the same co-substrate conditions (**Table B2**). This reduction in titre can be related with high peroxide level observed for WFO (58 mEG/kg) when compared with SBO (<10 mEq/kg), which is inhibitory for yeasts grow and protein synthesis (Picazo and Molin, 2021). Nevertheless, the acidic value of WFO (4.67 mg KOH/h) is 74% higher than the value observed for SBO, which indicates that it is more degraded (less content in TAG and more of FFA and MAG), and consequently more quickly consumed by yeast cells. Since MAG and FFA can enter directly in cells, without the need for prior lipase activity, WFO can be consumed more quickly than SBO. This phenomenon has been previously reported by Petar Keković in his PhD studies (Keković, 2022), where he demonstrated that higher productivities are obtained when using oils partially degraded into FFA. This can also explain why MELs titers plateau after the 10thy day of

cultivation, with no further consumption of residual lipids. These findings suggest opportunities for expanding the use of different residual oils in bioprocesses for MELs production, as it will be described in the following sections.

B.1.4.4 Bioprocess intensification towards MELs production: The use of co-cultivation strategy in bioreactors

In the previous sub-sections, it was studied the use of co-substrate strategy to improve MELs titres/productivities while keeping high MELs purities. However, the productivity values obtained are still extremely low (~ 0.84 g/L/day, **Table B2**) when compared with other values reported in literature (13.92 g/L/day, (Rau et al., 2005b)). Considering that both strains exhibit lipase activity of 0.4 IU/mL on day 1 of cultivation and WFO is quickly consumed, it was decided to explore the co-substrate strategy using both carbon sources from the time 0 of cultivation (40 g/L of glucose and 20 g/L of WFO).

With this condition (**Figure B*4***), it was achieved final MELs titres of 24.2 ± 0.3 and 20.2 ± 3.8 g/L for *M. antarcticus* and *M. bullatus*, respectively. Following the previous results (**Figure B*2***), the co-substrate strategy here implemented for *M. antarcticus*, led to an increase in 15% of MELs titres and a reduction of residual lipids in 81%, when compared with the use of sole WFO (same concentration of carbon, 2.6 M). For *M. bullatus,* there no significative differences were observed in the obtained titres in both conditions. Nevertheless, with this approach (using co-substrate strategy in the beginning of fermentation) the productivity increased from 0.84 g/L/day to 2.0 and 2.4 g/L/day, for *M. bullatus* and *M. antarcticus*, respectively.

These finding are supported by the conclusions achieved by Wada et al. (Wada et al., 2020). In this study the authors have studied the expression of different genes for different categories (energy production, conversion, ect), discovering that in the presence of a hydrophobic carbon source, genes involved in central metabolic pathways (glycolysis and TCA cycle) are highly expressed, which might indicate that the carbon assimilation into MELs is be quicker (see **Figure A*2*)**. However, more studies are required to prove this.

From this point, in all fermentation reported in next sections, hydrophilic and hydrophobic carbon sources are used in the beginning of cultivation.

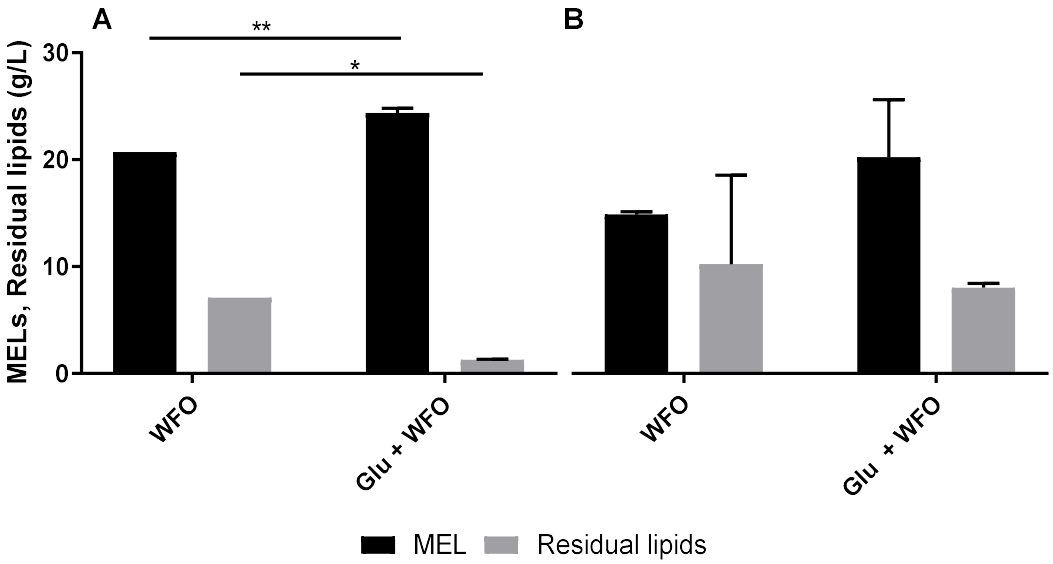


Figure B4: Final MELs titres (black bars) and residual lipids (grey bars) for *M. antarcticus* PYCC 5048T (A) and *M. bullatus* PYCC 5535T (B) using sole WFO as carbon source (40 g/L) or 40 g/L of glucose and 20 g/L of WFO as carbon source in the beginning of fermentation. Cultivation was carried during 10 days at 27 ºC, and with an agitation of 250 rpm.

Achieving the best condition to boost MELs titres, the next is to involve the use of bioreactors. However, there are still some experiments that can be performed in shake-flask, especially understanding how oxygen impacts final MELs titres/productivities. For that, using the previous established condition (40 g/L of glucose and 20 g/L of WFO in the beginning), it was varied the ratio headspace/medium (0.25; 1.5; 4 and 9), by using equally shake flasks (250 mL of total volume).

In **Table A4** it is possible to observe that only a few studies report the production of MELs in bioreactors using *M. antarcticus,* even though if higher titres are achieved with this strain (as previously observed in **Figure B*4***. This is mainly due to the formation of agglomerates during cultivation (see **Figure B*5*** A), leading to an increase of viscosity and complexity of fermentation broth rheology. In this regard, from this point it was decided to only use *M. bullatus* as main microorganism, while still developing and improving the best conditions (SOP) to produce MELs in bioreactors.

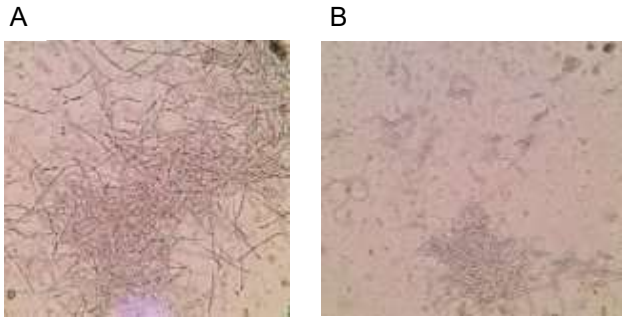


Figure B5: Microscope pictures (objective of 100x) took to cultivation of *M. antarcticus* (A) and *M. bullatus* (B). These cultures were cultivated for 7 days using glucose (40 g/L) and WFO (20 g/L) as carbon sources).

In the first two conditions, where the ratio volume headspace/volume medium is lower (0.25 and 1), most of glucose was not consumed, presenting low consumption rates (0.06 and 0.13 g/L/h respectively; see **Table F1**). Moreover, the production of biomass was also affected in these two conditions, and the maximum yield was 1.3 and 1.6-fold lower, respectively, than the conditions with higher ratio (4 and 9). Since some conditions had MELs beads, as previously reported (**Figure F*1***), for all the conditions was extracted MELs/residual lipids from the fermentation broth and the final titre and yield are represented in **Figure B6**. Interestingly, as the ratio volume headspace/volume medium increases, the final titre of MEL increases, until to a maximum of 26 g/L for the ratio 9, with statistically differences between all the conditions. However, the consumption of residual lipids only differs in the first two conditions with lower ratio (0.25 and 1), and after this, there is not statistically differences between the conditions (ratio 1, 4 and 9). In **Figure F*2*** it is possible to observe growth, MELs and residual lipids values through days.

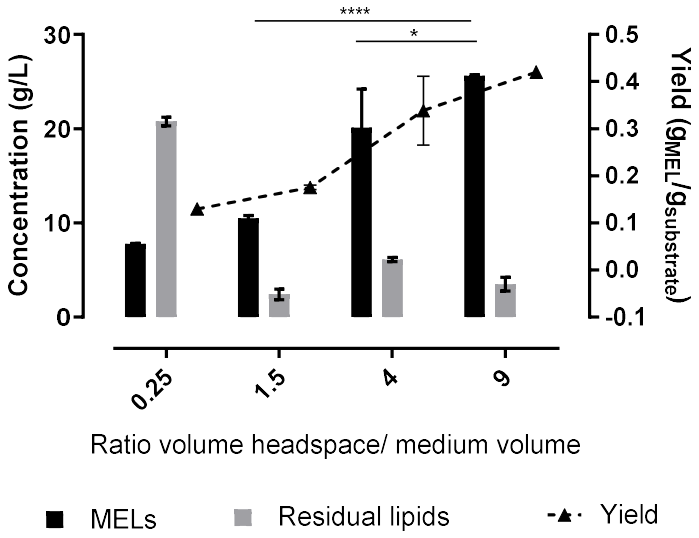


Figure B6: MELs titres (black bar), residual lipids (grey bar) and MEL yield (black dashed line with triangles) for *M. bullatus* PYCC 5535^{T} cultures incubated during 7 days at 27 ºC using 40 g/L of glucose and 20 g/L of WFO as carbon source, at different volume headspace and medium volume ratios (0.25, 1.5, 4 and 9 for a working volume of 200, 100, 50 and 25 mL, respectively). In the top of the top of each column, different uppercase and lowercase letters represent significant variations in MEL titre and residual lipids titres, respectively, between all the conditions. The difference represented have a p-value higher than 0.05.

Revealing the effect of oxygen in MELs production, before starting any experiments in bioreactor, it was studied volumetric mass transfer coefficient (kLa) for different agitations (400, 600 and 800 rpm) and different vvm (1, 1.5 and 2 vvm) using all the components of the culture medium (see section 2.3), in the presence and absence of oil, as represented in **Figure B7**, to better understand the phenome of oxygen mass transference inside the bioreactor.

At lower agitation speed (400 rpm), there were no significant differences as vvm increases. Although, as the agitation increases (from 400 to 800 rpm), the variations in kLa using different vvm became statistically significant. The maximum kLa (85 ± 1 h^{-1}) is reached using 800 rpm and 2 vvm, which is logical since higher agitation provides more energy to break larger air bubbles since (increasing the diameter, as vvm increases). Interestingly, when oil is presented in the medium, there is a drastic decrease in kLa for lower agitation (1.3-fold lower, in the condition of 1vvm and 400 rpm). Nevertheless, with increasing agitation, the differences in kLa are almost no relevant, and for 800 rpm and 2 vvm, the difference is meaningless.

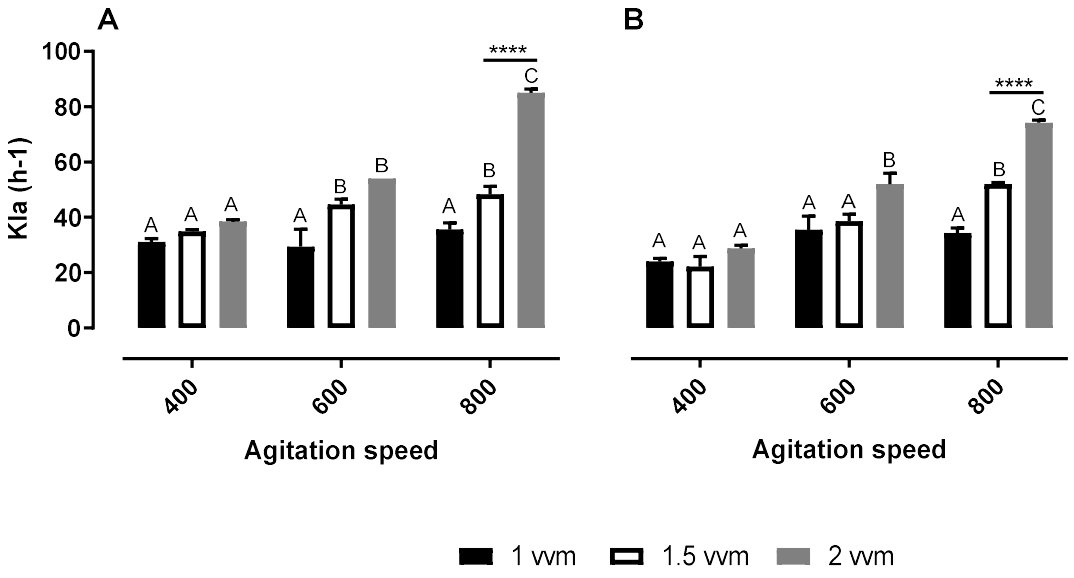


Figure B7: Determination of kLa using different agitations (400, 600 and 800 rpm) and aeration rates (1, 1.5 and 2 vvm) in the absence (A) and presence (B) of WFO (20 g/L). All tests were performed, using the components of the culture medium (Glucose, mineral salts and YE), as described in material and methods. In the top of the top of each column, different letters represent significant variations between different vvm, for the same agitation, with a p-value higher than 0.05.

Therefore, in order to achieve high oxygen transfer in the reactor, it is necessary to use 800 rpm and 2 vvm, as agitation and airflow, respectively. In this regard, two different operating conditions were initially studied and compared,: 1) The traditional use of a cascade system for agitation, 150-500 rpm, and the airflow between 0.5 and 1 vvm (**Figure B8** A, D); 2) Allow higher agitation speed and aeration, using a cascade system of 150-800 rpm and the aeration of 0.5-2 vvm (**Figure B8** B, E);. Both conditions started with D-glucose and WFO in the beginning (40 and 20 g/L, respectively), and included two feeds of 20 g/L of WFO on different days (day 4/8 and 3/6, respectively).

Analysing the patterns, it became clear that increasing the oxygenation conditions required readjusting the feeds, since foam started to appear earlier (day 2 or 3 of fermentation) when compared with the condition using only 500 rpm and 1 vvm (4th day 4 of fermentation). Foam appearance can be caused by several factors, such as cell death, protein precipitation, and others, but it can be an indication of exhaustion of the hydrophobic source, indicating that a new

feed of oil can be performed, acting as fresh carbon source and foam control agent. Remarkably, simply increasing oxygenation conditions resulted 29% increase in productivity (5.4 and 4.2 g/L/day, respectively), and the 14% increase in purity (see **Table B3**). Subsequently, using the improved aeration conditions, a feeding strategy consisting on semi-continuous WFO feeds, 6.66 g/L every day, was studied (***Figure B8*** C, F). Such strategy did not improve final productivity and had similar values to the ones obtained by the condition with lower oxygenation (cascade system using 150-500 rpm of agitation and 0.5-1 vvm of airflow; see **Table B3**). This might be explained by the ratio C/N, as some studies have already reported that high MELs titres are achieved using a high ratio C/N (N. T. Faria et al., 2014). Therefore, using larger feeds of WFO (20 g/L,) instead of smaller daily feeds (6.66 g/L), results in a higher C/N ratio in the first condition, which might activate the metabolic pathways for MELs production. However,, more studies are required to prove this.

A mass balance on carbon was also performed (see **Figure F*3***), revealing that the optimal condition for MELs production (800 rpm, 2 vvm and 2 feeds of WFO) resulted in lower emissions of CO_2 and lower biomass, compared to conditions with lower oxygenation (500 rpm and 1 vvm) or similar operating conditions but with a different feeding regime (small feeds of WFO every day). Since MELs production requires good oxygenation conditions, it is likely that portion of the carbon is being redirected to MELs biosynthesis. Nevertheless, further studies are required to confirm this, particularly using labelled carbon to trace the carbon pathway.

Even though the condition of 800 rpm and 2 vvm resulted in higher MELs productivity part of the volume evaporated by the end of the fermentation (see **Figure F*4*** F,I) when compared with the reactor using lower oxygenation (**Figure F*4*** C, F). This might be a result of using harsh condition such as the high airflow of 2 vvm. In the future, knowing already the kLa value for the optimal condition to achieve high titres of MELs, other experiments can be conducted to avoid high airflow while maintaining the same kLa, such as: 1) using a larger working volume and more impellers (work on-going); 2) using more or different impellers (e.g marine impellers instead of Rushton).

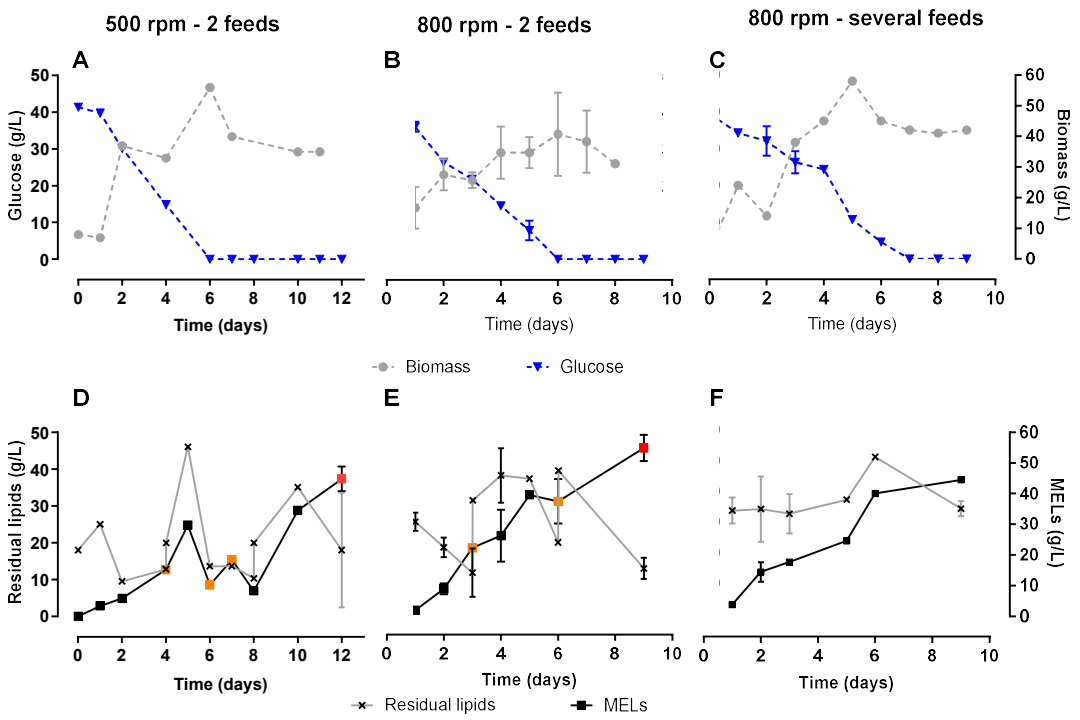


Figure B8: MELs production cultivating *M. bullatus* PYCC 5535^{T} on 40 g/L of glucose and 20 g/L of WFO, in bioreactor (1-L). A,D) **T**he cascade system for agitation varied from 150-500 rpm, and aeration rate from 0.5-1 vvm, with two feeds of 20 g/L of WFO at day 4 and 8, extending the fermentation for 12 days. The last two cases (B, C, E, F), the operation conditions were equally, were the agitation varied from 150-800 rpm, and aeration rate from 0.5-2 vvm, and only the feed strategy were altered: B, E) giving two pulses of 20 g/L of WFO at day 3 and 6; and C, F)giving small pulses (6.66 g/L of WFO) every day, stopping at day 6.

When comparing the developed here with the study performed by Rau et al. (Rau et al., 2005b), it is evident that MELs productivity it is still 56% lower (6.12 and 13.9 g/L/day, respectively). However, as previously discussed, these authors use large amounts of carbon sources (glucose, 50 g/L; SBO, 186 g/L; YE, $NaNO_3$, 14 g/L) reporting a final yield of 0.92 $g_{MELs}/g_{substrate}$. This value implies that only 8% of substrate is used for other products resulting from yeast metabolism (biomass, CO_2 emissions, residual lipids, proteins, ect), which seems unrealistic.

In this regard, an attempt was made to determine the yield in mols of C. This yield (as stated in **Table B*3***), it is calculated considering all carbon used, including the carbon present in YE (~40%). Surprisingly, when determining this yield, there are no differences between the yield calculated from values obtained by Rau et al. (0.62 mol of C $_{MELs}$/mol of $C_{substrate}$) and the best yield obtained

in this section (0.6 ± 0.1 mol of C $_{MELs}$/mol of $C_{substrate}$). Furthermore, even when comparing with studies that scaled up MELs production until demonstration scales (1 m^3) (Shen et al., 2024), there are no differences, which validates the process for the upcoming sections.

Table B3: Maximum MELs obtained (g/L); yield of MELs produced ($g_{MELs}/g_{Substrate}$), maximum productivity (g/L/day) and purity (g/g) in cultivations of *M. bullatus:* the cascade system for agitation varied only from 150-500 rpm, and aeration rate from 0.5-1 vvm, with two feeds of 20 g/L of WFO at day 4 and 8, extending the fermentation for 12 days. The last two cases the operation conditions were equally, were the agitation varied from 150-800 rpm, and aeration rate from 0.5-2 vvm, and only the feed strategy were altered: 1) giving two pulses of 20 g/L of WFO at day 3 and 6; and 2) giving small pulses (6.66 g/L of WFO) every day, stopping at day 6. It is also compared with values reported in literature. *n.a* – not available; *values calculated and not provided in the study.

Parameters	**Work developed in this chapter**			**References**	
	500 rpm: 2 feeds	**800 rpm: 2 feeds**	**800 rpm: several feeds**	(Rau et al., 2005b)	(Shen et al., 2024)
MELs $_{max}$ (g/L)	50.23 ± 2.43	59.71 ± 8.48	48.45 ± 0.15	165	61
Residual lipids (g/L)	18.04 ± 11.04	13.42 ± 1.04	35.76 ± 1.12	*n.a*	*n.a*
Biomass$_{max}$ (g/L)	22.18 ± 0.57	12.21 ± 1.31	18.5 ± 0.2	10.5	*n.a*
Y $g_{MELs}/gs_{ubstrate}$ (g/g)	0.41 ± 0.07	0.60 ± 0.08	0.48 ± 0	0.92	0.6
Yield $mol_{MELS}/mol_{substrate}$ (mol/mol)	0.17 ± 0.01	0.27 ± 0.10	0.05 ± 0	0.53*	*n.a*
Yield in C (mol of C $_{MELs}$/mol of $C_{substrate}$)	0.49 ± 0.03	0.60 ± 0.10	0.47 ± 0	0.62*	*n.a*
Productivity (g/L/day)	4.2 ± 0.12	6.1 ± 0.36	5.4 ± 0.12	13.9	6.1
MELs purity (g/g)	0.7 ± 0.1	0.8 ± 0.3	0.6 ± 0.1	*n.a*	*n.a*
Fermentation (days)	12	9	9	9	9

Biomass max – maximum biomass cell dry weight (g/L); MELs max – maximum MELs produced (g/L); Y MELs/Substrate – maximum MELs yield ($g_{MELs}/g_{substrate}$); Yield in C: mols of C in MELs/(mols of C in substrate + mols of C in yeast extract); MELs purity (g/g) at the end of the fermentation- Ratio of g of MELs to the sum of g of MELs and residual lipids

B.1.5 Final remarks

In the literature MELs production have been relied in two main strategies: 1) The use of hydrophilic substrates, allowing to achieve high purities, but low titres, and 2) The use of hydrophobic carbon sources which allows to achieve high titres but at the expense of low purities.

Therefore, in this section it was developed a novel strategy for MELs productions that combines of hydrophilic and hydrophobic carbon sources. It was observed that using 40 g/L of D-glucose (hydrophilic) and 20 g/L of WFO (hydrophobic) resulted in high productivities (2.0 and 2.4 g/L/day, for *M. bullatus* and *M. antarcticus*, respectively). Additionally, it was discovered that MELs production is totally and increases linear $V_{headspace}/V_{culture\ medium}$ volume (0.25, 1.5, 4 and 9),

Then the objective then shifted to applying the co-substrate strategy developed in shake flask to bioreactors. Due to issues with agglomerates when *M. antarcticus,* it was decided to use *M. bullatus* as case study. Therefore, initially was tested the effect of varying operational parameters, where it was discovered that just by varying agitation (from 500 to 800 rpm) and air-flow (1 to 2 vvm) the productivity increases in 45% (4.2 ± 0.12 and 6.1 ± 0.4 g/L/day, respectively).

Using the best operational parameters, it was attempted a different feed regime (daily feeds of 6.66 g/L of WFO until day 6, instead of 2 large feeds of 20 g/L of WFO), however the final productivity was 12% lower (5.4 ± 0.12 g/L/day).

This approach effectively resolved issues like foaming, without requiring large amounts of vegetable oil. Remarkably, when using 2 large feeds, the yield obtained is 0.6 ± 0.1 mol of C_{MELs}/mol of $C_{substrate}$,, corresponding to one of the highest yields reported in the literature, with the use of only 80 g/L of WFO (low amount for what is reported in others studies)

This section was important to establish a foundation for MELs bioprocess, which will be further explored in the following sections.

B.2 MELs production using cheese-whey as medium replacer and carbon source

B.2.1 Outline

In the previous section, it was shown that combining hydrophilic and hydrophobic carbon sources, can enhance MELs titres while maintaining high purities, and replacing vegetable oils (VO) with waste frying oils (WFO) does not impact the final titre. Nevertheless, the use of glucose as carbon source, followed by the rest of mineral medium it is one of the major bottlenecks since it can be cost-prohibitive and not sustainable. Therefore, it is here reported for the first time β-galactosidase production by *Moesziomyces spp* from different sugars (D-galactose, D-glucose and D-lactose), with D-galactose being the best β-galactosidase inducer, with 11.2 and 63.1 $IU/mg_{biomass}$, for *M. bullatus* 5535^T and *M. antarcticus* 5048^T, respectively. The production of this enzyme allows to break down D-lactose and thus to produce MELs directly from D-lactose or cheese whey (CW, a cheese industry by-product). Remarkably, when CW was used as sole media component (carbon and mineral source), in combination with WFO, MELs productivities were very close (1.40 and 1.31 g_{MELs}/L/day) to the ones obtained with optimized medium containing yeast extract (1.92 and 1.50 g_{MELs}/L/day), both for *M. antarcticus* and *M. bullatus,* respectively. The low cost, facile and efficient process, which generates large amounts of MELs potentiate the industrialization of the process here described on this thesis.

B.2.2 Introduction

As previously described (see **Table A3** and **Table A4**), most of the studies reporting MELs production in shake flask and bioreactor, makes use of large amounts of vegetable oils, but also other components, such as YE, sodium nitrate and so on. However, in an economical perspective, this is not sustainable, therefore, in this regard, attention should be directed to, among others, the cultivation media and substrates fed to MELs production fermentations. The use of an enriched medium in different nutrients is a constraint for the total price of the process (around 21% of total OPEX, operational expenditure) (Dhanarajan and Ramkrishna, 2014). Specifically, the use of SBO as a main substrate can constitute a threat for food availability and prices. Note that SBO is being directly produced from food crops, requiring large arable land area for its cultivation (Anto et al., 2020).

Substrate and media components selection is, therefore, of paramount importance to develop a successful bioprocess. Substrate composition determines carbon source type, nitrogen type, C/N ratio, content on other elements (salts, metals) and presence of eventual inhibitory products. Therefore, while substrate availability can affect process sustainability, substrate composition will greatly impact on microbial production. As shown in **Table A3** and **Table A4**, it was already attempt the production of MELs using agro-industrial residues, such as: 1) crude glycerol (Liu et al., 2011); 2) sugarcane juice (T. Morita et al., 2011); 3) wheat straw (N. Faria et al., 2014), ect. However, the use of such substrates often requires an additional substrate pre-treatment step, which contributes to increases the total production cost. Additionally, these additional steps may lead to the presence of potentially inhibitors to cell growth and MELs production (e.g polyphenols or acids), and/or results on low MELs final titres (Santos et al., 2019).

In a consolidated bioprocessing strategy (CBP), a single microorganism ensures enzyme production for substrate hydrolysis and bioconversion of released sugars into bio-based products. The natural or engineered microbial capacity of producing own cellulolytic and/or hemicellulolytic enzymes can improve the economy of the lignocellulose bioconversion processes by reducing and/or optimizing the use of commercial enzyme cocktails or even eliminating this significant operating cost in wastes/residues with complex carbon sources and its biorefining. In this regard,

previous work has shown *Moesziomyces* spp. ability to produce MELs directly from xylan, due to the cellulase-free xylanases production, but not from the complete lignocellulosic material (N. Faria et al., 2014; Faria et al., 2019). The ability of *Moesziomyces* spp**.** (formerly classified as *Pseudozymas* spp**.** due to their capacity to produce a broad spectrum of enzymes) to hydrolyze complex substrate structures makes them a promising model organism for the valorization of agro-industrial substrates with diverse compositions. Likewise, this study investigates the potential ability of *Moesziomyces* spp. to provide the tools to design CBPs based on D-lactose rich substrates, i,e. the production of enzymes able to hydrolyse lactose and ability to use not only D-Glucose, but also D-galactose, the resulting sugar monomers, as carbon sources. Cheese whey (CW) is explored as a promising D-lactose rich alternative substrate CW, a by-product of cheese production, is highly available as dairy industry is one of the major sources production of industrial effluents in Europe (40 million tons per year) (Demirel et al., 2005; Zotta et al., 2020). Moreover, CW discharge into the environment its restricted due to the low biodegradability of whey (Smithers, 2008) and its high chemical oxygen demand (COD). Along with a carbon source (D-lactose), CW composition includes minerals and other trace elements that may enrich a cultivation media for microbial production. This study brings new perspectives to the field of food by-products valorisation, while targeting biosurfactants production and their possible different biotechnological applications under circular economy concepts.

B.2.3 Material and Methods

B.2.3.1 Yeast strains, substrate, and cultivation conditions

Moesziomyces antarcticus PYCC 5048^T and *M. bullatus* PYCC 5535^T were obtained from the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal. Strains were plated in YM Agar (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, D-glucose 10 g/L and agar 20 g/L) and incubated for 3 days at 30°C. Stock cultures were prepared by propagation of yeast cells in liquid media described above for the inoculum and stored, in 20% (v/v) glycerol aliquots, at -80 °C. An inoculum was prepared by transferring the stocks cultures of *M. antarcticus* and *M. bullatus* into an Erlenmeyer flask with 1/5 working volume (50 mL) of medium containing 0.3 g/L $MgSO_4$, 3 g/L of $NaNO_3$, 0.3 g/L KH_2PO_4, 1 g/L yeast extract (YE), 40 g/L D-glucose, and incubating at 27 °C, 250 rpm, for 48 h. Then 10% (v/v) of inoculum was added into an Erlenmeyer flask with 1/5 working volume (50 mL) of cultured medium. All cultures were carried out in biological duplicates and incubated at 27ºC, 250 rpm, for 10 days.

The culture medium consisted for a first set of assays on mineral medium (0.3 g/L $MgSO_4$, 0.3 g/L KH_2PO_4 at initial pH 6.0) supplemented with 1 g/L YE, in the presence or absence of further nitrogen source (3 g/L $NaNO_3$), using 40 g/L of different carbon sources, i.e. D-glucose, D-galactose, or D-lactose. The concentration of 3 g/L of $NaNO_3$ and 40 g/L of a hydrophilic sugar follows the reference value of previous studies (N. T. Faria et al., 2014). A second set of assays explore the use of cheese whey (CW) (rich in D-lactose) alone (without any mineral media or other supplement) vs. CW in the aforementioned mineral medium with YE and $NaNO_3$. A final set of assays is started with D-glucose, D-lactose or CW as carbon source and addition of 20 g/L of waste fried oil (WFO), a lipid-rich source, in the presence of absence or the aforementioned mineral media with YE and $NaNO_3$. The selection of concentration of 20 g/L of WFO follows the results achieved on **section B.1**. CW was initially pre-treated as followed: after received, it was heated until 90ºC and kept at such temperature for 15 min, then centrifuged at 10 000 rpm for 10 min, the supernatant collected, and the pellet discarded. CW is not only a source of carbon, but also of nitrogen, phosphorous, other mineral and trace elements. Characterization of CW before

and after pasteurization is presented in **Table F2**. Cultivations were performed at least in two replicates.

B.2.3.2 Growth and biomass determination

Yeast growth was determined by measuring the cell dry weight (CDW), periodically, during fermentation time. CDW was determined from 1 ml of culture broth by centrifugation at 10 000 rpm for 6 min, followed by cell pellet washing with deionized water (twice) and drying at 60 ºC for 48 h. The supernatant collected was used to determine substrate consumption, extracellular protein and extracellular β-galactosidase activity.

B.2.3.3 Enzyme activity assays

Extracellular and intracellular β-galactosidase activities were determined in 1 ml of culture broth, after centrifugation at 10 000 rpm for 6 min, and separation of the supernatant (used for extracellular activity and protein determination) and pellet. The pellet was washed with deionized water (twice) and used for intracellular activity determination, as described below.

Extracellular β-galactosidase activity was determined by following an enzymatic assay adapted from the one described by Karasová et al (Karasová et al., 2002). The substrate used for the enzymatic assays was o-nitrophenyl-β-D-galactopyranoside (ONPG), and the activity was determined by measuring the release of o-nitrophenol from ONPG. All enzymatic activities were carried out in a 96 well-plate, and the reaction mixture was composed by: 20 mM of ONPG dissolved in 50 mM acetate buffer (pH 5.2). To initiate the enzymatic assay, 90 µL of ONPG, 20 mM solution and 10 µL of the supernatant were added to the 96 well plate. The reaction mixture was incubated at 37 ºC for 15 min and stopped by adding 200 µL of 10% (w/w) Na_2CO_3. The o-nitrophenol was measured at 420 nm in a microplate spectrophotometer (MultiskanTM GO, ThermoFisher Scientific). One unit (U) of β- galactosidase activity is defined as the amount of enzyme releasing 1 µmol o-nitrophenol per min. Intracellular β-galactosidase was determined after the incubation of washed cells with Y-PER™ (Yeast Protein Extraction Reagent, Pierce, Thermo Scientific, USA). The cell crude extract was used in the enzymatic assay, as described before. The lipolytic enzymatic assays were performed as previously described (Gomes et al.,

2011). The substrate used for the enzymatic assays was p-nitrophenyl butyrate. All enzymatic activities were carried out in triplicates, in a 96 well plate, and the reaction mixture composition was: 2.63 mM of p-nitrophenol butyrate dissolved in 50 mM acetate buffer (pH 5.2) and 4% of triton-X-100.

To initiate the enzymatic assay, 90 µL of p-nitrophenol butyrate 2.63 mM solution and 10 µL of the supernatants diluted was added to the 96 well plate. Then the reaction mixture was incubated at 37 °C for 15 min, and after that, the reaction was stopped by adding 200 µL of acetone. The released p-nitrophenol was quantified in a microplate spectrophotometer (MultiskanTM GO, ThermoFisher Scientific), at 405 nm. One unit (U) of lipase activity is defined as the amount of enzyme releasing 1 µmol p-nitrophenol per min.

B.2.3.4 Substrate and product quantification

The quantification of D-glucose, D-lactose and D-galactose were performed using HPLC. Culture broth samples were centrifuged at 10 000 g for 6 min, the supernatants filtered through a 0.22 µm-pore size-filter and injected into a HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany) and an Rezex ROA Organic Acid H+ column (300 mm× 7.8 mm, Phenomenex, Torrance, CA, USA), at 65°C. A aqueous solution of sulfuric acid (5 mM) was used as mobile phase at 0.5 mL/min.

The consumption of oil (triacylglyceride – TAG) in the culture samples was also analysed by HPLC, as described by Badenes et al (Sara M. Badenes et al., 2010), 500 µL of each sample was retrieved and mixture with 58.5 mM of 1 µL acetic acid and 499 µL n-hexane. Then it was centrifuged at 10 000 rpm for 2 minutes, and the organic phase was extracted and injected into another HPLC system (Hitachi LaChrom Elite), equipped with a Chromolith Performance RP-18 endcapped (100mm x 4.6mm x 2µm) column, an auto sampler (Hitachi LaChrom Elite L-2200), a pump (Hitachi LaChrom Elite L-2130) and a UV detector (Hitachi LaChrom Elite L-2400) set up at 205 nm. The flow rate was set up at 1 mL/min and the injection volume was 20 µL. Three mobile phases were employed: phase A consisted of 100% acetonitrile, phase B consisted of water 100% and phase C comprising a mixture of n-hexane and 2-propanol (4:5, v/v). Quantification was

carried out using calibration curves of Glyceryl trioleate and Glyceryl trilinoleate (>98 %, Sigma-Aldrich GmbH) for TAG.

MELs were quantified as previously described, through GC analysis of methyl esters generated by methanolysis of freeze-dried biological samples (1 mL) (N. Faria et al., 2014). For the cases where beads enriched in MELs were observed and no representative sample could be retrieved, the total fermentation broth was extracted, using equal volumes of ethyl acetate to the broth volume in three successive extractions, the solvent evaporated in a rotavapor (Buchi R3, Switzerland) at 40 ºC and 240 mbar, the bottom phase collected and weighted and 50 mg of it retrieved and submitted to methanolysis and GC analysis as previously described (N. Faria et al., 2014).

B.2.3.5 Statistical analysis

Statistics were performed, using Graph-pad, by analysis of variance (two-way ANOVA) and p-values of the differences between groups were corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p \leq 0.05$.

B.2.4 Results and discussion

B.2.4.1 D-Lactose as a sole carbon and energy source for MELs production – the role of endogenous β-galactosidase

The ability of *Moesziomyces* spp. to grow and produce MELs using D-lactose (40 g/L) as a sole carbon source was evaluated for *M. antarcticus* PYCC 5048^T and *M. bullatus* 5535^T. In this regard, yeast cells were directly inoculated in media containing D-lactose. Mineral media with YE was used for these set of experiments. In addition, nitrogen source (3 g/L of $NaNO_3$) supplementation was investigated (**Figure B9**), since it is known that the absence of nitrogen promotes MELs production, however its presence is crucial for amino acid synthesis and consequently, enzyme production.

Cell growth was monitored by the determination of the CDW. Both strains were able to grow using D-lactose as carbon source, regardless the nitrogen source supplementation (**Figure B9** A, B). After 10 days of cultivation, cell biomass values of 18.0 ± 4.0 and 13.5 ± 1.5 g/L were obtained for *M. antarcticus* and *M. bullatus*, respectively, in the presence of nitrogen source. Interestingly, for both strains, there is not significant differences in the final cell biomass with or without nitrogen supplementation.

Comparing D-lactose consumption rate in both strains, *M. bullatus* cultivation stands out, with no differences regardless the nitrogen supplementation (0.38 ± 0.02 and 0.39 ± 0.04 g/L/h, without and with nitrogen supplementation, respectively) (**Figure B9** D). In *M. antarcticus*, an increase on sugar consumption rate was observed with nitrogen supplementation, from 0.24 ± 0.00 to 0.32 ± 0.02 g/L/h (**Figure B9** C), but still lower than the ones observed in *M. bullatus* cultivations and with an adaptation to the substrate (D-lactose) observed in the first day of cultivation, where only the residual D-glucose present in the cultivation media seems to be consumed. It is important to note that although the relatively high values reported for the D-lactose consumption rate, D-galactose slightly accumulates in the broth. In *M. bullatus* cultivation without nitrogen supplementation, more than 10 g/L of D-galactose were accumulated at day 4, which were

consumed until the end of the cultivation (10 days). In the case of *M. antarcticus*, a maximum of 6 g/L of D-galactose were accumulated at day 7, but not fully consumed, with 4 g/L of D-glucose and 4 g/L of D-galactose left in the end of the cultivation. With nitrogen supplementation, there was no D-galactose accumulated at day 7 and 10 of *M. antarcticus* cultivation, after an accumulation of 10.6 g/L at day 4.

Direct MELs production from D-lactose was observed in all conditions tested. Interestingly, although a lower substrate consumption rate reported above, the cultivation conditions using no supplementation of inorganic nitrate source ($NaNO_3$) rendered higher MELs titres, analogous effects of addition of $NaNO_3$ on MELs production by these yeasts have been previously reported for D-glucose and D-xylose (N. T. Faria et al., 2014). Similar MELs titres were achieved for the two yeasts after 4 days of culture. However, after 10 days cultivation, *M. antarcticus* reached 4.93 ± 0.53 g/L and *M. bullatus*, 2.20 ± 0.10 g/L. With $NaNO_3$ supplementation, the final MELs titre was 2.93 ± 0.53 and 0.15 ± 0.02 g/L for *M. antarcticus* and *M. bullatus,* respectively (where maximum MELs titre on *M. bullatus* cultivation was reached at day 4, a maximum of 1.91 ± 0.01 g/L) (**Figure B9** A, B). Again, when these yeasts were fed with xylan and the xylanolytic capability of these yeasts was required for hydrolyse of the xylan into D-xylose, *M. bullatus* shows to be a more efficient MELs producer (Faria et al., 2015). In this study, both strains are able to produce β-galactosidase (**Figure B9** E, F), with higher β-galactosidase extracellular activity obtained when additional nitrogen source is added. Still, even without the addition of $NaNO_3$ the enzymatic activity seems to be high enough to promote D-lactose hydrolysis into its monomers D-galactose and D-glucose, which are consumed to support cell growth and MELs production. The high standard deviations found on the β-galactosidase extracellular activity profiles for *M. bullatus*, (40 g/L of lactose, without NaNO3), are indicative of culture behaviour variability as enzyme production was halt after 1 day in culture for one of the replicates (**Figure F6**).

The maximum MELs titres using D-lactose were about 1.8-fold lower, for both strains, when compared to previous studies using the same strains cultivated in D-glucose; 4.9 and 3.4 g/L of MELs was reported for *M. antarcticus* and *M. bullatus*, respectively, in similar C/N ratio (N. T. Faria et al., 2014). Nevertheless, higher biomass values were observed when D-lactose was used (1.7 and 1.2-fold higher, respectively). These results may indicate that the cell is dispending more

energy to hydrolyse lactose, which is reflected in the use of more carbon source for production of building blocks, enzymes and biomass, rather than MELs.

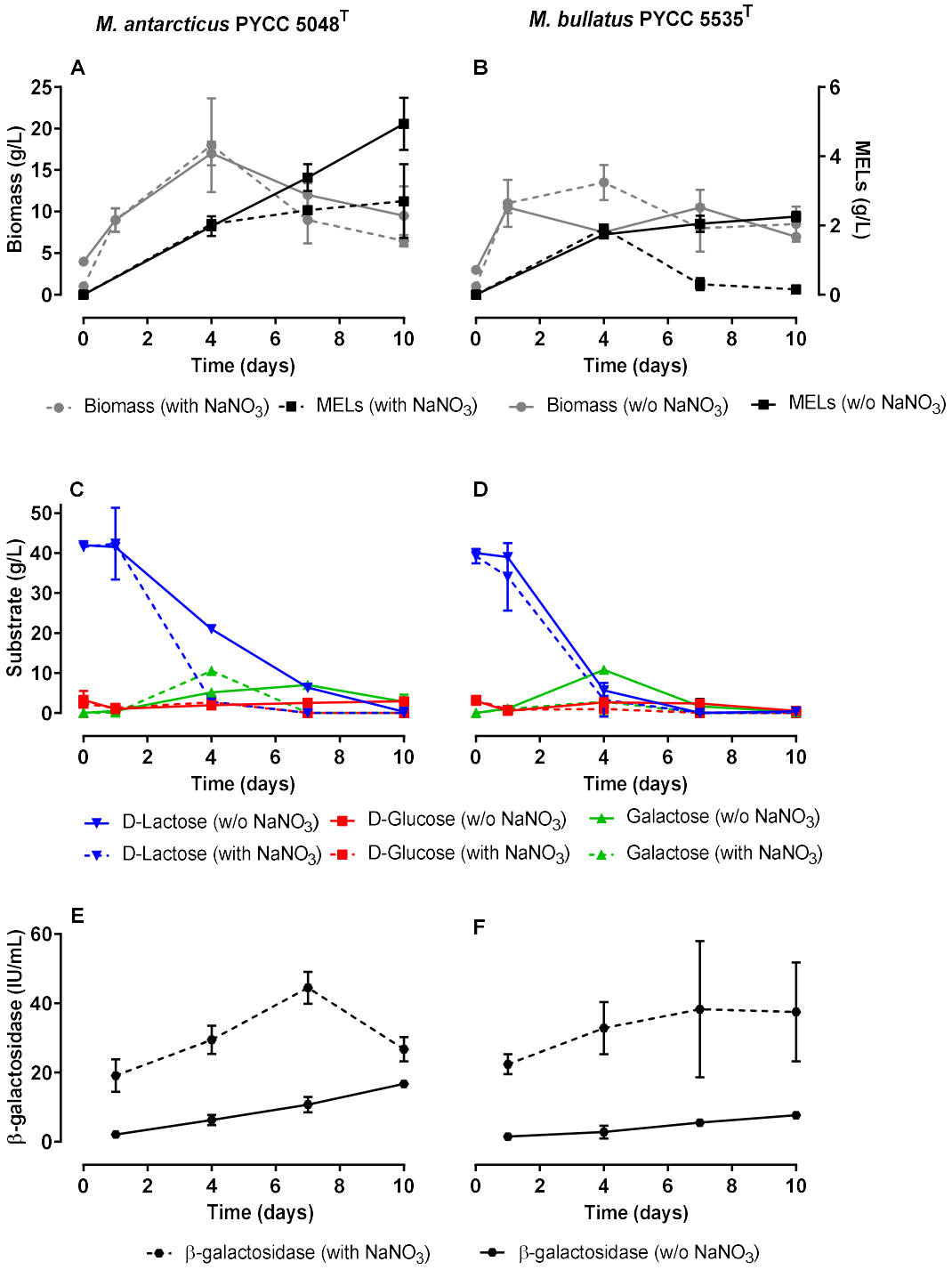


Figure B9: Cultivation of *M. antarcticus* PYCC 5048^T (A, C, E) and *M. bullatus* PYCC 5535^T (B, D, F) in D-lactose (40 g/L), mineral medium with YE and in the presence or absence of NaNO3 (dashed line and filled line, respectively), during 10 days at 27 ºC. Yeast biomass (circles) and MELs production (squares) (A, B); Carbon source profiles (Lactose, galactose and glucose represented with inverted triangles, triangles, and squares, respectively) (C, D); Extracellular β-

galactosidase profile activities (circles) (E, F). Standard deviations values lower than 1 g/L and **1** IU/mL are not represented.

B.2.4.2 Characterization of β-galactosidase profiles using different substrates.

The hydrolytic potential of *M. antarcticus* and *M. bullatus*, regarding extracellular β-galactosidase activity, was evaluated. Following the trends observed for D-lactose consumption (**Figure B9** C, D), supplementation of nitrogen source resulted in higher β-galactosidase activity, 2.6 and 4.2-fold higher than for cultures without $NaNO_3$ supplementation, for a maximum of 44.5 ± 3.3 and 37.5 ± 10 IU/mL, obtained with *M. antarcticus* and *M. bullatus*, respectively.

Previous studies with *M. antarcticus* and *M. bullatus* have shown that the detection of enzymatic activities is highly dependent on the substrate used. Lipase activity can be detected when *M. antarcticus* was grown on D-glucose (Morita et al., 2007c), while xylanase activity was not detected, but only when cultured in pentose-based sugars (Faria et al., 2019). In this regard, the induction of β-galactosidase activity by using the D-lactose monomers, D-glucose and D-galactose (separated and mixed), was evaluated, supplementing the medium with nitrogen source (3 g/L of $NaNO_3$) (**Figure B10** and **Table F4**). The results of D-galactose cultivation for both strains, without addition of $NaNO_3$, are presented as supplementary data (**Figure F9** and **Table F4**). Analysing the values obtained, we observed that when D-galactose and D-glucose were mixed, the volumetric activity values were similar to the ones obtained with D-lactose (around 30-50 IU/mL) (**Figure B10** and **Table F4**). Surprisingly, when D-galactose was used as a sole carbon source, the highest volumetric activity was obtained, 12.8 and 8.3-fold higher than the ones obtain for cultures using D-lactose as substrate, reaching a maximum of 505.2 ± 3.1 and 127 ± 31.2 IU/mL, for *M. antarcticus* and *M. bullatus* cultures, respectively. Even though a positive effect of D-galactose was observed for β-galactosidase activity, it seems that this substrate does not promote extensive yeast growth, as the maximum biomass reached to such cultures was 1.8-fold lower when than the one obtained for D-lactose based cultures (**Table F4** and **Table F5**). In

the presence of both monomers (D-galactose and D-glucose), the values of biomass were comparable with D-lactose cultivations.

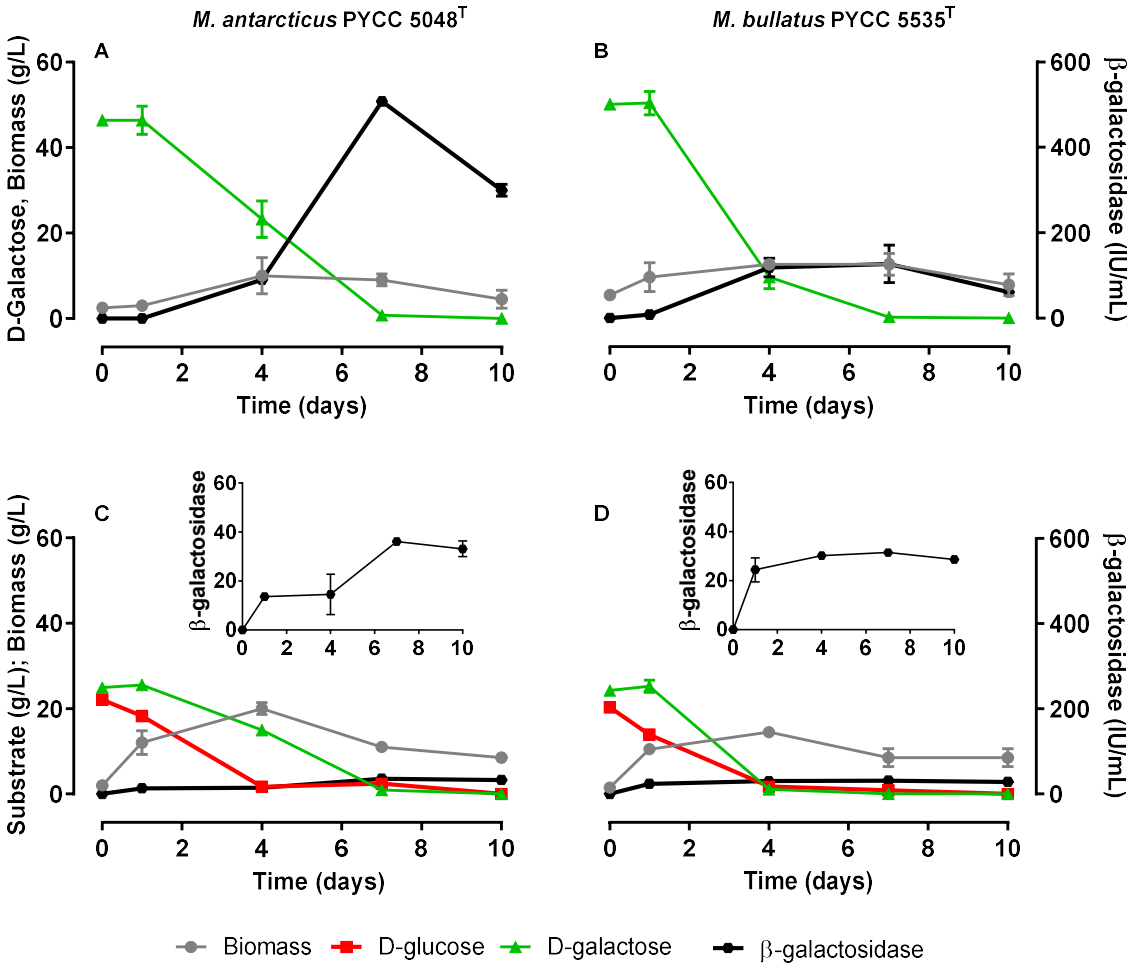


Figure B10: Cultivation of *M. antarcticus* PYCC 5048^T (A, C) and *M. bullatus* PYCC 5535^T (B, D) in D-galactose (40 g/L) (A, B) and a mixture of D-glucose (20 g/L) with D-galactose (20 g/L) in mineral medium with YE and $NaNO_3$ (C, D), during 10 days at 27 ºC. For all graphics the patterns of yeast biomass (circles), Carbon source profiles (D-glucose and D-galactose consumption represented with squares and triangles, respectively) and β-galactosidase production (circles) are shown. Standard deviation lower values than 1g/L and 10 IU/mL (or 1 IU/mL for inserted figures) are not represented.

In fact, both strains had shown different patterns regarding sugar consumption, when D-glucose and D-galactose were used as carbon source. Sugar consumption trends in *M. bullatus* cultivations were similar regardless the substrate used. In fact, both D-glucose and D-galactose were fully consumed at day 4 of cultivation. *M. antarcticus* had shown a preference for D-glucose,

and at day 4 of fermentation, while most of D-glucose is consumed, only 5 g/L of D-galactose were consumed.

In this regard, the fast consumption rate of D-lactose and limited D-galactose accumulation in the media contrasts with the lower consumption rate of D-galactose, when used as sole carbon source (**Figure B9** and **Figure B10**). Therefore, the presence of additional β-galactosidase mechanisms (other than extracellular) was investigated. Namely, intracellular β-galactosidase activity was estimated in samples collected at day 2 and 4 of cultivation of *M. antarcticus* and *M. bullatus* using D-lactose, D-galactose and mixtures of D-glucose and D-galactose (**Figure B11**, **Table F6**). Notable, all the conditions showed the presence of significant intracellular β-galactosidase activity, but at day 4 its values were lower than the extracellular β-galactosidase activity, with exception for *M. antarcticus* cultures on D-galactose (**Figure B11** C). When D-galactose was used as substrate, initially at day 2 of fermentation (**Figure B11** A, B, **Table F6**), the intracellular and extracellular volumetric activity were, respectively, 8 and 18-fold lower for *M. antarcticus* (**Figure B11** A) than for *M. bullatus* cultivations (**Figure B11** B), but at day 4 of fermentation (**Figure B11** C, D, **Table F6**), the intracellular specific activity for *M. antarcticus* became 5-fold higher than the values obtained with *M. bullatus.* The volumetric activities values obtained for cultures using D-lactose as carbon source or its monomers mixture, were similar among them and significantly lower than the ones obtained when using D-galactose as sole carbon source. However, for day 2 in *M. antarcticus*, the intracellular volumetric activity was virtually zero (0.03 ± 0.02 IU/mL) for mixture of monomers cultures, and, although still low, it was two orders of magnitude higher (1.2 ± 0.2 IU/mL) in D-lactose cultures. These results may indicate that the induction of β-galactosidase is promoted by the metabolites driven from metabolization of D-galactose in both strains, which explains the low activities when it is used D-lactose and the monomers.

From our literature search, the only specie, more closely related with the strains used in this work, that is able to produce β-galactosidase in presence of galactose was *Hypocrea jecoina* (*Basidiomycota)*. Fekete et al (Fekete et al., 2007) have shown that the ability of this strain to grow in D-lactose, is strongly dependent on the formation of an extracellular glycoside hydrolase (6H) family 35 β-galactosidase encoded by *Baga1* gene. Additionally, the authors also had shown

the importance of D-galactose degradation into galactitol, to induce β-galactosidase expression. In this regard, it was performed a Blast-P (computational tool used to compare different proteins, provided by NCBI – National Centre for Biotechnology Information) of this protein from *H. jacoina* (glycoside hydrolase (6H) family 35 β-galactosidase) against *M. antarcticus* and *M. bullatus* sequences. A glycosidase hydrolase, family GH35, and a hypothetical protein PaG_04781 were found (93% of coverage for both), for *M. antarcticus* and *M. bullatus*, respectively. The protein found for *M. antarcticus* belongs to a family of hydrolases (GH1, GH2, GH35 and GH42) (Lombard et al., 2014). From those hydrolases, GHx and GHy have β-galactosidase activity. The discovery that this type of proteins can also be expressed in *M. bullatus* has the potential to identify a new β-galactosidase or β-galactosidase producer.

Until date, the highest specific activity reported in literature for β-galactosidase is 4.2 IU/$mg_{biomass}$ (obtained after cell permeabilization with isoamyl alcohol), using *Kluvyromyces lactis* (Dagbagli and Goksungur, 2008), one of the yeast industrially used for the production of the enzyme. Using D-lactose, the same substrate used in the study of *K. lactis*, a specific activity of 5.1 ± 0.8 IU/$mg_{biomass}$, with *M. antarcticus*, is achieved, representing 44.5 ± 3.3 IU/mL of volumetric activity. Remarkably, using *M. antarcticus* and D-galactose as carbon source, it was achieved the value for β-galactosidase specific activity, at day 4, considering intracellular activity (57.2 ± 15.5 IU/$mg_{biomass}$, and 525.9 ± 16.3 IU/mL), and the highest β-galactosidase activity reported in this work, extracellular, at day 7, reaching 63.1 ± 6.1 IU/mg (505.2 ± 3.1 IU/mL), which is 15-fold higher than the reported value (4.2 IU/$mg_{biomass}$).

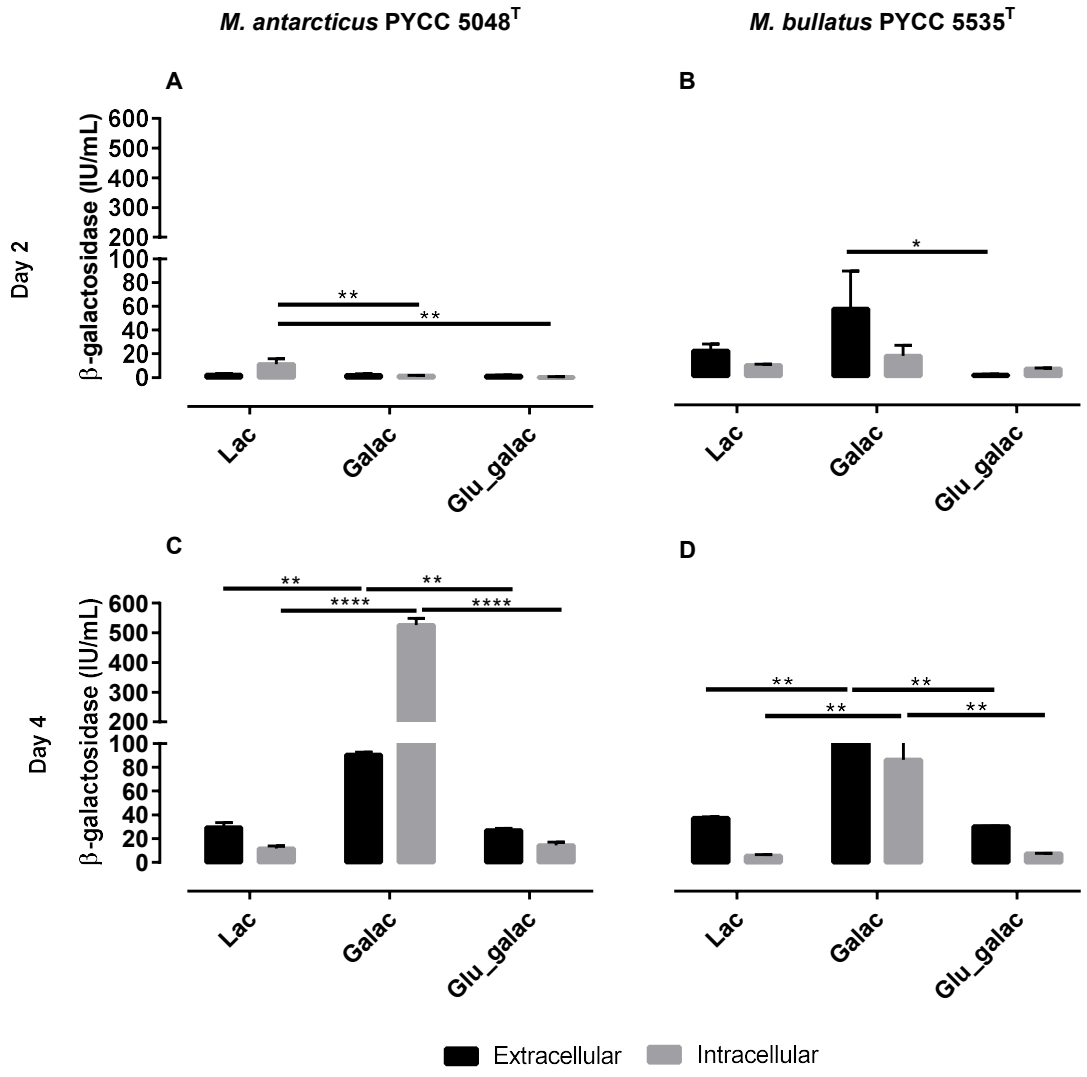


Figure B11: Intracellular (grey) and extracellular (black) β-galactosidase activity determined at day 2 (A, B) and 4 (C, D) for *M. antarcticus* PYCC 5048^T (A, C) and *M. bullatus* PYCC 5535^T (B, D) cultivated in 40 g/L of D-galactose (A, B), 40 g/L of D-lactose (c; d) and 20 g/L of D-galactose and 20 g/L of D-glucose (e; f) as carbon sources in mineral medium with YE and NaNO3. * $p \leq 0.05$; ** $p \leq 0.003$; **** $p < 0.0001$.

B.2.4.3 Exploring *Moesziomyces* spp. as MELs producer from renewable substrates

So far, MELs production efficiency from D-lactose (4 g/L, **Figure B9**) was similar to the one observed when D-glucose was used as carbon source, highlighting the efficient hydrolytic capacity of both *M. antarcticus* and *M. bullatus.* Cheese whey, a D-lactose-rich by-product of dairy products (see **Table F2** for detailed composition of CW), was explored as a potential complex cultivation media (mineral medium, YE and $NaNO_3$) for *M. antarcticus* and *M. bullatus* cultivations, first alone, and then in combination with WFO, a lipidic rich carbon source.

Direct conversion of cheese-whey into MELs

The use of CW as sole nutrient source was first assessed against conditions where CW was used in mineral media with YE (**Figure B12** and **Table F7**). The experiments were carried out during 10 days at 27 ºC and 250 rpm, using CW (30 % v/v), corresponding to a 40 g/L of D-lactose in the cultivation media. Both yeasts were able to grow in CW (**Figure B12** A, B), but slower when compared with cultures on refined D-lactose in mineral media with YE (**Figure B9**). However, only *M. antarcticus* produced lower biomass when grown in CW if compared with D-lactose (11.5 and 11.0 g/L, Table F6, using CW as sole media component or in mineral media with YE, respectively, compared to 18.0 g/L, **Table F5**, of biomass when using D-lactose in mineral media with YE).

D-lactose consumption rate was higher in *M. antarcticus* than *M. bullatus* when cultured in CW (0.41 and 0.32 g/L/h, respectively) (**Figure B12** C, D). However, the direct use of CW alone (without mineral media nor YE supplementation) reduced D-lactose consumption rate in *M. antarcticus* cultivations (from 0.41 to 0.25 g/L/h) (**Table F7**). Also, an accumulation of D-galactose was observed, reaching 10 g/L of D-galactose at day 4. In opposition, in *M. bullatus* cultivations the accumulation of D-glucose and D-galactose was not observed (**Figure F10**). The emphasized efficient mechanisms for lactose assimilation were verified, especially in *M. bullatus* with CW, even on the absence of addition of mineral media and YE addition. The hydrolytic potential of *M. antarcticus* and *M. bullatus* was evaluated regarding the extracellular β-galactosidase activity

(**Figure B12** C, D). Interestingly, in spite of CW do not present measurable D-galactose, β-galactosidase activity using this substrate was estimated to be 10-fold higher than the one for D-lactose cultures. Therefore, enzyme production by the yeast and activity may benefits from other compounds, that not D-galactose, such as additional nitrogen, trace elements and ions potentially provided by the CW (**Table F2**). Also, the use of mineral media induces higher extracellular β-galactosidase activity, for both strains, but more pronounced in *M. antarcticus,* at day 4 (305.2 IU/mL).

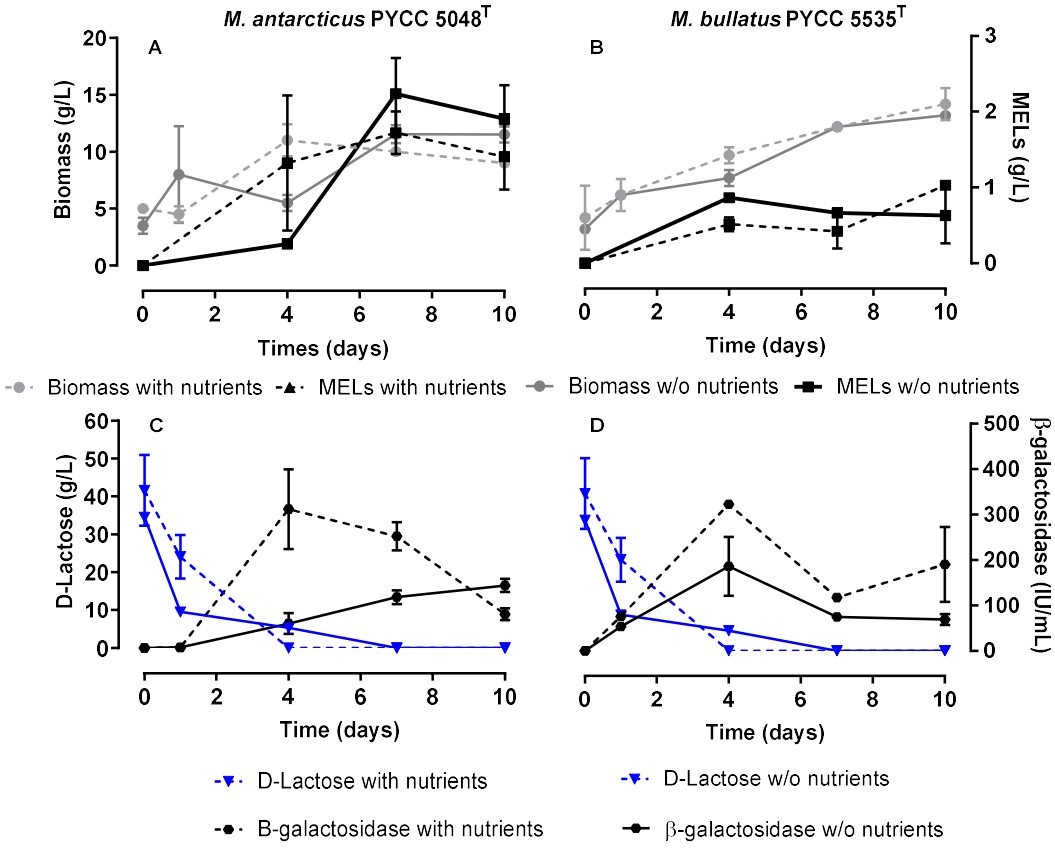


Figure B12: Cultivation of *M. antarcticus* PYCC 5048 T (A, C) and *M. bullatus* PYCC 5535 T (B, D) in CW (40 g/L), during 10 days at 27 ºC in the presence and absence of mineral medium with YE (dashed line and filled line, respectively), on both cases with addition of $NaNO_3$. Yeast biomass (circles) and MELs production (squares) (A, B); Carbon source (inverted triangles) and extracellular β-galactosidase activities profiles (circles) (C, D). The D-glucose and D-galactose

monomers profiles are represented in Figure F4, in attachments (section F). Standard deviations values lower 1 g/L are not represented.

The direct MELs production from CW was observed (**Figure B12** A, B). Although the lower D-lactose consumption rate in cultivation of *M. antarticus* using CW without addition of mineral media and YE, MELs production was higher in such conditions (1.94 g/L), while the use of *M. bullatus* rendered the lowest MELs value, 0.77 g/L. Nevertheless, MELs obtained from CW cultures (without mineral media nor YE) was consistently lower if compared with the use of D-lactose as carbon source in mineral medium with YE (**Table F5**). The carbon chain length of MELs acyl groups obtained was similar to the ones found both in D-lactose and D-glucose, mainly composed of C10:n and C12:n (data not shown).

Considering the fact that 65 % of world population are lactose intolerants, β-galactosidase plays a crucial role in dairy industry by producing lactose-free products, a market in expansion with a CAGR (compound annual growth rate) of 11 % and evaluated at USD 4.69 billion in 2015 (Pre COVID-19) (Maida, 2016; Saqib et al., 2017). In this regard, these results achieved with *M. antarcticus,* especially the values achieved when using CW as sole carbon source and medium component (137.54 ± 10.42 IU/mL), can open new perspectives and studies for this type of industry.

Towards sustainable MELs production – combination of CW with WFO

CW was successfully assessed as sole media component for MELs production. Nevertheless, as observed for other sugar-based carbon sources, MELs production was rather limited. As previously described in section B1, MELs productivities increase by adding a relatively amount of a lipidic rich carbon source, however such strategy relies on the lipolytic activity of *Moesziomyces spp.* Therefore, the enzymatic activity of lipases for D-glucose, D-galactose, D-lactose and CW (**Figure F7**), in the absence or presence of mineral media supplemented with YE and $NaNO_3$, as well a mixture of D-galactose and D-glucose in mineral media with YE and $NaNO_3$ (**Figure F8**) were determined. The results obtained showed lipase activity profiles for the cultures on CW and on D-glucose to be very close.

In this regard, using the condition developed on section B1 (**Figure B7**), three carbon sources such as, D-glucose (40 g/L), D-lactose (40 g/L) or CW (40 g/L in D-lactose), were assessed in combination with WFO (20 g/L), with or without mineral media, YE nor $NaNO_3$ supplementation, for both *M. antarcticus* and *M. bullatus.* The results are resumed on **Table B4**. The biomass and MELs production as well as sugar and residual lipids profiles for cultures using WFO combined with CW (**Figure B13**) or refined sugars (**Figure F*11*** and **Figure F12**) in the presence (**Figure B13** A, B) or absence (**Figure B13** C, D) of mineral medium, YE and $NaNO_3$.

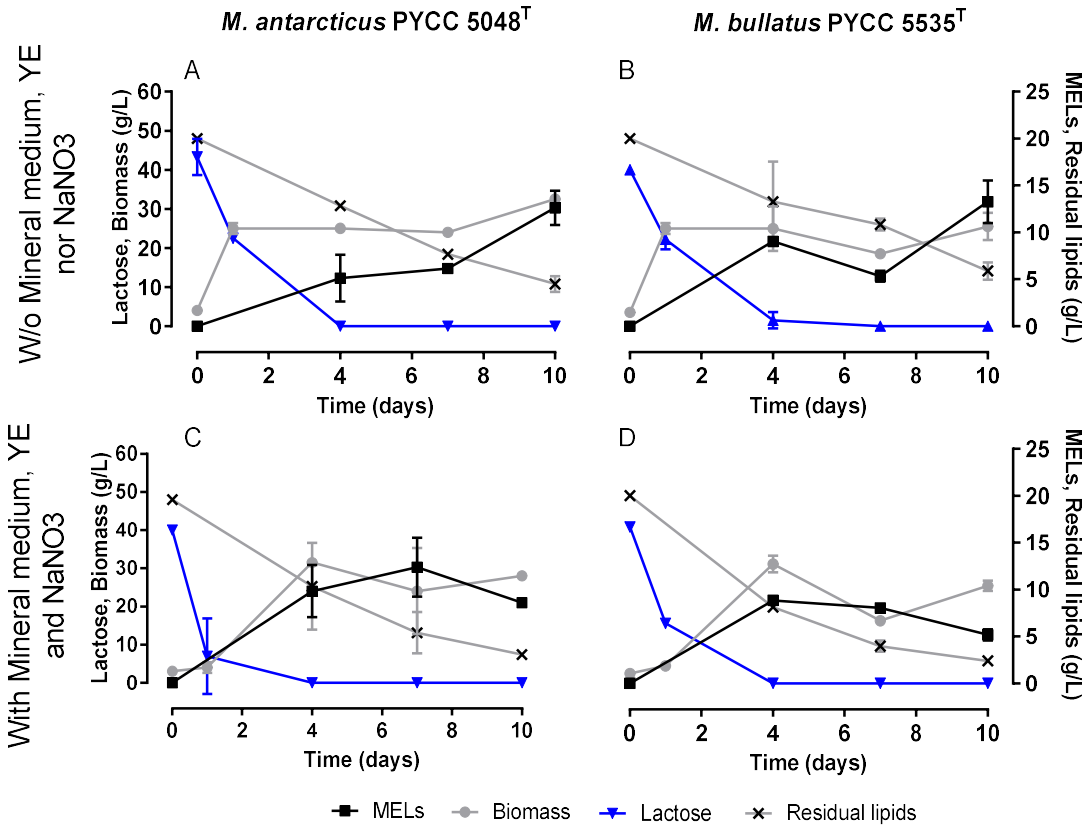


Figure B13: Cultivation of *M. antarcticus* PYCC 5048^T (A, C) and *M. bullatus* PYCC 5535^T (B, D) in CW (40 g/L) and WFO (20 g/L), during 10 days at 27 ºC, in the absence (a, b) and presence of mineral medium with YE and NaNO3 (C, D). For all graphics the patterns of yeast biomass (circles) and MELs production; D- lactose (inverted triangles) and residual lipids (driven from WFO, crosses) consumption, are shown. The red point indicates the appearance of beads enriched in MEL and residual lipids. Standard deviations values lower 1 g/L are not represented.

Table B4: Rate of consumption (Rs); maximum biomass produced; yield (Yx/s); Maximum MELs obtained (g/L); yield of MELs produced ($g_{MELs}/g_{Substrate}$), maximum productivity (g/L/h) and purity ($g.g^{-1}$) for *M. antarcticus* PYCC 5048^T and *M. bullatus* PYCC 5535^T using a sugar base carbon source (40 g/L), i.e. CW, D-lactose or D-glucose, and WFO (20 g/L). in the presence or absence of mineral media with YE and $NaNO_3$.

Condition	Parameters	***M. antarcticus*** PYCC 5048^T			***M. bullatus*** PYCC 5535^T		
		CW	D-lactose	D-glucose	CW	D-lactose	D-glucose
Mineral media with YE and $NaNO_3$	rs (g/L/h)	0.34 ± 0.02	0.32 ± 0.09	0.18 ± 0.02	0.37 ± 0.00	0.24 ± 0.00	0.17 ± 0.00
	$Biomass_{max}$ (g/L)	31.5 ± 0.5 (Day 4)	15.5 ± 0.50 (Day 10)	23.0 ± 0.1 (Day 4)	30.5 ± 1.5 (Day 4)	12.8 ± 2.2 (Day 10)	20.0 ± 0.0 (Day 7)
	$Y_{Xmax/S}$ (g/g)	0.52 ± 0.01	0.25 ± 0.01	0.38 ± 0.02	0.49 ± 0.02	0.21 ± 0.04	0.32 ± 0.03
	$MELs_{max}$ (g/L)	12.63 ± 2.28 (Day 7)	13.54 ± 1.25 (Day 10)	19.21 ± 0.02 (Day 10)	8.93 ± 0.07 (Day 4)	11.26 ± 0.95 (Day 10)	15.02 ± 0.99 (Day 10)
	$Y_{MELs/Substrate}$ (g/g)	0.21 ± 0.04	0.22 ± 0.02	0.31 ± 0.02	0.15 ± 0.03	0.18 ± 0.02	0.25 ± 0.02
	$Productivity_{max}$ (g/L/day)	1.80 ± 0.33	1.35 ± 0.13	1.92 ± 0.24	1.28 ± 0.01	1.13 ± 0.12	1.50 ± 0.10
	MELs purity (g/g)	0.74 ± 0.01	0.94 ± 0.01	0.89 ± 0.02	0.68 ± 0.02	0.94 ± 0.01	0.87 ± 0.01
Mineral media without YE nor $NaNO_3$	rs (g/L/h)	0.39 ± 0.02	0.00	0.00	0.37 ± 0.01	0.00	0.00
	$Biomass_{max}$ (g/L)	32.5 ± 0.5 (Day 10)	13.0 ± 3.0 (Day 1)	12.0 ± 0.0 (Day 10)	25.5 ± 2.5 (Day 10)	10.0 ± 0.0 (Day 1)	7.5 ± 1.5 (Day 10)
	$Y_{Xmax/S}$ (g/g)	0.53 ± 0.01	0.21 ± 0.05	0.20 ± 0.02	0.42 ± 0.04	0.16 ± 0.03	0.12 ± 0.02
	$MELs_{max}$ (g/L)	13.98 ± 0.06 (Day 10)	7.38 ± 0.16 (Day 10)	5.41 ± 0.15 (Day 10)	13.10 ± 1.77 (Day 10)	2.53 ± 0.02 (Day 10)	2.53 ± 0.02 (Day 10)
	$Y_{MELs/Substrate}$ (g/g)	0.23 ± 0.00	0.12 ± 0.02	0.09 ± 0.02	0.21 ± 0.03	0.04 ± 0.02	0.04 ± 0.02
	$Productivity_{max}$ (g/L/day)	1.40 ± 0.01	0.74 ± 0.02	0.54 ± 0.02	1.31 ± 0.18	0.25 ± 0.02	0.25 ± 0.01
	MELs purity (g/g)	0.77 ± 0.04	0.34 ± 0.02	0.24 ± 0.01	0.72 ± 0.02	0.11 ± 0.01	0.11 ± 0.01

$Biomass_{max}$ – maximum biomass cell dry weight (g/L); r_s – sugar consumption rate (g/L/h); $Y_{Xmax/S}$ – maximum biomass yield (g/g); $MELs_{max}$ – maximum MELs produced (g/L); $Y_{MELs/Substrate}$ – maximum MELs yield (g/g); MELs purity (g/g) at the end of the fermentation- Ratio of g of MELs to the sum of g of MELs and residual lipids

As expected, when following the strategy to combine WFO and D-glucose or D-lactate, the use of mineral medium with YE supplementation was crucial to obtain high MELs productivity (**Figure F*11***). Indeed, when culture medium was not supplemented (**Figure F12**), D-lactose and D-glucose are not consumed, while a fraction of WFO lipids are consumed and MELs were produced, but at low concentrations (5.41 ± 0.15 and 2.53 ± 0.02 g/L for *M. antarcticus* and *M. bullatus*).

Remarkable, high MELs productivities were reached using CW and WFO solo, without using mineral media nor YE. Such values definitively represent a significant increase in the product yield and productivity, when compared with the sole CW utilization (**Table F7**). Impressively, the level of MELs production using only CW and WFO (titres of 13.98 ± 0.06 g/l and 13.10 ± 1.77 g/L and productivities of 1.40 ± 0.01 and 1.31 ± 0.18 g/L/h for *M. antarcticus* PYCC 5048^T and *M. bullatus* PYCC 5535^T), close to the ones obtained using D-glucose in mineral media with YE supplementation (titres of 19.21 ± 0.02 g/l and 15.02 ± 0.99 g/l and productivities of 1.92 ± 0.24 and 1.50 ± 0.10 g/L/h for *M. antarcticus* PYCC 5048^T and *M. bullatus* PYCC 5535^T), represents a potential route to circumvent the use of refined or expensive substrates, including D-glucose, $NaNO_3$, $MgSO_4$, KH_2PO_4 or YE. The co-utilization of WFO seems to positively impact on D-lactose consumption for cultures with CW (**Figure B12** and **Figure B*13***) or D-lactose (**Figure B*13*** A and B vs **Figure B9**), emphasizing the role of CW as a media substitute. Later, it will be discussed the economical impact of replacing culture medium by CW in MELs bioprocess (see **section C.2**).

Although *M. antarcticus* cultivations using CW and WFO reach similar maximum MELs titres (13-14 g/L) regardless the conditions tested (**Table B4**), very different cultivation profiles were observed (**Figure B13**). Noteworthy, the cultivations in CW and WFO with mineral media and YE supplementation reached the highest MELs at day 7 (12.63 g/L), with a decrease after that time point (**Figure B13** C) while in cultivations with no supplementation, the highest MELs value (13.98 g/L) is obtained only at day 10 (**Figure B13** A). The decrease observed for MELs after day 7, in conditions using the culture medium, may result from the depletion of carbon sources, and consequent starvation of *M. antarcticus* and *M. bullatus*, suggesting energy storage as a possible biological function for MEL.

The values obtained for biomass at day 1 were higher for cultures of CW and WFO alone, i.e. without mineral media and YE supplementation, but no significant further increases in cell biomass over time (except between days 7 and 10, **Figure B13** A) were observed. When mineral media with YE was added, biomass increased until day 4, where it reached its highest value (31.5 g/L). Another interesting achievement was the faster consumption of D-lactose and lipids observed for *M. antarcticus* cultivations supplemented with mineral media and YE, where after 1 day of cultivation less than 10 g/L of D-lactose was present (**Figure B13** C vs. **Figure B13** A). *Moesziomyces bullatus* cultivations followed the same trends observed to *M. antarcticus* for the same conditions, but with lower sugar and lipids consumption. Importantly, when *M. bullatus* is cultivated in CW and WFO in mineral media and YE, the maximum MELs is produced early, at day 4, but at a lower value of 8.93 g/L; while cultures without mineral media or YE supplementation reached a MELs titre of 13.1 g/L MELs, but only at day 10.

Until now, most of the studies performed to optimize MELs production required a complex mixture of nutrients, that increase the final cost of the process. For example, Beck A and Zibek S (Beck and Zibek, 2020) have performed a study testing different medium components, aiming to achieve fast biomass growth (around 0.16-25 h^{-1}), without affecting MELs production. However, medium formulation continues to be a bottleneck in the fermentation process, as the use of cofactors and vitamins increase the process complexity and final manufacture cost. Furthermore, in most of the studies using renewable substrates, there is the need to continuous supplement together the carbon source and medium components (such as YE, mineral salts), representing additional cost increases.

So far there is only one study in literature, reporting the production of MELs using CW, although, in such study is reported the pre-treated of CW with a commercial β-galactosidase (Dzięgielewska and Adamczak, 2013). Here, for the first time, it is observed the capacity of *Moesziomyces* spp. to produce β-galactosidase, and consequently, it is reported the production of MELs using only CW or using two industrial residues (CW and WFO), without using any pre-treatment, mineral medium nor YE. These results are very promising, since they show the capacity of CW to replace the whole medium, allowing to overcome one of the major bottlenecks in industrial MELs

production. In this regard, more studies are being conducted, especially using fed-batch fermentation in bioreactors to increase MELs titres.

B.2.5 Final remarks

Remarkably, it was observed the capacity of *M. antarcticus* 5048^T *and M. bullatus* 5535^T to produce β-galactosidase from D-lactose and D-galactose. The highest extracellular β-galactosidase activity was observed when D-galactose was used as sole carbon source, 60.3 and 11.2 $IU/mg_{biomass}$, using *M. antarcticus* 5048^T and *M. Bullatus* 5535^T, respectively.

Cheese whey, one the major residues produced in Europe, is rich in lactose. Considering the discover that the efficient MEL producers, *Moesziomyces* spp., are also able to produce β-galactosidase, CW was assessed as carbon source. CW was used as sole media component, directly used with no further addition of any other organic nor inorganic media component. Furthermore, when CW was used alone with supplementation of 20 g/L of WFO, a lipidic rich substrate to boost MELs production, productivities of 1.3 and 1.4 $g/L.day^{-1}$ for *M. Bullatus* 5535^T and *M. antarcticus* 5048^T, were obtained, respectively. These productivities values are only 13% - 26% lower than the ones previously obtained for an optimized medium using refined D-glucose, WFO, YE and inorganic sources of nitrogen and phosphate. Those are promising results on further bioprocess development due to the potential to perform cultivations using low-cost and renewable raw materials instead of complex media with refined components, solving one of the current bottlenecks for sustainable microbial surfactants production.

B.3 The use of olive pomace residues as carbon source towards MELs production

B.3.1 Outline

The EU has declared the bio-based products sector a priority area; thus, microbial surfactants (mannosylerythritol lipids, MELs) are interesting alternatives to fossil-driven surfactants. However to achieve this objective and fostering a bioeconomy, it is required the valorisation of different agro-industrial residues, promoting a circular economy.

In this regard, apart from cheese/milk industry, the olive oil industry is also one of the most important industries throughout south Europe. Nevertheless, this industry generates large amounts of residue (olive pomace or pomace oil), which are mainly used for low-value applications.

Here, the valorisation of olive oil residues for the production of MELs is explored, by *Moesziomyces* spp., towards sustainable bioeconomy concepts. The use of pomace oil, a non-edible oil, as alternative substrate to common oils (waste frying oils) led to an increase in productivity of 40% (from 1.98 ± 0.04 to 2.78 ± 0.09 g/L/day). The scale-up of the process resulted in a productivity of 7 g/L/day, with a purity of 89%.

These results are promising, since its one of the highest productivities obtained in MELs production, with MELs crude extract at high purity and ready to be used in different applications, without the step of purification which potentially leads to a decrease in overall costs of the process.

B.3.2 Introduction

Since 6000 thousand years ago, with the discovery of olive oil, this product has been a key ingredient of the Mediterranean diet owing to its excellent properties (e.g., anti-cancer, antioxidative, etc.). Therefore, this industry is a significant productive sector in European union (EU), mainly in Mediterranean basin ("History of Olive Oil," 2015), where it is estimated that EU is responsible for 67% of worldwide production and 53% of total consumption of olive oil (Europea Comision, 2021).

The olive oil extraction process operates continuously with 2 or 3-phase decanter systems (Gullón et al., 2020). Both systems generate a large waste volume with a high organic load and phytotoxicity due to the presence of fat, lipids, and polyphenols (Ochando-Pulido et al., 2018). While, the 3-phase system, uses small area, having a centrifugation step that generates 3 fractions: liquid olive oil, olive pomace residues (OP) and liquid oil mill wastewater (OMW, ca 80-120 L/100 kg olives); the two-phase uses less water, but yields a semi-solid residue (moisture, ca 70wt%) of higher complexity and pollutant load with high concentrations in solid, fat, lipids, carbohydrates, and polyphenols (Dermeche et al., 2013). Consequently, different studies have been conducted to reduce the environmental toxicity of these streams and recover high-value compounds, such as biodiesel (Yücel, 2011) or pharmaceutical compounds (e.g., cosmetics), by extracting polyphenols (Galanakis et al., 2018). However, most of these streams, especially OP residues, have been used for low-value applications, such as energy generation (e.g., thermal energy and electricity generation) (Berbel and Posadillo, 2018), mainly due to the absence of industrial bioprocess/methods capable of use these stream and produce high-value compounds (e.g polyphenol content variability and additional complexity posed to olive oil mill operation) (Roselló-Soto et al., 2015).

As described in previous sections, one of the bottlenecks of MELs bioprocess is the fermentation step, due to the low productivities (12 g/L/day) obtained compared with SLs (88 g/L/day), and the large amounts of medium components required to achieve this productivity (~186 g/L of SBO; 40 g/L of glucose and 14 g/L of YE) (Rau et al., 2005b). If in **section B.2**, the objective was to study the replacement of entire culture medium and hydrophilic carbon source

(glucose), by cheese-whey (industrial residue of milk industry), here, the objective will be to study the replacement of hydrophobic carbon source.

Therefore MELs bioprocess was developed by exploring the use of different residues from olive oil industry (**Figure B*14*),** with the objective of increasing productivity and sustainability of the MELs process, by adding value to one of the wastes more produced in the EU.

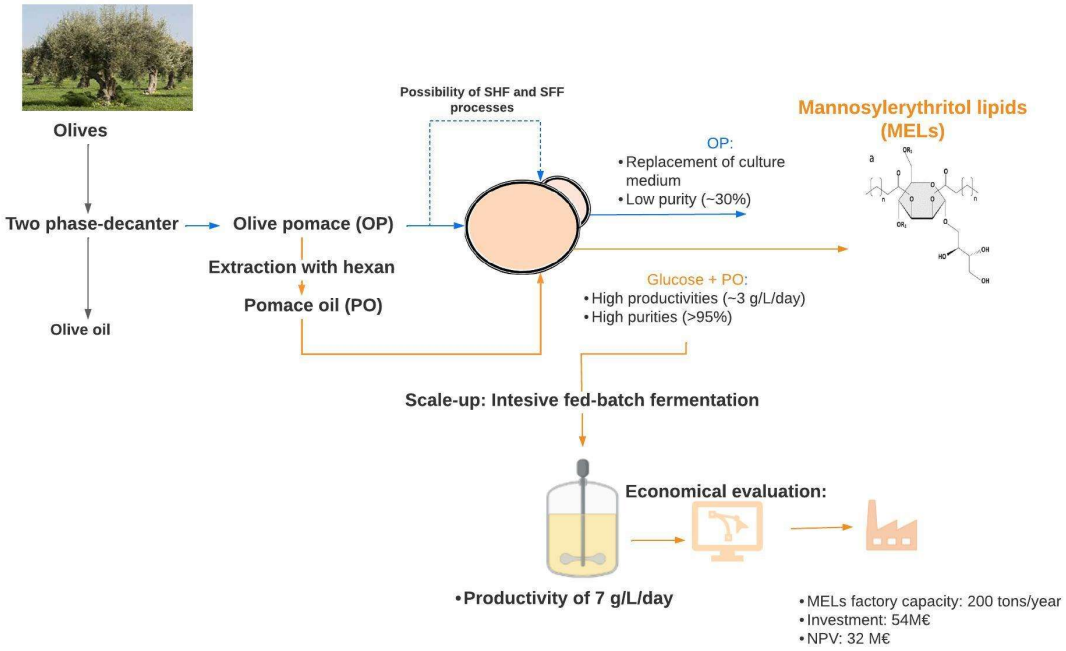


Figure B14: Scheme-representation of the work performed using different residues from olive oil industry for MELs production.

B.3.3 Materials and Methods

B.3.3.1 Yeast strains, substrate, and cultivation conditions

Moesziomyces antarcticus PYCC 5048^T and *M. bullatus* PYCC 5535^T were obtained from the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal. The strains were plated on YM Agar (yeast extract 3 g/L, malt extract, peptone 5 g/L, D-glucose 10 g/L and agar 20 g/L) and incubated for 3 days at 30 °C. Stock cultures were prepared by propagation of yeast cells in liquid media as described above for the inoculum and stored in 20% (v/v) glycerol aliquots, at -80 °C. An inoculum was prepared by transferring the stocks cultures of *M. antarcticus* and *M. bullatus* into an Erlenmeyer flask with 1/5 working volume (50 mL) of medium containing 0.3 g/L $MgSO_4$, 3 g/L of $NaNO_3$, 0.3 g/L KH_2PO_4, 1 g/L yeast extract (YE), 40 g/L D-glucose, and incubating at 27 °C, 250 rpm, for 48 h. Then, 10% (v/v) inoculum was added to an Erlenmeyer flask with 1/5 working volume (50 mL) of the culture medium. All cultures were performed in biological duplicates and incubated at 27ºC, 250 rpm for 10 days.

In the first set of assays, we tested the use of olive pomace (OP) residues 10% w/v) without any pretreatment, varying the presence of medium components: 1) Employing no culture medium, 2) exclusively using OP residues followed by the addition of 3 g/L of $NaNO_3$; 3) and using the entire culture medium components. Different percentages of pomace residues (5, 10, and 15%) were tested without the addition of any culture medium components.

In another set of experiments, the oil present in OP residues was extracted (see **section B.3.3.5**) and tested for MELs production, being compared with reference oils (olive oil (OO) and waste frying oils (WFO)). Two types of conditions were tested:1) starting with 40 g/L of hydrophobic source and 2) starting with 40 g/L of glucose and 20 g/L of hydrophobic source, with both conditions using the culture medium components described above. All experiments were performed in biological duplicates.

B.3.3.2 Bioreactors conditions and parameters

Moesziomyces antarcticus PYCC 5048^T and *M. bullatus* PYCC 5535^T were cultivated in a 2-L bioreactor (New Brunswick^{TM} Bioflo^R/CelliGen^R 115), using 1.5L of working volume, with the same medium as described in **section B.3.3.1**. Fermentation was started with 40 g/L of glucose and 20

g/L of PO, followed by three feeds of 20 g/L of PO on days 2, 4, and 6, and cultivation time was 10 days, where samples were taken every day for further analysis of cell dry weight, MELs, and residual lipid quantification. The temperature was set at 27 °C and the pH was not controlled throughout the days. The dissolved oxygen (DO) was set up at 15%, controlled with a cascade system, by varying the agitation and air flow rate (vvm), between 150-800 rpm and 0.5-1.5 vvm, respectively.

B.3.3.3 Growth and biomass determination

Depending on the type of substrate used, due to the presence of different residues, two different methods were used to follow yeast growth. In the presence of OP residues, growth was followed by using the method of colony forming units (CFU); for the rest of the experiments carried out with different types of oils, growth was determined by measuring the cell dry weight (CDW) periodically during fermentation. CDW was determined from 1 mL of culture broth by centrifugation at 10 000 x rpm for 6 min, followed by washing twice with deionized water (twice) and drying at 60 °C for 48 h.

B.3.3.4 Chemical characterization of olive pomace residues

Chemical characterization of olive pomace residues were performed in collaboration with an external laboratory (Laboratório Nacional de Energia e Geologia, LNEG). As deeply described by Fernandes et al., (Silva-fernandes et al., 2015) all materials were initially ground in a knife mil, and their moisture content was determined by ovendrying at 105ºC to constant weight. All samples were subjected to acid hydrolysis. Initially using 72% (w/w) of sulfuric acid for 60 min at 30ºC, and then diluting to a final value of 4% (w/w), allowing the hidrolysis to occur for 60 min at 121ºC. The samples were analysed for different components (see **Table B5**): Glucan; Xylan; Arabinan; Acetyl groups; Klason lignin and ashes. The methods used to quantify each components are described in Fernandes et al., (Silva-fernandes et al., 2015).

B.3.3.5 Pomace oil and crude MELs extraction

For extraction of pomace oil (PO) from olive pomace residues (OP), hexane was used at a ratio of 1:1 ($g_{olive\ pomace\ residues}/g_{hexane}$).. The organic phase was evaporated in a rotary evaporator

(Bucher) at 40ºC using 335 mbar to recover hexane and obtain pomace oil. This step was performed three times.

On the final day(s) of fermentation, beads enriched in MELs were observed (see **Figure F15**); therefore, the samples taken were considered as not representative of the titres of MELs and residual lipids.. Therefore, extraction was carried out by adding an equal volume of ethyl acetate (1:1 v/v) to the fermentation broth and mixing, allowing the creation of two phases. The organic phase was then retrieved and evaporated in a rotary evaporator at 40 °C using 240 mbar to recover ethyl acetate and obtain a crude fraction of MEL and residual lipids. This procedure was performed 3x times. A sample of crude MEL was weighted (~20-50 mg) following the procedure described in **Section B.3.3.6**.

B.3.3.6 Substrate and product quantification

The quantification of D-glucose was performed using HPLC. Culture broth samples were centrifuged at 10000 rpm for 6 min, and the supernatants were filtered through a 0.22 µm-pore size-filter and injected into an HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany) and a Rezex ROA Organic Acid H+ column (300 mm× 7.8 mm, Phenomenex, Torrance, CA, USA) at 65°C. Sulfuric acid (5 mM) was used as mobile phase at 0.5 ml/min.

The consumption of TAG in the culture samples was also analysed using HPLC, as described by Badenes et al. (Sara M. Badenes et al., 2010). 500 µL of each sample was retrieved and mixture with 1 µL of acetic acid 58.5 Mm and 499 µL n-hexane. Then, it was centrifuged at 10000 rpm for 2 min, and the organic phase was extracted and injected into the HPLC system equipped with a Chromolith Performance RP-18 endcapped (100 mm x 4.6 mm x 2µm) column, an auto sampler (Hitachi LaChrom Elite L-2200), a pump (Hitachi LaChrom Elite L-2130), and a UV detector (Hitachi LaChrom Elite L-2400) set up at 205 nm. The flow rate was set at 1 mL/min and the injection volume was 20 µL. Three mobile phases were employed: phase A consisted of 100% acetonitrile, phase B consisted of water 100% and phase C consisted of a mixture of n-hexane and 2-propanol (4:5, v/v). Quantification was performed using the calibration curves of glyceryl trioleate and glyceryl trilinoleate (>98 %, Sigma-Aldrich GmbH) for TAG.

MELs were quantified as previously described by GC analysis of methylesters generated by methanolysis of freeze-dried biological samples (1 mL) or in cases where beads enriched in MELs were observed and no representative sample could be retrieved, approximately 50 mg of the evaporated organic phase from the extraction of the total broth, using equal volumes of ethyl acetate to the broth volume (N. Faria et al., 2014).

B.3.3.7 Thin Layer Chromatography (TLC)

TLC was performed only for the qualitative evaluation of the type of MELs produced and the identification of β-carotene and chlorophyl. An aliquot (0.5-1.0 mg (dw) of each sample to be assessed was placed in a different lane of an aluminum TLC sheet pre-coated with a silica gel 60 layer (Macherey-Nagel Alugram Xtra SIL G/UV254). Two different mixtures of solvents were used: 1) a mixture of chloroform, methanol, and water in a ratio of 6.5, 1.5, and 0.2, was used for the separation of different MELs congeners (Morita et al., 2007a); 2) a mixture of hexane (70%) and acetone (30%) was used to identify chlorophyll and β-caratone, as previously reported by Bacher et al. (Bacher, 2016). After elution, a solution of α-naphthol in sulfuric acid, comprising naphthol (1.5 g), ethanol (51 mL of ethanol, water (4 mL), and sulfuric acid (6.5 mL of sulfuric, was sprayed on the TLC sheet, which was heated at 100 °C for 1 min, allowing visualization of the eluted compounds.

B.3.3.8 Statistical analysis

Statistical analyses were performed using GraphPad by analysis of variance (one- and two-way ANOVA), and p-values of the differences between groups were corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p < 0.05$

B.3.4 Results and Discussion

B.3.4.1 Assessment of olive pomace (OP) as carbon source and medium replacer

During the olive oil extraction, different types of residues are generated, including olive leaves, olive pomace (OP), and pomace oil (PO, after extraction from OP residues). These residues exhibit different potentials for use in MELs fermentation, therefore, in this study was considered to use two different residues as main carbon sources: 1) OP residues, without any pre-treatment; and 2) PO, after the extraction from OP residues, using hexane as the organic solvent.

The ability *of Moeszymioces* spp. to grow in OP residues (10% w/w) was assessed in *M. antarcticus* PYCC 5048^T and *M. bullatus* 5535^T. Initially, keeping the percentage of OP residues constant, the effect of culture medium components was evaluated by varying the presence of different culture medium components, such as: 1) Employing no culture medium components; 2) exclusively using an inorganic nitrogen source ($NaNO_3$); and 3) using the entire culture medium components.

Considering that OP residues have a variety of detritus (e.g., small stones), cell dry weight (CDW) was determined by measuring the colony forming units (CFUs). In this regard, it was possible to observe (**Figure B15** A, B) that both strains were able to grow on these residues, indicating that these residues are not toxic to yeast cells. Furthermore, considering the production of MELs, for both strains, the use of solo OP residues when compared to other conditions (exclusively using an inorganic nitrogen source or the whole culture medium), led to fairly high titres of MELs (4.71 ± 0.47 and 3.71 ± 0.00 g/L, for *M. bullatus* and *M. antarcticus,* respectively). Nevertheless, for *M. antarcticus*, the use of glucose led to higher titres than using solo OP residues (6.0 and 3.7 g/L of MELs, respectively), while for *M. bullatus*, it was the opposite (0.85 ± 0.00 g/L and 4.71 ± 0.47, respectively). This discrepancy on MELs production for these two strains was previously observed, while *M. antarcticus* seems to be a more efficient MELs producer from D-glucose and D-xylose, *M. bullatus* was reported to accumulate more storage lipids and be slower on their use for MELs production (N. T. Faria et al., 2014). This phenomenon was also observed in **section B.1**.

To discover the potential of using OP residues as a carbon source and as a medium replacer, the next experiment consisted at increasing the percentage of residues (5, 10%, and 15% w/v). While for *M. antarcticus* (**Figure B15** C) there are significant differences on final MELs titre between 5 and 15% of OP residues, for *M. bullatus* (**Figure B15** D**)** none of the conditions have shown significant differences. Interesting, the final concentration of residual lipids tends to increase and, when using 15% of OP residues (~36.56 g/L of residual lipids), only half of these lipids are consumed in both strains. These results can be explained by the high viscosity of OP residues (~ 180 cp), as observed by Battista et al. (Battista et al., 2016), which leads to a low gas transference of oxygen, resulting in low MELs titres and lipid consumption. These results are supported by the small experiment performed in **section B1** to prove the importance of oxygen in MELs bioprocess (**Table F1**)

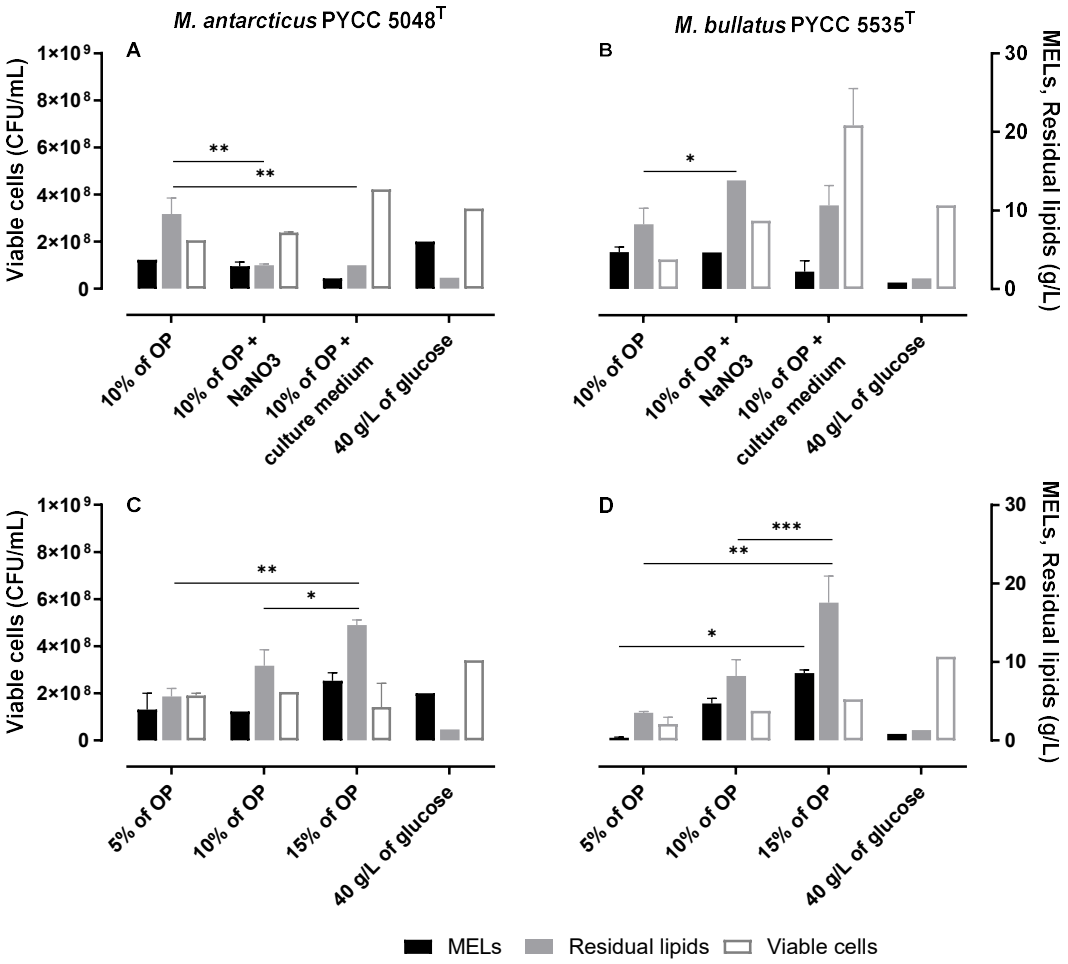


Figure B15: Values of MELs titre, viable cells and residual lipids at day 11 for *M. antarcticus* PYCC 5048^T (A, C) and *M. bullatus* PYCC 5535^T (B, D) 10% (w/v) of pomace residues directly, or in combination with only $NaNO_3$, or with all entire culture medium (A, B); Olive pomace residues (5, 10 and 15% w/v) without using any additional nutrients (C,D). * $p \leq 0.0032$; ** $p \leq 0.0021$; *** $p < 0.0002$. All conditions were performed in biological duplicates, with the exception of glucose fermentations (condition already accessed in different studies; see **section B.1 and B.2**).

Additionally, the composition of OP residues was analysed before and after fermentation for both strains (**Table B5**), to determine whether yeast could deconstruct the lignocellulosic structure. Analysing the results obtained, it is evident that there was sugar no consumption of the sugars available in lignocellulosic structure, and the observed increase in concentration is simply due to the evaporation of culture medium during the cultivation period. However it is known that these strains have the capacity to produce a wide range of enzymes (e.g lipases, xylanases), as shown by Faria et al. (Faria et al., 2019). The lack of sugar consumption can be explained by the high viscosity of OP residues in the culture medium (~ 180 cp, . (Battista et al., 2016)), which inhbits the action of enzymes.

Nevertheless, these results are promising and pave the way for research involving simultaneous saccharification and fermentation (SSF) and/or separated hydrolysis and fermentation (SHF). In fact, there are already STR equipped with additional equipment on the top of reactor, where solid residues can be added and crushed, feeding the system. This approach could be a potential future direction to consider, using OP residues (after oil extraction) as a replacement for glucose.

Table B5*:* Analysis of different components (Gn – Glucan; Xn – Xylan; Arn – Arabinan; Acet – Acetyl groups; LK – Klason lignin) present in olive pomace residues (10% of OP without addition of culture medium) for different samples: Before fermentation, after 11 days of fermentation for *M. antarcticus* PYCC 5048T and *M. bullatus* PYCC 5535T, and after extraction with hexane for retrieving the oil present in the residues.

Sample	Components analysed (g/100 g of dry weight)						
	Gn	Xn	Arn	Acet	LK	Ash	Others
Initial	7.5 ± 1.3	15.3 ± 0.1	1 ± 0.1	4.6 ± 0.2	56 ± 2	1.4 ± 0.1	14.3
After fermentation (*M. antarticus)*	10.4 ± 0.5	18.4 ± 1.1	1.6 ± 0.2	5.1 ± 0.1	49.9 ± 1.4	1.3 ± 0.1	13.4
After fermentation (*M. Bullatus)*	10.5 ± 0.4	12.7 ± 5	1.2 ± 0	2.7 ± 1.4	52.9 ± 6.2	1.1 ± 0.1	18.8
After extraction with hexane	10.4 ± 0.3	15.4 ± 3.6	1 ± 0.1	4.1 ± 1.1	53.8 ± 7.2	0.9 ± 0.2	14.4

B.3.4.2 Assessment of MELs production using pomace oil (PO)

While the use of OP residues allowed the production of MELs without the use of any culture medium components, the final purity was very low (~30%), increasing the complexity of separating MELs from residual oils and, consequently, the final costs of the process. In this regard, the olive oil present in these residues was extracted with hexane (section **B.3.3.5**) to obtain pomace oil (PO) without any detritus. Even though if it is known that hexane is not the most green solvent, it was decided to use it, since the objective is only to extract as much as possible PO. In future works will need to be carried out to replace the use of hexane by others organic solvents (i.e, methanol).

Three different types of oils were assessed: waste frying oil (WFO), PO, and olive oil (OO), under two different conditions:1) using only 40 g/L of each type of oil as main carbon source (**Figure F13** and **Table B5)**; and 2) a Combination of D-glucose (40 g/L) and 20 g/L of each type of oil (**Figure B16** and **Table B5)**, followed by culture medium components.

Whereas condition 1 follows the standard conditions used in the literature for MELs production (Kitamoto et al., 1998), condition 2 was chosen based on previous results obtained in **section B. 1,** the co-combination of glucose and WFO can boost the productivity of MELs and the consumption of residual lipids more than when just using solo WFO (see **Figure B7**). In fact, comparing the productivity under both conditions (**Table B5)**, regardless of the type of oil used, the productivity was always higher when using this co-substrate strategy.

As expected, the combination of D-glucose with WFO (similar to common vegetable oils), led to higher titres (21.8 ± 0.42 and 16.98 ± 0.39 g/L, for *M. antarcticus* and *M. bullatus*, respectively), when compared with PO (2-fold higher) and OO (1.3-fold higher). Interestingly, while using WFO **(Figure B16** C, D) or OO (**Figure B16** E, F**)**, the residual lipids are consumed throughout 10 days, however for pomace oil (**Figure B16** A, B), residual lipids are totally consumed in 2 days, leading to a maximum productivity obtained for MELs (2.78 ± 0.09 and 2.24 ± 0.17 g/L/h, for *M. antarcticus* and *M. bullatus*, respectively). The final lower titre (after 10 days) observed for conditions using PO, was due to substrate availability, where after day 4, for both strains, there was no substrate available (neither glucose or PO), leading to starvation of yeasts and consequently consumption of MELs (suggesting energy storage as possible biological MELs function). Furthermore, even for biomass production, when PO was used as the hydrophobic carbon source, the maximum was achieved on day 2 (~24 g/L), whereas for conditions using WFO or OO, the maximum was achieved around day 7.

To understand the fast consumption of PO in contrast to WFO and OO, the components present in every type of oil were analysed (**Figure F14**). Whereas WFO (like OO) is mainly composed of triacyclglycerols (~77%) and oleic acid (~19%), PO is composed of oleic acid (~45%) and glyceryl monooleate (~35%). As well described in **section A1**, the mechanism of action by *Moesziomyces* spp. to produce MELs from hydrophobic substrates, consists in the production of lipases that will cleave the structure of oils, forming glycerol and fatty acids (mainly monoglycerides and free fatty acids) that enter the cell. These molecules then undergo partial β-oxidation (known as the chain-shortening pathway) and are incorporated into MELs, as demonstrated by Kitamoto et al. (Kitamoto et al., 1998). Therefore, having a more degraded oil (higher percentage of fatty acids, such as PO) will increase the productivity, since the action of lipase is not required, and fatty acids can go directly for partial β-oxidation and then into MELs production. These assumptions were also observed by Petar Keković, where he observed that methyl esters and free-fatty acids are quicker converted and incorporated into MELs than using common vegetable oils (Keković, 2022), when used as main carbon source, leading to higher productivities.

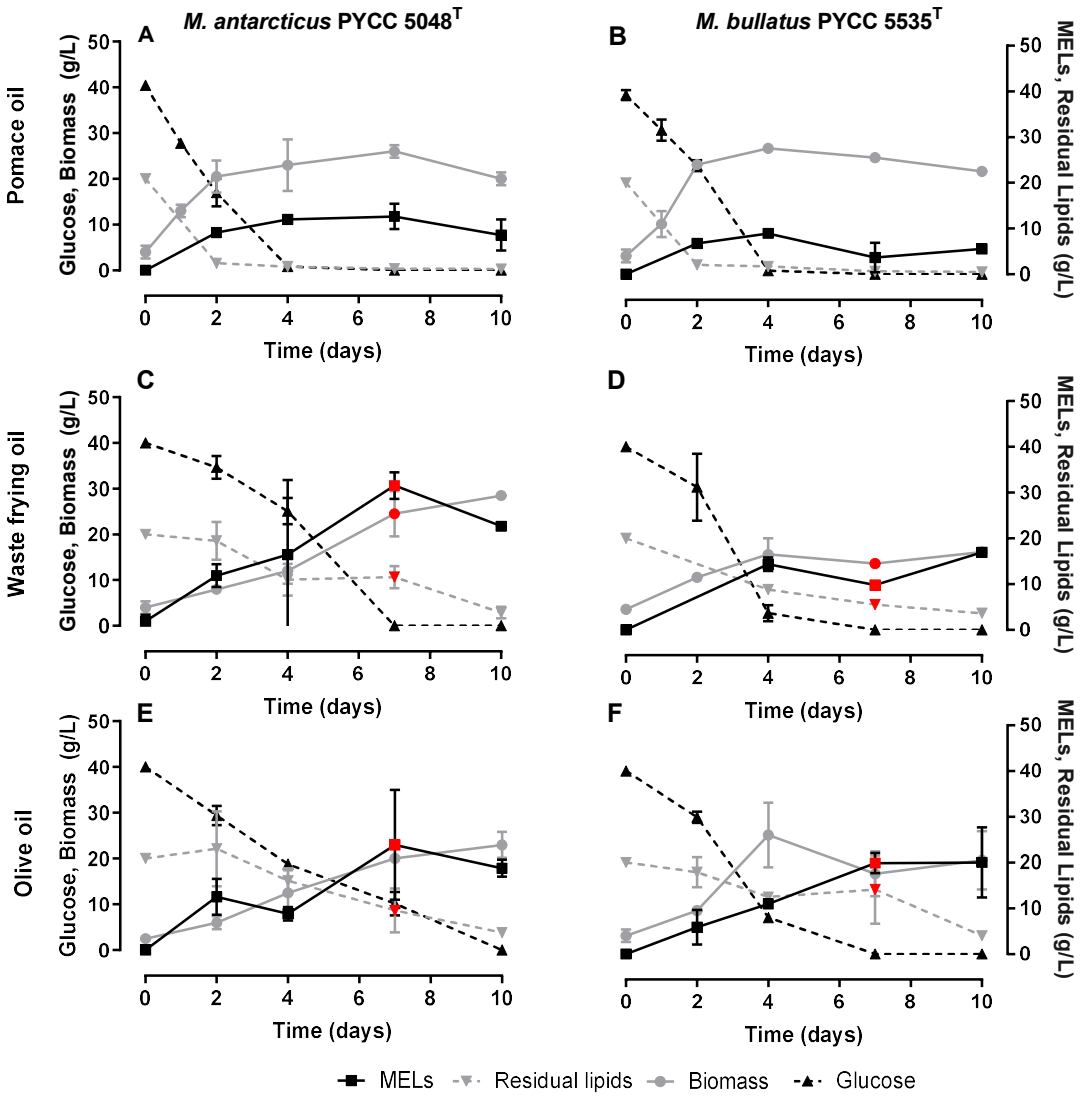


Figure B16: Cultivation of *M. antarcticus* PYCC 5048^T (A, C, E) and *M. bullatus* PYCC 5535^T (B, D, F) in 20 g/L of pomace oil (PO) (A, B), WFO (C, D) and processed olive oil (E, F), all in combination with 40 g/L of glucose, during 11 days at 27 ºC and 250 rpm in the presence mineral medium, YE and $NaNO_3$. The red point, at specific days, represent the appearance of beads enriched in MEL and residual lipids. Standard deviation lower than 1 g/L are not represented.

Table B6: Maximum values for MELs and residual lipids titre (g/L), MELs yield ($g_{MELs}/g_{Substrate}$), productivity (g/L/day) and purity (%) for *M. bullatus* PYCC 5535^T and *M. antarcticus* PYCC 5048^T using solo olive pomace (OP) residues with different concentrations (5%, 10% and 15%), 40 g/L of hydrophobic substrate (Pomace oil (PO); Waste fried oil (WFO) and Olive oil (OO)) and 40 g/L of glucose with 20g/L of hydrophobic substrate. For the conditions using OP residues, it was estimated the amount of pomace oil present (as described in **section B.3.3.2**).

Strain	Hydrophobic substrate	Strategy	OP used (g)	PO (g)	PO (g/L)	MEL $_{max}$ (g/L)	Residual lipids (g/L)	Yield $_{max}$ (g/g)	Productivity $_{max}$ (g/L/day)	Purity (%)
M. antarcticus	OP	5%	3.25	0.61	12.18	3.96 ± 1.46 (Day 10)	5.63 ± 0.71	0.32 ± 0.12	0.36 ± 0.13	39.89 ± 6.19
		10%	6.49	1.22	24.36	4.86 ± 1.15 (Day 10)	9.55 ± 1.43	0.14 ± 0.01	0.31 ± 0.03	26.12 ± 0.86
		15%	9.74	1.83	36.56	6.83 ± 0.06 (Day 10)	14.71 ± 0.46	0.21 ± 0.02	0.69 ± 0.07	34.00 ± 1.44
	PO	[40 PO:0]	-	2	40	16.81 ± 0.52 (Day 7)	0.43 ± 0.07	0.42 ± 0.01	2.4 ± 0.07	97.49 ± 0.47
		[40 glu: 20 PO]	-	1	20	11.12 ± 0.36 (Day 4)	0.82 ± 0.03	0.19 ± 0.01	2.78 ± 0.09	93.17 ± 0.01
	WFO	[40 WFO:0]	-	-	-	16.55 (Day 11)	1.55	0.41	1.50	91.44
		[40 glu: 20 WFO]	-	-	-	21.8 ± 0.42 (Day 11)	2.9 ± 0.9	0.36 ± 0.01	1.98 ± 0.04	88.42 ± 3.02
	Olive oil	[40 OO:0]	-	-	-	11.61 ± 0.45 (Day 11)	6.82 ± 2.11	0.29 ± 0.01	1.06 ± 0.04	63.89 ± 6.43
		[40 glu: 20 OO]	-	-	-	17.9 ± 1.31 (Day 11)	3.84 ± 0.11	0.3 ± 0.02	1.63 ± 0.12	82.29 ± 0.65

Strain	Hydrophobic substrate	Strategy	OP used (g)	PO (g)	PO (g/L)	MEL $_{max}$ (g/L)	Residual lipids (g/L)	Yield $_{max}$ (g/g)	Productivity $_{max}$ (g/L/day)	Purity (%)
M. bullatus	OP	5%	3.25	0.61	12.18	0.35 ± 0.05 (Day 10)	3.54 ± 0.1	0.03 ± 0	0.03 ± 0	9.03 ± 1.39
		10%	6.49	1.22	24.36	4.71 ± 0.47 (Day 10)	8.24 ± 1.45	0.19 ± 0.02	0.43 ± 0.04	36.64 ± 1.80
		15%	9.74	1.83	36.56	8.61 ± 0.28 (Day 10)	17.57 ± 2.4	0.24 ± 0.01	0.78 ± 0.03	33.12 ± 2.33
	PO	[40 PO:0]	-	2	40	13.09 ± 0.53 (Day 7)	0.71 ± 0.13	0.33 ± 0.01	1.87 ± 0.08	94.86 ± 1.06
		[40 glu: 20 PO]	-	1	20	8.96 ± 0.67 (Day 4)	1.73 ± 0.14	0.15 ± 0.01	2.24 ± 0.17	83.85 ± 0.05
	WFO	[40 WFO:0]	-	-	-	13.21 (Day 11)	11.27	0.33	1.20	53.96
		[40 glu: 20 WFO]	-	-	-	16.98 ± 0.39 (Day 11)	3.65 ± 0	0.28 ± 0.06	1.54 ± 0.04	82.32 ± 0.31
	Olive oil	[40 OO:0]	-	-	-	12.97 ± 1.81 (Day 11)	5.39 ± 1.39	0.22 ± 0.03	1.18 ± 0.16	71.08 ± 2.54
		[40 glu: 20 OO]	-	-	-	12.81 ± 1.81 (Day 11)	4.03 ± 0.58	0.21 ± 0.02	1.16 ± 0.16	76.10 ± 0.08

MELs $_{max}$ – maximum MELs produced (g/L); $Y_{MELs/Substrate}$ – maximum MELs yield (g/g); MELs purity (g/g) at the end of the fermentation- Ratio of g of MELs to the sum of g of MELs and residual lipids.

B.3.4.3 Development of a fed-batch cultivation in bioreactor, using pomace oil (PO)

As described in **section A1**, of the major bottlenecks in the MELs bioprocess is the fermentation process in bioreactors (less than 20 articles producing MELs in bioreactor, see **Table A4**) due to the complexity of the process, which is often associated with formation of foam. Hence, discovering the capacity of using PO to obtain high productivities, the next step involved the process development in bioreator.

Following the studied strategies on bioreactors and results obtained in **section B.1** (**Figure B8**), a 2-L bioreactor, in fed-batch fermentation mode, was performed for both strains (**Figure B17** and **Table B7)** starting with 40 g/L of glucose and 20 g/L of PO, followed by three feeds of 20 g/L of PO, that would be added in specific days based in two conditions: 1) No residual lipids in the culture medium; or 2) In the moment of foam appearance.

However, both strains exhibited different behaviours, and for *M. antarcticus*, the last feed was at day 6, and for *M. bullatus, it* was at day 7. This can be explained by the high capacity of *M. antarticus* to produce lipases over *M. bullatus* (see **Figure F*8***). Additionally *M. antarcticus* has a more efficient metabolism for MELs production, something transversal throughout this thesis, results in different final MELs titres (37.5 and 70.2 g/L for *M. Bullatus* and *M. antarcticus*, respectively) and purities (55.7 and 89.7 % for *M. Bullatus* and *M. antarcticus*, respectively).

Moreover, as previously discussed, the fast consumption of this oil also leads to high biomass productivity, and after 4 days, it is achieved at approximately 40 g/L of biomass for both strains. The continuous growth of biomass observed (**Figure B17** A, B**)** after day 4 was due to the presence of beads enriched in MELs and residual lipids (**Figure F15,** marked at red points the days that appeared), leading to a nonhomogeneous sample and avoiding the separation of these beads from the final pellet. Furthermore, when beads are present in the fermentation broth, the titre of MELs is not representative of the real value, explaining the rise in titre from days 9 to 10 (when beads disappear).

Remarkably, using *M. antarcticus,* a final productivity of 7 g/L/day was achieved, which was only 41% lower than the best productivity reported in the literature for the MELs bioprocess (12 g/L/h)

(Rau et al., 2005b). Note, that in this study, it was used large amounts of soybean oil (SBO) (approximately 186 g/L, 2-fold higher), D-glucose (approximately 50 g/L), 14 g/L of yeast extract (YE, 14-fold higher), and mineral medium (including sodium nitrate), which compromises the economy and sustainability of the process. Additionally, the productivity achieved here, is the highest obtained on this thesis, but also among the works reporting the use of industrial streams for MELs production. In this regard, these values were used to perform an economical evaluation of a MELs process, using PO as a hydrophobic carbon source (see **section C.2).**

Interestingly, the colour of the extracted MELs is green rather than orange, commonly reported in previous studies (**Figure F16**). This may be due to the presence of chlorophyll in the OP, which is also extracted with the pomace oil. This was proved when TLC was performed (**Figure F17**), where the band corresponding to MELs is in the same position as the band observed for OP. In parallel, a different TLC **(Figure F18)** was performed and optimized for the detection of chlorophyl and β-carotene, where it is possible to observe two intense bands for PO, in the same position for MELs, suggesting that MELs incorporate these pigments (also extracted with OP).

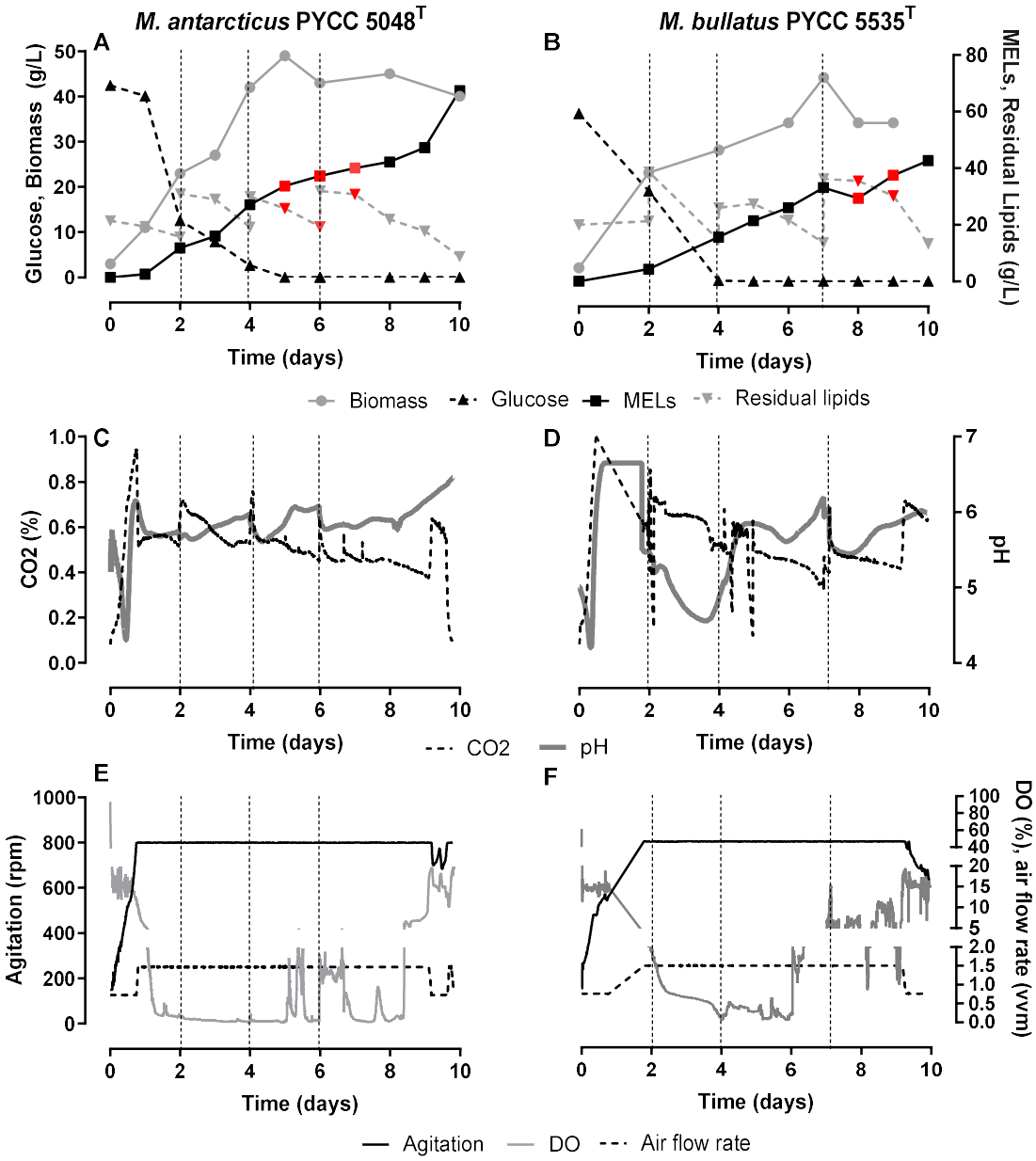


Figure B17: Cultivation of *M. antarcticus* PYCC 5048^T (A, C, E) and *M. bullatus* PYCC 5535^T (B, D, F) in bioreactor, starting with 40 g/L of glucose and 20 g/L of pomace oil in the beginning of fermentation, followed by three feeds of 20 g/L of pomace oil at day 2, 4 and 6. A, B) yeast biomass, glucose consumption, MEL production and residual lipids consumption; C, D) % of CO2 and pH through the days; E, F) Agitation, Dissolved oxygen (DO) and air flow rate (vvm). Each vertical line represents a feed of 20 g/L of pomace oil. The red point, at specific days, represent the appearance of beads enriched in MELs and residual lipids. Standard deviation lower than 1 g/L are not represented.

Table B7: MELs production in bioreactor using PO as main substrate for *M. bullatus* PYCC 5535^{T} and *M. antarcticus* PYCC 5048^{T}. Maximum biomass produced and MELs titre (g/L); yield of MEL produced (gMELs/gSubstrate); Purity (%), productivity (g/L/day).

Parameters	*M. Bullatus*	*M. antarcticus*
Biomass (g/L)	45.00	43.00
MELs (g/L)	37.54	70.20
Purity (%)	55.38	89.71
Total substrate added (g)	100.00	100.00
Yield (g/g)	0.38	0.58
Productivity (g/L/day)	3.75	7.02

Biomass - biomass cell dry weight (g/L); MEL – maximum MEL produced (g/L); $Y_{MEL/Substrate}$ – MEL yield (g/g); MEL purity (g/g) at the end of the fermentation- Ratio of g of MEL to the sum of g of MEL and residual lipids.

B.3.5 Final remarks

In order to react to actual climate change, one of the milestones is the creation of a circular economy by reducing waste and creating high-value products from leftovers of different industries. For the first time, the use of different olive oil residues (OP and PO) for the production of MELs was assessed.

Interestingly, it was found that OP residues can be used as carbon source and medium replacer, and the maximum titre is achieved using 15% of OP residues (8.61 ± 0.28 and 6.83 ± 0.06 for *M. Bullatus* and *M. antarcticus*, respectively), although, MELs purity are low (~ 30%), which will increase the difficulty of separating MELs from residual lipids. Hence, when extracting PO from OP residues, it is obtained high productivities (2.78 ± 0.09 and 2.24 ± 0.17 g/L/h, for *M. antarcticus* and *M. bullatus*, respectively) with high purities (< 95%). The scale-up of the process (2-L bioreactor) and the use of a fed-batch fermentation, allowed to achieve one the highest productivities reported here on this thesis but also in literature for MELs (7.1 g/L/day when using *M. antarcticus*). Future work is being carried out to replace glucose with lignocellulosic residues left after oil extraction, testing SHF and SSF processes.

Section C: MELs bioprocess integration

C.1 Development of a nanofiltration technology for MELs purification

Author contribution:

This section was developed in co-operation with Dr Petar Keković, and the part of the experimental work related to methanol, TAG extraction and activated carbon decolouration was performed by him.

C.1.1 Outline

Glycolipid biosurfactants are the most prominent group of microbial biosurfactants, comprising rhamnolipids (RMs), sophorolipids (SLs) and mannosylerythritol lipids (MELs). Usually large amounts of hydrophobic substrates (e.g. vegetable oils) are used to achieve high titres (~ 200 g/L) of a crude product with low purity at values limited of 50-60 %, contaminated with unconsumed triacylglycerol, residual free fatty acids (FFA) and monoacylglycerides (MAG). The methods reported for the removal of these contaminants use a mixture of organic solvents, compromising solvent recyclability and increasing final process costs. This study reports, for the first time, an innovative downstream method for MELs, in which 90% of the triacylglycerols are separated from the crude MEL mixture in a first stage, using methanol, and the other lipid derivatives (free fatty acids, mono- and diacylglycerols) are removed by organic solvent nanofiltration (OSN).Three commercially available membranes (GMT-oNF-2, PuraMEm-600 and DuramMem-500) and several home-made membranes, casted from 22, 24 or 26% (w/v) polybenzimidazole (PBI) solutions, were assessed for crude MELs purification by diafiltration. A final purity of 87-90% in MELs was obtained filtering 2 diavolumes (DV) of methanol or ethyl acetate solutions through a PBI 26% membrane, resulting in MELs losses of 14.7 ± 6.1 % and 15.3 ± 2.2 %, respectively. Higher biosurfactant purities can be archived using the PBI 26% membrane at higher DV, but at costs of higher product losses. Namely, in MeOH the use of 6 DV leads to losses of 32% for MELs and 18% for SLs. To obtain MELs at reagent grade, with purities equal or higher than 97%, a two sequential cascade filtrations approach was implemented using the commercial membrane, GMT-oNF. In such process, MELs with 98% purity was obtained at the cost of 11.6% MELs losses. Finally, decolouration, important in some applications, was successfully assessed using activated carbon. Overall, this study reports a unique solution for microbial biosurfactants production, with minimal products losses, enabling solvent recycling and potentially reducing costs of the process.

C.1.2 Introduction

Microbial biosurfactants, as previously described in **section A.1**, have a wide range of advantages over chemical surfactants, especially the higher biodegradability and less toxicity to humans and environment, and as show in **section B,** they can be produced from renewable sources. However, the market of microbial biosurfactant (USD 400 million) it's still not competitive with surfactants market (USD 45 billions), indicating that scalable fermentations and downstream processes are sub-optimized, and more studies are required. In fact, high productivity has been achieved for SLs (57.6 g/L/day) (Wang et al., 2020), but so far, most of the studies that achieved high purities (<97%) for MELs and SLs used a high volume of mixtures of solvents, not allowing solvent recyclability. Indeed, inefficient and costly downstream processing strategies are, according with some authors, the main driver of production costs for emerging bioproducts (Campos et al., 2013). Specifically for RMs, a recent review claims that up to 80% of total production cost is allocated to downstream processing (Sekhon Randhawa and Rahman, 2014).

These type of glycolipids biosurfactants can be produced from a variety of substrates, including carbohydrate-based materials, lipids, as well as other compounds, such as hydrocarbons and glycerol. (Marchant and Banat, 2012). While the highest productivity in RMs production is achieved using carbohydrate-based substrates that can be combined with hydrophobic substrates (Camilios-Neto et al., 2010), MELs and SLs production uses substantial amounts of hydrophobic substrates (Rau et al., 2005b; Shah et al., 2007), leading to a low purity of the final product (~30-40%), contaminated with TAGs or lipid derivatives not consumed during the fermentation process. Separating those hydrophobic contaminants from the produced MELs and SLs is challenging, due to the formation of stable emulsions and other supramolecular structures (e.g biosurfactant/water/oil systems) (Worakitkanchanakul et al., 2009). Therefore, the typical crude biosurfactant obtained is product, that for the most of the envisaged uses, has a low quality and applicability (Van Bogaert et al., 2007).

The processes reported for harvesting the biosurfactant fraction from the culture broth are based on an organic solvent extraction, using tert-butyl methyl ether (MTBE), ethyl acetate (EtOAc), and other solvents; or sedimentation/decantation, often coupled with heating/boiling (Worakitkanchanakul et al., 2009). Example of chromatography column based processes for

MELs purifications are able to achieve high product purities (> 97%), however the amount of MELs recovered was limited to only 4 % (Kim et al., 1999) or 50 % (Morita et al., 2007a), using as eluents chloroform mixtures with methanol (MeOH) or acetone, respectively. Both of this studies report the use and disposal of large volumes of solvents, (Smyth et al., 2010) particularly toxic and carcinogenic and of difficult recyclability, namely due to the formation of stable azeotropes (Shephard et al., 2016). An example of an organic solvent extraction method for MELs extractions is reported in the study by Rau et al. (Rau et al., 2005a), which uses sequential liquid-liquid extraction employing 1:2 of n-hexane, 1.6:1 of MeOH, 3:1 of MTBE and 3:1 of cyclohexane (v/v of fermentation broth), but able to recover 8% of the MELs produced. The MTBE used in the initial extraction has the potential to be recycled by distillation, but the other solvents would require challenging separation and should be considered waste. In another study, Shen et al. (Shen et al., 2019) achieved 80% of MELs recovery employing 2.5:1 of MeOH and 3:1 n-hexane (v/v of fermentation broth) using solvent shifts (i.e. avoiding the need of solvents to be completed evaporated prior to addition of new ones). Still, again solvents recyclability is hampered by formation of complex mixtures and stable azeotropes.

The innovative proposed process in this section, involves the use of organic solvent nanofiltration (OSN), with the goal of removing lipid derivatives (FFA, MAG) smaller than MELs, RMs and SLs molecules. The separation by OSN of molecules of similar molar mass is challenging (TAGs: 870-930 Da; SL ~ 706 Da; RL ~ 650 Da; MELs ~ 676 Da). Therefore, prior to the OSN stage, the larger TAGs are removed by solvent selective dissolution, based on the solubility differences between MELs and TAGs in a selected solvent. Finally, AC was used to remove colour impurities generated during the fermentation from the MELs, resulting in a pure product with light coloration. The downstream process is presented schematically in **Figure C1**. The downstream processing proposed in this work provides a unique solution for the purification of microbial biosurfactants produced from hydrocarbon and lipid-based substrates, with emphasis on avoid of use of solvent mixtures, allowing solvent recovery and reuse.

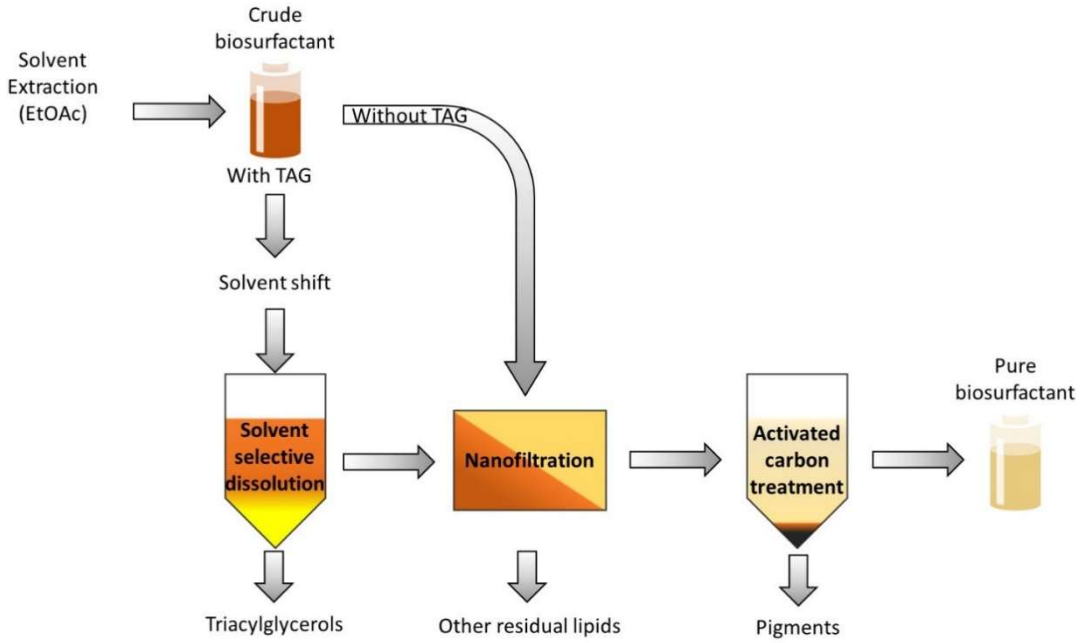


Figure C1: Schematic representation of proposed downstream process for glycolipid biosurfactants produced from hydrocarbon- and lipid-based substrates.

C.1.3 Material and Methods

C.1.3.1 Microbial biosurfactants

MELs used in this study were produced by *Moesziomyces antarcticus* PYCC 5048^{T} (CBS 5955) and *M. bullatus* PYCC 5535^{T}, provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal, following previously established fermentation medium and conditions (Rau et al., 2005b). Yeasts were cultivated in a 2-L bioreactor (New Brunswick^{TM} Bioflo^{R}/CelliGen^{R} 115), using 1.5 L of working volume, with a medium composition described elsewhere in **section B.** The temperature was set up at 27 ºC and the pH was not controlled. The dissolved oxygen (DO) was set up for 15%, varying the agitation and the air-flow rate in the ranges from 150-800 rpm and 0.5-1.5 vvm, respectively. After 12 days of cultivation, the broth was extracted three times with EtOAc in equal volumes to the fermentation broth and the solvent was evaporated and recovered using a rotavapor. The composition of crude MELs vary widely with the feed strategy used. Typical examples are described on **Table C1.** Here we consider three case studies that include: (A) the standard co-substrate feeding strategy optimized to render a TAG free product, as described in **section B 1.1** (Faria et al., 2023; Nascimento, 2017); (B) a non-optimized co-substrate feeding strategy (Keković, 2022), yielding crude MEL with high content on FFA and MG, but negligible TAG; and (C) the feeding strategy based on one single addition of a vegetable oil at day zero, yielding a crude MEL with TAG (Marek Adamczak and Bednarski, 2000).

Note that such compositions are presented for indicative purposes as the exact values vary from batch to batch. Therefore, when a crude MELs of different compositions are used, the initial MELs purity, before separation step, are indicated in the respective data set. SLs were provided by Holiferm, UK and also used in separation strategies development.

Table C1: Composition of crude MELs obtained in the end of fermentation, based on the feed strategy employed.; Glu – Glucose; SBO – Soybean oil; FFA- Free fatty acids; MAG – Monoacylglycerides; DAG – Diacylglycerides; TAG – Triacylglycerols

Feed strategy	Time (days)	MELs (%)	FFA (%)	MAG (%)	DAG (%)	TAG (%)
A) 40 g/L Glu (day 0) + 20 g/L SBO (day 0, 4)	9	84.6	6.9	8.3	-	-
B) 40 g/L Glu (day 0) + 20 g/L SBO (day 4, 7)	12	62.0	11.0	23.0	2.0	-
C) 80 g/L SBO (day 0)	6	56.3	3.3	6.4	0.2	33.8

C.1.3.2 Lipid hydrolysis as MELs extracts contaminants

Commercial immobilized CAL-B (Novozym® 435, Novozymes, Denmark) enzymes, diluted in MilliQ water, were used to the hydrolysis of lipids and its derivatives. This mixture was kept at 50 °C, mixed with a magnetic stirrer at 400 rpm up to 24 h, when the organic phase was collected. This mixture of lipids and lipid derivatives was characterised, and mixed with biosurfactants in different ratios, to simulate the extracts collected from fermentations with residual lipid impurities.

C.1.3.3 Analysis of FFA mono-, di- and triacylglycerides concentration

Fatty acids, mono-, di- and triacylglycerides contents were was determined by high-performance liquid chromatography (HPLC), following a method developed by Badenes et al (Sara M Badenes et al., 2010). Samples of supernatant (200 µL) were mixed with 1 µL of acetic acid 58.5 mM and 799 µL of n-hexane and centrifuged at 10000 rpm for 2 minutes. The organic phase was recovered and used for HPLC analysis using a Chromolith Performance RP-18 endcapped (100 mm x 4.6 mm x 2 µm) column with a UV detector at 205 nm. The injection volume was 20 µL. Three mobile

phases, at 1 mL/min, were employed: phase A = acetonitrile 100%, phase B = water 100% and phase C = n-hexane/2-propanol (4:5, v/v).

C.1.3.4 Analysis of MELs, SLs and residual lipids concentrations

MELs and residual lipids from the feed, permeate and retentate solutions were quantified after solvent evaporation, following the methods previously described, through GC analysis of methyl esters generated by methanolysis of the solutes (N. Faria et al., 2014). SLs were measured by MS-HPLC as described elsewhere (Ribeiro et al., 2012).

C.1.3.5 Thin Layer Chromatography (TLC)

Solvents, such as isopropanol (IPA), chloroform, tert-butyl methyl ether (MTBE), methanol (MeOH), ethyl acetate (EtOAc), dichloromethane (DCM), n-hexane, water, ethanol (EtOH) and acetone, in a closed TLC development chamber. The standard solvent mixture used for separation of different MELs homologues includes the use of a solvent system of chloroform/MeOH/water (6.5:1.5:0.2) as eluent. Precoated aluminium TLC sheets with a silica gel 60 coating were used (Macherey-Nagel Alugram Xtra SIL G/UV_{254}) with 0.5-1.0 mg (dw) of the analysed sample being placed for each lane on the TLC. To visualization of the compounds, a developing solution of α-naphthol in sulfuric acid (1.5 g of naphthol, 51 mL of ethanol, 4 mL of water and 6.5 mL of sulfuric acid) was sprayed and the plate was heated over 100 °C for 1 minute. Further assessment of separation of MELs from TAGs, based on the difference of their solubility in a selected solvent, is described on the results and discussion section.

C.1.3.6 Membrane preparation

A home-made polybenzimidazole (PBI) organic solvent membrane (OSN) was manufactured by phase inversion technique. Celazole® S26 solution (26 wt% PBI, 1.5 wt% LiCl in N,N-dimethylacetamide (DMAc), PBI Performance Products Inc., USA) was diluted with DMAc (Panreac, Spain) to 22, 24 and 26 wt% PBI concentration. The solution was mechanically stirred at 60 rpm overnight to obtain a homogeneous dope solution, with additional 24 hours unstirred for the removal of air bubbles. The resulting solution was first manually casted using a home-made casting knife, height of 250 μm, on the top of a non-woven Polyolefin Novatexx 2471 (Freudenberg Filtration Technologies, Germany), selected to provide additional mechanic support

of the PBI membranes, without significant addition of mass transfer resistance. The film obtained was then immersed in a distilled water precipitation bath (1 hour, three times), and then in an IPA (Carlo Erba, Spain) bath (1 hour, three times) for water removal. The obtained membranes were kept on IPA until further use. All the processes were performed at room temperature and 40% humidity.

The crosslinking of the above described OSN membranes was also considered following a post-manufacture treatment of the membrane. A crosslinking solution was prepared by mixing of 3% of α,α,-Dibromo-p-xylene (Sigma Aldrich) with acetonitrile. The membranes prepared and stored in IPA were immersed and agitated in the crosslinking solution, for 24 h at 80 °C under reflux. After 24 h, the reaction was stopped and the membrane was washed three times with IPA, to remove residues of crosslinker solution and solvent. All membranes were storage in IPA. The cross-linked PBI membranes are labelled as PBI-X.

C.1.3.7 Microbial biosurfactants purification by OSN

The commercially available OSN membranes assessed in this work were (i) the GMT-oNF-2 (Borsing GmbH), DuraMem-500 (Evonik) and PuraMem© 600 (Evonik), with molecular cut-off (MWCO) of about 600, 500 and 600 Da, respectively; and (ii) the home-made PBI and PBI-X OSN membranes, made as previously described.

The filtrations were performed on a dead-end Sterlitech HP 4750 Stirred Cell, fitted with a circular OSN membrane with an area of 14.6 cm^2. Duplicates were performed using different membrane samples, which were preconditioned by filtering pure solvent (~400 mL), until a constant solvent flux was obtained. A pressure of 15 bar was applied using pressurized nitrogen gas and all experiments were performed under magnetic stirring of 300 rpm at room temperature. Concentrations of MELs and residual lipids on OSN studies were assessed by GC as previously described. The experimental strategy followed uses crude MELs, with typical compositions based on the one described in **Table C1**. Crude MELs were dissolved in either EtOAc or MeOH at 50 g/L, and 50 mL of the solutions obtained were used as feed solution for MELs rejection estimation and MELs purification by diafiltration.

The solvent flux (ϕ) was estimated by with **Equation *3***, on the basis of membrane area (Am), filtration time (t) and permeate volume (V_P). The membrane rejection (R) was estimated by

Equation *4*, considering solute concentrations in the feed (Cf) and permeate (Cp), after permeation of 50% of the feed volume in concentration mode.

$$\phi = \frac{V_P}{A_m \times \mathrm{t}}$$

Equation 3 : Solvent flux determination.

$$\mathrm{R} = 1 - \frac{C_P}{C_F}$$

Equation 4 : Membrane rejection determination.

MELs purifications were assessed in diafiltration mode, allowing to retain the product, as smaller lipidic molecules where pushed through the OSN membrane. A HPLC pump Series I, Scientific Systems Inc. was used to add fresh solvent (EtOAC or MeOH) as required to keep the retentate nanofiltration cell volume constant at a value of 50 mL, compensating for the volume leaving the system through the permeate. Diavolume (DV) is defined as the volume of fresh solvent added by the retentate constant volume (i.e. the initial volume of crude MELs solution submitted to OSN). Samples were collected to measure the solute concentrations on the retentate (C_R) and permate (C_P), after addition of 2, 4 and 6 DV of solvent, i.e. after permeation of 100, 200 and 300 mL of permeate. MEL losses or contaminants removal can be calculated by **Equation *5***, according with membrane rejection to the solute and DV used.

$$\frac{C_R}{C_F} = e^{-DV(1-R)}$$

Equation 5 : Diavolumes determination, based in the concentration of retentate, feed and the rejection coefficient.

C.1.4 Results and discussion

On this section a novel multi-step downstream process it is proposed here for the purification of glycolipid biosurfactants produced from lipid-based substrates, including 3 stages: 1st selective a first stage combining TAG selective removal from glycolipids; 2nd using OSN for molecular separation of the glycolipid from smaller lipidic impurities and 3rd a stage of product decolouration using AC. This final stage was selected to not be shown here, and more information can be found in PhD thesis by Petar KeKović (Keković, 2022) and in the article, already published by the same authors (Nascimento et al., 2023).

OSN is here assessed with the aim to remove free fatty acids and monoacylglycerols of smaller molecule size. Those are predominant contaminants on longer fermentations, or the ones optimized to avoid overfeeding of vegetable oils used as substrates (e.g. **Table C1**, strategy A), which have a low content on TGA. For such case studies, downstream route can employ ethyl acetate (EtOAc) as the same solvent for crude MELs extraction from the fermentation broth and in the OSN.

Noteworthy, TAGs have similar or larger molecular weights than glycolipids biosurfactants. Therefore, for fermentations with large contaminations on TAG (e.g. **Table C1**, strategy B), a stage prior to OSN to remove these large molecules are advisable. Such separation has been attempted by gravimetry for SLs (Dolman et al., 2019), but such process is not efficient for MELs. In the current study, TAG removal from MELs by solvent selective dissolution was studied. Again, ideally, it was also attempted the use of same solvent on TAG removal and in OSN stages.

C.1.4.1 Selection of solvent for MELs separation from TAGs

Solvent interaction with MELs and lipids

The first study assessed the interaction of organic solvents with MELs and TAGs aiming at their efficient separation by thin-layer chromatography (TLC). Soybean oil (SBO) is mainly comprised by TAGs and thus the TLC reference values (Rf) for SBO and with ~85% purity (**Table C1**, feeding strategy A, without TAG present) in different solvents were measured. The results for the tested eluents are represented in **Table C2**. TLCs can be found in supplementary data of the article (Nascimento et al., 2023).

The higher the differences between Rfs values (ΔRf), the higher the differences in solvents affinity to MELs and TAGs and so, higher potential of using such solvent for the separation of these compounds. Dichloromethane (DCM) and chloroform present the higher ΔRf, but given their poor environmental performance, the extracting solvent was selected from the best performing non-halogenated ones, i.e. ethanol (EtOH), methanol (MeOH) and tert-butyl methyl ether (MTBE). Ideally, the selected solvent should be possible to be produced from renewable resources in a sustainable manner, and importantly to be able to separate MELs and TAGs by dissolving and eluting one of the compounds at high concentrations, while not solvating the other, promoting its precipitation.

Table C2: Rf values of MELs and SBO for different organic solvents. Solvents with ΔRf higher than 0.50, indicating a superior separation between MELs and SBO, are highlighted in green.

Eluent	Rf SBO	Rf MELs	ΔRf
IPA	0.79	0.58	0.21
Chloroform	0.80	0.00	0.80
MTBE	1.00	0.39	0.61
MeOH	1.00	0.60	0.60
EtOAc	0.94	0.67	0.27
DCM	0.89	0.00	0.89
Hexane	0.00	0.00	0.00
Water	0.00	0.00	0.00
EtOH	0.00	0.73	0.73
Acetone	0.85	0.56	0.29

The role of methanol in MELs separation from TAGs

MeOH was selected as the best candidate for separation of TAG and MELs, in spite of other solvents have presented higher ΔRf, considering chloroform or DCM have been exclude due to their toxicity, chlorohydrocarbons nature and volatility. MTBE and EtOH were not efficient on discriminating between TAG and MELs on their elution (Alder et al., 2016; McGarity, 2004). On the other hand, MeOH dissolves MELs and free fatty acids completely (data not shown) while almost no TAG can be dissolved in it, enabling the separation. Indeed, the solubility of SBO in MeOH was negligible (< 1 g/L), however separation efficiency drops with higher concentrations of MELs, as the biosurfactant and the oil probably form macromolecular structures, stabilizing the oil in the methanol solution.

The removal of TAGs from MELs, exploring the different compound solubility in MeOH, was then studied. Crude MELs (feeding strategy B) and SBO, accounting to a total mass of 8.3 g, were vigorously mixed in 20 mL of MeOH. This mixture represents a case study where MELs are highly contaminated with TAGs, indeed the solute mass fractions of MEL and TAG were similar (~39% each), and the remaining solute fraction 22% accounts for ~ 7% free fatty acids (FFA), ~14% monacylglycerides (MAG), and ~1% diacylglycerides (DAG). The mixture was then submitted to a mild centrifugation (2 min at 4000 rpm) to speed up phase separation. The top solution, enriched in MELs, was separated, and kept as purified fraction, while TAGs were predominantly left in the bottom layer, a precipitate (**Table C*3***). Additional washings can be performed to increase triacylglycerol removal, with additional MELs losses.

Importantly, with a single MeOH washing step, is it possible to remove almost 94% of TAGs from the crude MELs, with a product loss of 12.3% (**Table C*3***). However, such separation its yet not optimized, and higher MELs purities can be achieved using higher solute concentrations, using lower volumes of MeOH. On such conditions, higher amounts of TAGs will precipitate, but eventually some additionally MELs will be lost to the bottom phase.

In this regard, MELs losses can be mitigated by using higher volumes of solvent or applying consecutive steps of MeOH washing for further retrieving the lost MELs from the bottom layer. Such strategy was experimentally applied by redissolved, in MeOH, the bottom layer obtained in

the previous step. The phases were separated as previously described. This was repeated two times. The values of the cumulative losses in MELs and removal contaminants were determined using **Equation *6*** and **Equation *7***, respectively.

Table C3: Cumulative MELs and contaminant losses and composition of combined top phases for consecutive step of MEL purification with MeOH by solvent selective dissolution).

Components		Initial sample	After 1st step	After 2nd step	After 3rd step
MELs and contaminants losses	**MELs Losses (%)**	-	12.3	5.9	4.1
	TAG removed (%)	-	94.7	91.2	90.6
Composition	MELs (%)	39.0	66.0	63.9	62.8
	TAG (%)	39.0	4.0	6.0	6.1
	FFA + MAG+DAG (%)	22.0	30.0	30.1	31.0

$$Cumulative\ MELs\ Losses\ (\%) = \left(1 - \frac{\sum_{i=0}^{n} MEL(g)_{Top\ phase,i}}{MEL(g)_{initial\ sample}}\right) \times 100$$

Equation 6 : Cumulative MELs losses (%).

$$Cumulative\ contaminant\ removal\ (\%) = \frac{\sum_{i=0}^{n} TAG(g)_{Top\ phase,i}}{TAG(g)_{initial\ sample}} \times 100$$

Equation 7 : Cumulative contaminant removal (%).

The results obtained point out that it is possible to reduce MELs losses to values of 3.55%, maintaining its purity at values above 90%. Importantly, FFA and MAG are preferentially dissolved together with MELs on the top MeOH solution. In the case of the experiment here performed, after removal of the TAGs, 30% of the total weight of the product are comprised of FFA and MAG (**Table C*3***). Therefore, there is the need to design a second separation stage to remove these contaminants.

C.1.4.2 Nanofiltration as new downstream route

The technological challenges for organic solvent nanofiltration (OSN) and aqueous stream nanofiltrations are slightly different. For aqueous systems one of the main issues is the fouling at

the membrane surface, due to the combined action of ions (e.g. Ca^{2+}) and of organic matter (e.g. driven from cells debris, proteins, etc that stay on aqueous phase). On the other hand, for OSN, the use of membranes stable on the solvents used, i.e. without swelling and able to maintain their performance, is of utmost importance. In the last two decade, the development of OSN membranes have been extensively evolved and they had been assessed for separation and purification of many compounds (Marchetti et al., 2014). Within the research group where I have developed my PhD studies, a special focus has been given to the separation and purification of API (Active pharmaceutical ingredients), especially using PBI membranes (F. A. Ferreira et al., 2019). Therefore, in the current study, it was decided to compare the efficiency of commercially available OSN membranes against home-made PBI membranes.

In this study, the OSN membrane is used to retain MELs (MW ranging from 580-650 Da), while smaller residual lipids, e.g. FA and MAG (280-350 Da) are pushed into the permeate. The diafiltration should be carried out in a solvent which, to facilitate its recycling, should be the same that is used on the previous step of the downstream process. Such solvents are either (i) MeOH, used for removal of TAGs (e.g. **Table C1**, feeding strategy A) or (ii) EtOAc used for MELs extraction from the fermentation broth (e.g. **Table C1**, feeding strategy B). This latter case is particularly relevant for fermentations optimized for complete TAG metabolization with residual smaller lipid derivatives (e.g FFA and MAG) left unconsumed.

In resume, the OSN membranes selected should be compatible with MeOH or EtOAc and have MWCO high enough to retain MELs. Therefore, considering the existing knowledge on OSN membranes, the membranes selected for this study were the three commercially available membranes, GMT-oNF-2 (based on polydimethylsiloxane, MWCO 600 Da), PuraMem 600 (polyimide, MWCO 600 Da) and the DuraMem-500 (crosslinked polyimide, MWCO of 500 Da) and home-made PBI membranes casted from solutions with 22, 24 and 26% PBI solutions, as prepared or cross linked. These membranes have been previously characterized by Razali et al. (Razali et al., 2017) for their permeability in different organic solvents, such as EtOAc, MeOH, toluene, and rejection of specific compounds with different molecular sizes.

In the current study, the membranes were assessed by estimating solvent fluxes, for EtOAc and MeOH, and determining membrane rejections for MELs and residual lipids. Those values were

then imputed for theoretical estimations of MELs losses and diavolumes needed to obtain MEL at a reagent grade of 97% (food grade), starting from a crude MEL with a purity of 85%. Finally, the more promising OSN membranes were experimentally assayed for crude MELs purification, removing the smaller lipidic derivatives by diafiltration in MeOH or EtOAc. A sequential cascade OSN system was also assessed, aiming at higher MELs purities, with the permeate of the first diafiltration fed to a second diafiltration to improve overall MEL recover yields.

Membrane screening

The solvent fluxes through the membranes were estimated by equation 2 (**Figure C2** and **Table F8**). In case of commercial membranes, it was interesting to observe that solvent fluxes of MeOH (5.1 of polarity index) and EtOAc (4.1 of polarity index) were negligible (lower than 0.3 $L.m^{-2}.h^{-1}$) for the GMT-oNF-2 (based on polydimethylsiloxane) and the DuraMem-500 (cross-linked polyimide) membranes, respectively. This is explained by the interaction of the solvent with the membrane, as discussed by Razali et al. (Razali et al., 2017), showing that the solvent-polymer interaction is crucial for the success of the filtration. Therefore, low permeability is observed for more polar solvents (i.e. MeOH) through hydrophobic membranes (GMT-oNF-2) or more apolar solvents (i.e. EtOAc) through hydrophilic membranes (e.g DuraMem-500). The usually used process for crosslinking polyimide make this polymer more hydrophilic. Therefore, a negligible EtOAc flux was observed for DuraMem-500 (crosslinked polyimide membrane), but a 25.5 $L/m^2/h^1$ of EtOAc flux was measured through PuraMem-600 (non-crossed linked polyimide membrane), under an applied pressure of 15 bar. The EtOAc fluxes of the home-made PBI membranes decrease, from 36 to 9 $L/m^2/h^1$, with increasing concentration of PBI casted solution from 22% to 26%. The observed MeOH fluxes are higher and more independent on PBI concentrations, ranging between 30.0 to 43.5 $L/m^2/h^1$, as PBI is a hydrophilic polymer, and so the interaction with MeOH is higher.

Retention of the larger product to purify is typically point out as the main decision criteria for selection of the membrane. However, as previously illustrated mathematically by Ferreira et al (Ferreira et al., 2020), the high rejection of impurities to be pushed through the membrane is also a main obstacle for efficient separations. While MWCO is a valid metric concerning the retention

of larger molecules, the permeation of the smaller ones depends on the shape of the retention curve, which is often not sharper enough for a successful separation. The rejection coefficients (R) were calculated (equation 3) for MELs and residual lipids (**Figure C2** and **Table F8**). A high-performance membrane is one that has a high rejection coefficient for MELs and a low rejection coefficient for residual lipids, allowing a better separation between the products presented in crude extracted MELs.

Concerning the use of commercially available membranes, the rejection of DuraMem-500 for residual lipids in MeOH is 77%, making this separation very challenging as it is difficult to push these solutes through the membrane. On the other hand, GMT-oNF-2 has a rejection of residual lipids of 32% in EtOAc, and thus it has the potential to separate them from MELs. Still the forecast product losses may be significant as this membrane presents a rejection to MELs of only 87.1%.

The PBI 22% membrane, with residual lipids rejection coefficients of 26.6% in MeOH and 32.6% in EtOAc has the potential to effectively remove these compounds residual lipids from MELs. However, again the rejection coefficients for MELs are low, at values of around 67.7% and 78.4%, respectively for MeOH and EtOAc. In order to increase MEL retention, a decision was made to increase the % of PBI in the casting solution and to assess the cross-linking of the membrane after its manufacture. The intend effect was observed, with PBI 24 and 26% membranes revealing higher MELs rejection (> 90%), with no significant differences between both solvents, EtOAc or MeOH (**Figure C2** and **Table F8**). However, the rejection coefficients for residual lipids also

increase significantly for PBI 24% and PBI 26% reaching values of 68% and 71% in EtOAc and 52% and 75 % in MeOH, respectively.

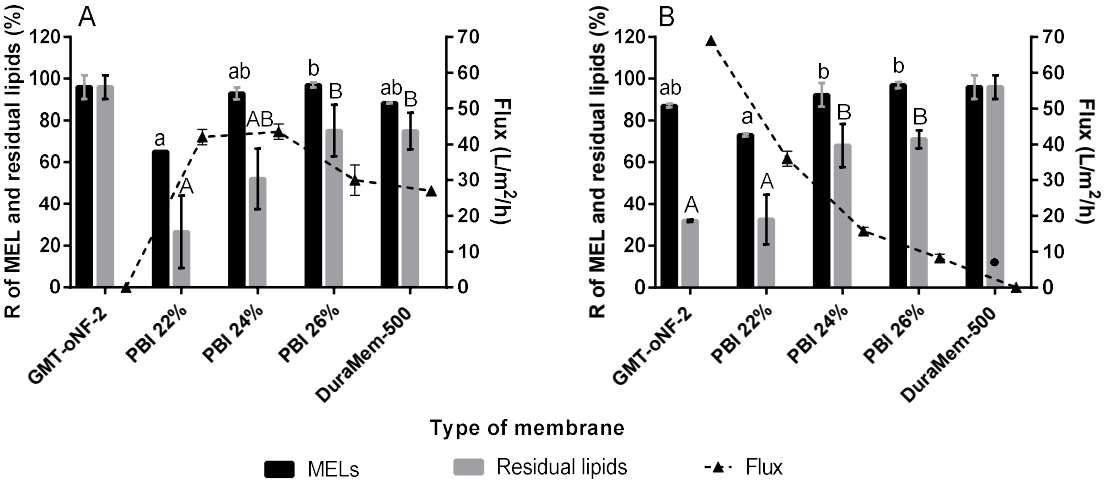


Figure C2: Rejection coefficient for MELs and residual lipids, and the solvent flux for each OSN membrane: PBI 22, 24 and 26%; GMT-oNF-2 (Borsig) and DuraMem-500 (Evonik) using MeOH (A) and EtOAc (B). Dots refer to filtrations that did not occur, since no solution has permeated. Different lowecase and upercase letters in each colunm represent significant variations in MELs and residual lipids rejections, respectively, for different membranes and the same solvent. Results for membranes highlighted in different letters have a p-value lower than 0.05.

Cross-linking PBI membranes typically decrease membrane MWCO and increase robustness, as shown by Valtcheva et al., (Valtcheva et al., 2015). As expected, also on this study the crosslink of PBI membranes lead, on most of the cases, to a decrease on solvent fluxes and increase on MELs rejections. However, PBI membranes crosslinking also leads to a significant increase on residual lipids rejection, an increase which was particularly stringent for PBI 22% (**Table F8**). Such effect is not beneficial for the intended separation and therefore the use of PBI crosslinked membranes was not further considered in this study.

Importantly, not that average rejection coefficients are estimated with significant associated standard deviations (**Table F8**), especially for MELs rejection. Such variation results from potential variations on pores size distribution on membrane active layers, due to slightly different environment conditions on the phase inversion process, leading to different rejections from batch to batch. Moreover, according with to fermentation variability, different MELs congeners and

residual lipids, which can be presented as FFA or MAG, will be fed to the filtration. While similar rejection was measured for MELs, regardless their fatty acid chain length, rejection for residual lipids present higher variabilities (**Figure F19)**.

Diavolumes strategy for MELs purification

After performing a membrane screening, MELs were purified following a diavolume (DV) strategy, aiming at retaining and purify MELs, while pushing the smaller lipids molecules through the OSN membrane. In this regard, the minimum theoretical DV and the MELs losses associated for each membrane were calculated, to achieve (at least) 97% of MEL purity, by considering the rejection coefficients values obtained for MELs and for residual lipids (**Figure C2**) as well as a crude MEL with 85% purity, broadly corresponding to the case studies identified on **Table C1** as “feeding strategy A” in EtOAc or "feeding strategy C”, after removal of TAGs by MeOH selective dissolution.

In **Figure C3** it is possible to observe that filtrations presenting lower theoretical MELs losses (ranging from 13-16% losses) are the ones using PBI 26% membrane, which theoretically requires 6 to 7 DV to reach the 97% MEL purity. This membrane was selected to be experimentally assessed under diafiltration mode.

The poorest performances are estimated for filtrations using PBI 22% and GMT-oNF-2 membranes, with MELs losses ranging from 65-80%. Nevertheless, since these OSN membranes (PBI 22% and GMT-oNF-2) have a higher MWCO than PBI 26%, lipids permeation is less challenging and only 4 DV are necessary to achieve 97% of purity. The use of such membranes could be interesting for a cascade membrane system, where MELs, lost to the permeate on the first step, would be recovered on a second step. Therefore, their performance, under diafiltration mode, was also experimentally assessed.

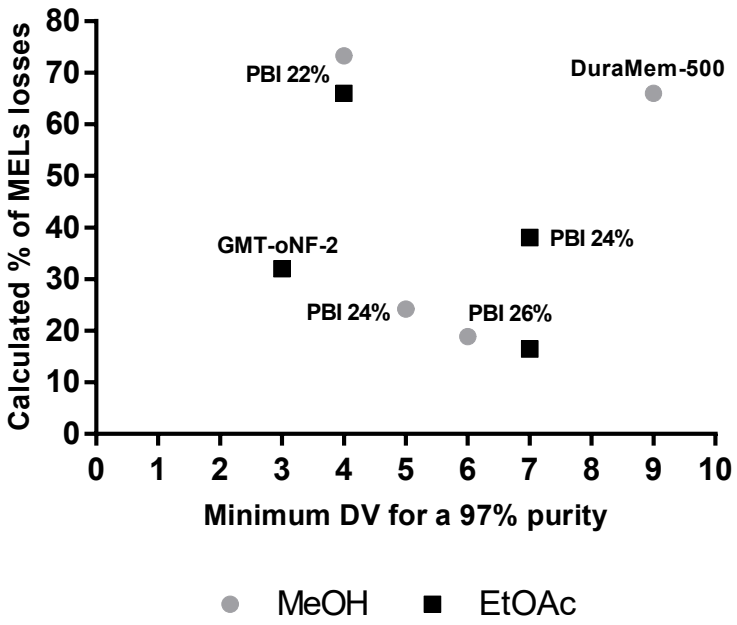


Figure C3: Theoretical minimum DV vs calculated MELs losses for each OSN membrane: PBI 22, 24 and 26%; GMT-oNF-2 and DuraMem-500, for MeOH (red circles) and EtOAc (grey squares) as organic solvents, to achieve at least 97% of MELs purity. All these results were calculated using equation 2 and the values shown in **Figure C2** and **Table F8**.

The experimental results for MELs purity and losses obtained for each DV used for PBI 22, 26% and GMT-oNF2 membranes are shown in **Figure C4**.

When using PBI 26%, the best performing membrane, there are no differences in the final MELs purity attained with MeOH and EtOAc, reaching more than 97% MELs purity and losses of 26.3% and 32.4% for EtOAc and MeOH, respectively, after 6 DV. These values are still high compared to the best value reported, 10% MELs losses, that uses several solvent extractions to reach similar purities (Shen et al., 2019).

Remarkably, after 2 DV of EtOAc, a diafiltration using GMT-oNF-2 or PBI 26%, resulted on a purity higher than 90%, with losses of 15.08 ± 3.10 % and 15.3 ± 2.2 %, respectively. Again, similar performance, corresponding to MELs losses of 14.7 ± 6.1 %, can be achieved by diafiltration of 2 DV of MeOH through the PBI 26%.

The strategy here presented can be an interesting purification platform for other biosurfactants, particularly glycolipids, that face similar challenges on the separation of lipidic fractions from the

glycolipids. As a proof of concept for others microbial biosurfactants, a solution of 50 g/L of crude SLs with 87% of purity was diafiltrated using PBI 26% with MeOH, as SLs do not dissolve well in EtOAc. After 6 DV it is possible to purify SLs (**Figure F20**), and with losses around 18%. A value lower than the one obtained for MELs losses after a 6DV diafiltration also performed in MeOH and using the PBI 26% membrane, which is expected considering the larger size of SLs (MW 650-685 g/mol).

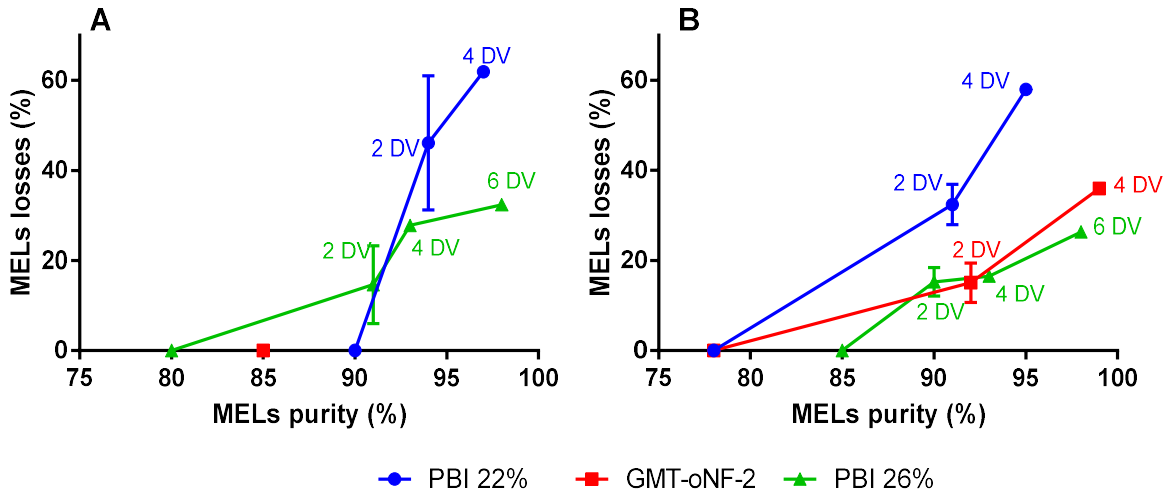


Figure C4: MELs purification through DV technology for different OSN membranes (PBI 22 (blue line with circles) and 26% (green line with triangles), and GMT-oNF-2 (red line with squares), using MeOH (A) or EtOAc (B) as organic solvents, for different DV (0, 2, 4, 6). All filtrations were performed with maintaining a volume of 50 mL and 15 bar of pressure.

Envisaging membrane cascade systems, when using GMT-oNF-2 or PBI 22% membrane, after diafiltrating 4 DV of EtOAc, 36.0 or 58.0 % of the MELs will be in the permeate, while the MELs on the retentate will have purities of 99% or 95%, respectively. For MeOH based diafiltrations, the PBI 22% with 2 DV could be used to obtain MELs in the retentate with 98% purity, while 62% of MELs will be in the permeate. MELs losses theoretically and experimentally estimated are quite similar for PBI 22% and GMT-oNF-2 (coefficients of variation of 3-12%), but more divergent for

the PBI 26% (coefficients of variation between 20-26%). A possible explanation is related with the fact that rejection coefficients were obtained in concentration mode with a decrease of the retentate volume to half of its initial value, while in diafiltration the retentate volume is kept constant.

Cascade system for reduction of MELs losses

As discussed, the use of the membranes assessed on this study are far from ideally for the separation intended. The membranes that provide high retentions to MELs (above 97%), also present high rejections for the lipids (above 70%), implying the need of using high DV, which over the extensive diafiltration leads to cumulative substantial losses in MELs to the permeate, as illustrated on the previous section for PBI 26%. A possible strategy to obtain high product purity with low losses would be the development of new membranes, with better performance, i.e. lower MWCO able of higher retentions of MELs and sharper rejection curves with lower rejections for lipids.

However, here it was followed a non-obvious approach, where it is suggested the use of OSN membranes, such as GMT-oNF-2 and PBI 22%, that have lower MELs rejections (67.7 to 87.1%), but also with lower rejections to the lipids (26.6-32.6%). In other words, to facilitate removal of lipids, we indulge a separation with high MELs losses to the permeate. Such strategy, if implemented on a single step, will lead to non-acceptable losses of MELs. Still, a two-step filtration cascade OSN membrane system, as described by Kim et al. (Kim et al., 2013), can be implemented, where the permeate of the first filtration is fed into a second filtration to recover the MELs, while the lipids is pushed into the permeate. Such system was assessed using the same membrane for the consecutive DV filtrations, as represented in **Figure C5**, which illustrates the two diafiltrations with an intermediate step of solvent evaporation to concentrate the permeate (with MELs and residual lipids). The solvent distilled, from both permeates, can be recycled as fed solvent into the diafiltration. The DV was selected to be the lowest number to achieve >95% of MELs purity, regardless the MELs losses, which for the selected membranes correspond to 4DV.

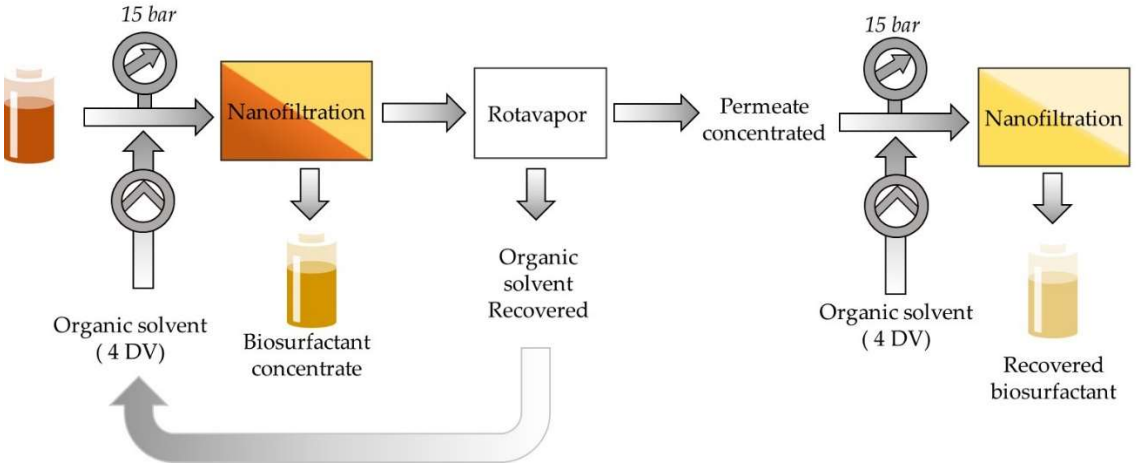


Figure C5: Overview of the cascade system studied, which included a first diafiltration, the recovery and concentration of the filtrate, and a second diafiltration using the filtrate as feed solution. Both diafiltrations used 4DV.

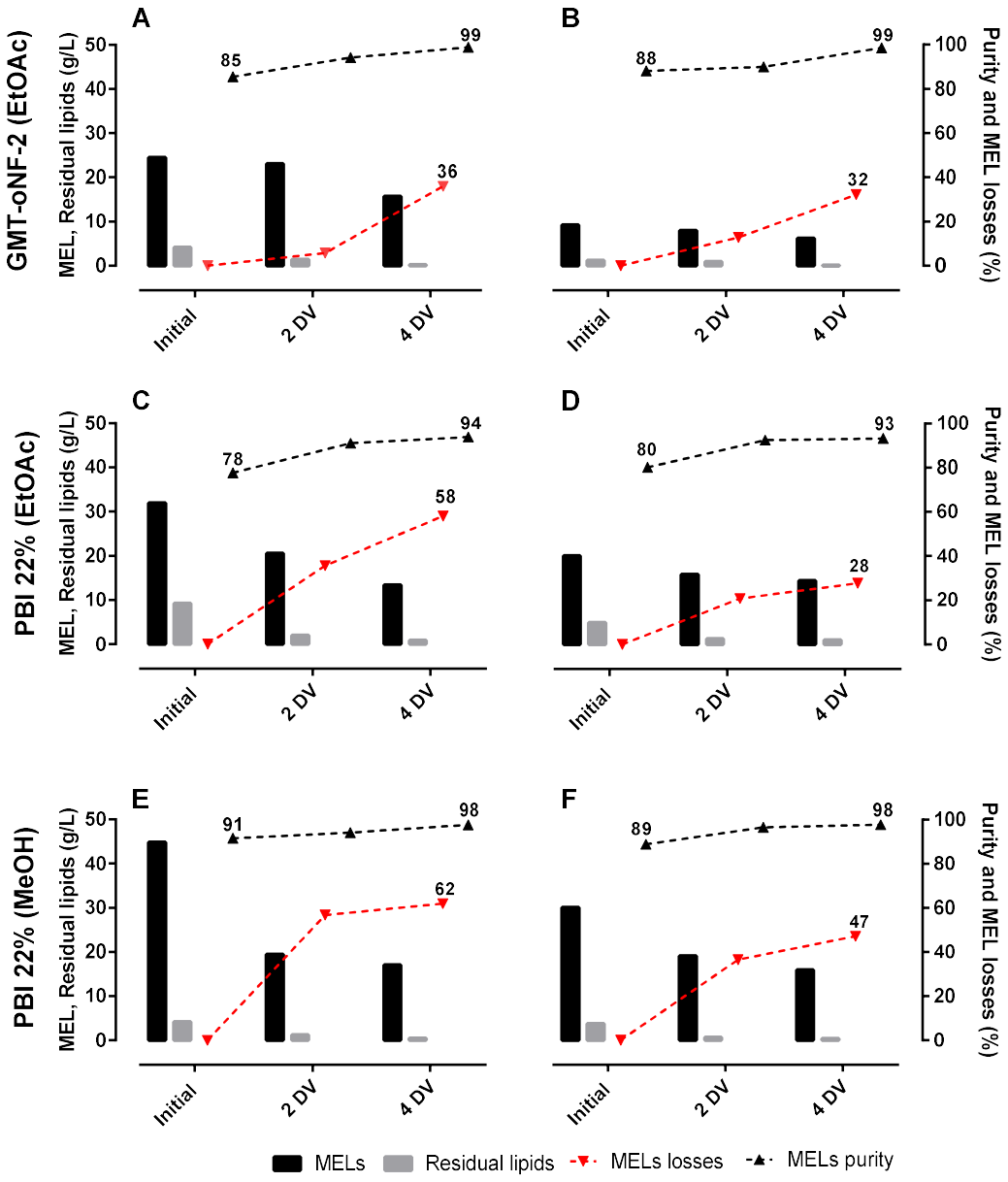


Figure C6: Cascade system using OSN membranes: GMT-oNF-2 (C, D, E and F) and PBI 22% using EtOAc (A, B, C, D) and MeOH (E, F) as organic solvents. After the first filtration (A, C, F) the permeate was concentrated and passed again through the same membrane (B, D, E). Each graphic represents the concentration of MELs in the retentate (black bar), residual lipids (grey bar), MELs losses (red line with inverted triangles) and purity (black line with triangles). For all filtrations used, the volume was kept at 50 mL, and 15 bar of pressure was applied.

In **Figure C6** shows MELs concentration on retentate for each DV, as well the purity and losses associated, for each cascade system. While the first diafiltration (**Figure C6 A; C and E)** and the second diafiltration (**Figure C6 B; D and F)** are represented on the left and right panels, respectively. The second filtration is fed with the concentrated permeate obtained in the first filtration**.** After the first diafiltrations with the membrane GMT-oNF-2, MELs losses were 36.0% with a purity of 98.3%. However, 66.8% of such MELs were recovered in the second diafiltration (**Figure C6** and **Table C4)**.

Remarkably, 88.4% of the MELs fed to cascade system is collected in the two retentates, which the combined solution presents a purity in MELs of 98.8%. Therefore, the second filtration allows to reduce cumulative MELs losses to only 11.6%, while attaining high purities, being this so far one of the best results obtained in the literature for MELs purification. Acceptable higher fluxes were obtained, at values of 63.45 L/m^2/h) for EtOAc permeation through GMT-oNF-2, pressurized at 15 bar. EtOAc is an appropriate solvent to extract crude MELs from the fermentation broth. Therefore, this approach is particularly adequate when coupled with our co-substrate feeding strategy A (**Table C1**) optimized to avoid the presence of TAGs at the end of the fermentation.

The experimental results on MELs purification using the two-diafiltration cascade system and a single diafiltration for PBI 22% are also shown in **Figure C4** and **Table C4**. PBI 22% is a membrane with lower rejections for MELs than GMT-oNF-2. Therefore, the MELs lost to the permeate in the first 4DV diafiltration are significantly higher for PBI 22%, at values of 58.0% and 61.9% in EtOAc and MeOH than when using the GMT-oNF-2, at a value of 36.0%. Consequentially, in MeOH, the cascade system (using the PBI 22% and 4 DV in each diafiltration) and the single diafiltration approaches (using the PBI 26% with 6 DV) lead to similar MELs purities (97.6% vs 97.1%) and losses (26.3% vs 32.4%). On the other hand, with EtOAc, the use of 4 DV in two consecutive diafiltrations with OSN 22% yields a MELs with 93.5% purity, a value below the target threshold. Therefore, there is no benefit of implementing the membrane cascade system using the PBI 22% membrane.

In resume, this study presents one of the best reported results for MELs purification in EtOAc using the GMT-oNF-2 membrane. This membrane can be used in a single 2 DV diafiltration or in

a 4 DV two-diafiltration cascade system to obtain MELs with 90% or 98% purities, respectively, with 15.1% and 11.6% of MELs losses.

For MeOH, the recommended membrane to be used is the PBI 26%, where a MELs purity of 90% or 97% can be reached using 2 or 6 DV in a single diafiltration, with MEL losses of 14.7% or 32.4%, respectively. The use of MeOH is important when TAGs are present in the crude MELs and the selective dissolution step for TAG removal needs to be included on the downstream route. However, the OSN operations are less efficient using MeOH, barely justifying the effort to implement a membrane cascade system, which requires more unit operations than the single membrane diafiltration. These results support the importance to implement feeding strategies that avoid the presence of TAGs at the end of the fermentation. Moreover, to support the ambition to further purify crude MELs in MeOH, membranes with different features, such as the PBI 24%, could be employed in the cascade system.

Table C4: MELs losses and purity (%) for different OSN membranes (GMT-oNF-2, PBI 22 and 26%) and both filtrations when using EtOAc or MeOH as organic solvents. In the cascade system it was used two diafiltrations with 4 DV in each (membranes GMT-oNF-2 and PBI 22%) and on the single diafiltration it was used 6 DV (PBI 26%).

Type of filtration	**Organic solvent**	**EtOAc**			**MeOH**	
	OSN	**GMT-oNF-2**	**PBI 22%**	**PBI 26 %**	**PBI 22%**	**PBI 26 %**
	Diavolumes used	**4 +4**	**4 +4**	**6**	**4 + 4**	**6**
1st filtration	MELs losses (%)	36.0	58.0	26.3	61.9	32.4
	MELs purity (%)	98.8	93.7	98.0	97.5	97.1
2nd filtration	MELs losses (%)	32.2	27.8	-	47.2	-
	MELs purity (%)	98.6	93.2	-	97.7	-
	MELs recovered (%)	67.8	72.2	-	52.8	-
Overall	MELs losses (%)	11.6	16.1	26.3	29.2	32.4
	MELs purity (%)	98.8	93.5	98.0	97.6	97.1

Comparison of different downstream process for microbial glycolipids purification

The novel downstream process here suggested is compared with other reported approaches for microbial biosurfactants purification in **Table C*5***. Several features of the process are highlight on such comparison, namely:

I. Type of solvent and volumes used, reported in relation to fermentation broth volume harvested;
II. Solvent recyclability using a simple distillation was assessed as easy (labelled as “Y”) when no solvent mixtures are formed, the solvent used is of low toxicity and the solvent has an acceptable low boiling point; or difficult (labelled as “N”) when solvent mixtures used form stable azeotropes, hindering solvent separation by simple distillation, which often ends up as waste streams;
III. Number of solvent shifts, i.e. number of times in the process that the total volume of the solvent is completely evaporated, and replaced completely by another solvent, which are high energy intensive steps; and
IV. Recovery efficiency, i.e. weight of product isolated on the end of the downstream route per weight of product produced in the upstream states, in percentage; and
V. Final purity, i.e. the total weight of product by total weight of product sample.

The method proposed in the present article enables theoretically complete reuse of solvent used in the downstream process, as mixing multiple solvents is avoided. On the case studies, in which TAGs are not present at the end of the fermentation, crude biosurfactant can be harvested by extraction from the fermentation broth with EtOAc (1:1, 3x times) and then purified by OSN, also in EtOAc, without any solvent shifts. For such case studies we do recommend the use of a cascade system (4DV + 4DV) using a GMT-oNF-2 membrane, followed by AC treatment, if colour removal is required (see article for more information or PhD thesis by Dr. Petar Keković). The whole process can take place on the same solvent, and if 90% of the solvent used is recycled, a ratio of 1.1:1 of total volume of EtOAc to fermentation broth will be used.

On the other hand, when residual TAG are present at the end of the fermentation, TAGs are extracted together with the biosurfactant by EtOAc from the fermentation broth (1:1, thrice). Thus,

for such case, a single solvent shift is necessary from EtOAC to MeOH (0.12:1) and additional MeOH (0.12:1, twice) will be used for removal of TAG by selective dissolution with minimal MELs losses. The three fractions of MELs in MeOH will be added together and additional methanol to obtain a 50 g/L crude MELs solution that will be fed to OSN. Removal of small lipidic contaminants will be performed by a single diafiltration using a 6 DV using a PBI 26%, followed by AC treatment, if colour removal is required. This process uses two solvents, EtOAc for the initial harvesting extraction and MeOH for the other unit operations, but do not yield solvent mixtures. When 90% of the solvent used is recycled, a ratio of 1:1 of total solvent volume to fermentation broth will be used. Furthermore, in the suggested process, the use of non-sustainable and toxic solvents is fully avoided.

Table C5: Efficiency of downstream methods (LLE – liquid-liquid extraction; CR – Crystallization, CP- Column purific amount of solvent required (volume of solvent/volume of fermentation broth), easiness to recycle solvent used (Y - for shifts, percentage of recovery and final purity (%). A /" " is used to separate information from different unit operations v omitted information in the source.

GB	Method	Solvent used	Solvent required (solvent volume/ broth volume)	Solvent recyclability easiness	No. of solvent shifts	Recovery (% w/w)	Final Purity (% w/w)	
RLs	LLE	EtOAc	4:1	Y	1	65.5	84.1	Acid p followe
	LLE + CP	EtOAc	2:1/NA *	N	3	89.4	90.7	LLE wit extrac *Solven for
		n-hexane	3:1/NA *	N				
SLs	LLE + CP	EtOAc	0.8:1/NA *	N	2	NA **	NA **	*Colum for cons **Reco
		2-propanol	0.2:1	N				

GB	Method	Solvent used	Solvent required (solvent volume/ broth volume)	Solvent recyclability easiness	No. of solvent shifts	Recovery (% w/w)	Final Purity (% w/w)	Comments	Ref.
		MeOH	NA *	N					
	LLE + CP	EtOAc	0.8:1/NA *	N	3	NA **	NA **	Oleic acid used as lipophylic substrate LLE with Ethyl acetate/2-propanol mixture, extract washing with hexane, followed by column chromatography *Solvent consumed for column purification not reported **Recovery rate and final purity not reported	(Daverey and Pakshirajan, 2010)
		2-propanol	0.2:1	N					
		n-hexane	1:1	Y					
		Chloroform	NA *	N					
		MeOH	NA *	N					
MELs	LLE	MTBE	3:1	Y	3	8	100	Multiple solvents used in subsequent extractions	(Rau et al., 2005a)
		MeOH	1.6:1	N					
		Cyclohexane	3:1	N					

GB	Method	Solvent used	Solvent required (solvent volume/ broth volume)	Solvent recyclability easiness	No. of solvent shifts	Recovery (% w/w)	Final Purity (% w/w)	Comments	Ref.
		n-hexane	1:1	N					
	LLE + CP Preparative HPLC	EtOAc	2:1	Y	3	4**	100	*Two column separations and preparative HPLC using $CHCl_4$/MeOH ratios 90/10, 95/5, 96/4. Unknown volumes, non-recyclable **Reported recovery based on mass of MELs after L-L extraction	(Kim et al., 1999)
		MeOH	1:1/ NA *	Y ; N *					
		Chloroform	NA *	N *					
	LLE + CP	EtOAc	1:1	Y	2	50**	100	*Column purification with gradient elution by $CHCl_4$/Acetone mixture (80:20, 40:60 and 0:100, v/v). Volume unknown, most likely immense, non-recyclable **Reported recovery based on mass of MELs after L-L extraction	(Morita et al., 2007a)
		Chloroform	NA *	N					
		- Acetone	NA *	N					
	LLE	MeOH	2.5:1	N	1	90	100	Multiple solvents used in subsequent extractions; solvent shifts avoided	(Shen et al., 2019)
		n-hexane	3:1	N					

GB	Method	Solvent used	Solvent required (solvent volume/ broth volume)	Solvent recyclability easiness	No. of solvent shifts	Recovery (% w/w)	Final Purity (% w/w)	Comments	Ref.
	LLE + OSN + AC treatment	*EtOAc*	2:1	Y	0	88.4	98.8	**Method reported in this section No TAG present. EtOAc used through all the process. Cascade system (4DV + 4DV), GMT-oNF-2 membrane. 90% of solvent recycled**	
	LLE + TAG removal + OSN + AC treatment	*EtOAc, MeOH*	1:1	*Y*	1	64.8	97.1	**Method reported in this section. TAG present. EtOAc used in extraction. MeOH used through the other steps of the process. Single diafiltration (6DV), PBI 26% membrane. 90% of solvent recycled**	

C.1.5 Final remarks

This study presents, for the first time, a unique method, based on OSN, for the purification of glycolipid biosurfactants that using only one solvent reaches 98% purity, with product losses of about 11.6% and the possibility of solvent recycling.

Different commercially available and home-made membranes (made of polybenzimidazole (PBI) were selected to retain MELs, while allowing permeation of smaller lipidic contaminants (FFA and MAG). None of the assessed OSN membranes presented interesting performance for the targeted separation. While, some have low rejections for MELs (67.7 to 87.1%), the ones with high retentions to MELs (rejections above 97%), also present high rejections for the lipids (above 70%), such is the case of the PBI 26% membrane. According with the model presented in our previous study [45], such membrane can be used to obtain a high product purity, but at costs of high product losses (20-40%) and use of high DV.

Different case studies were evaluated. A first case study highlights the importance to optimize fermentations to avoid the presence to triacylglycerols (TAGs) in the end of the fermentation. In such case, only one solvent, EtOAc was used for the whole process. The PBI 26% can effectively be used to in diafiltrations with 2 DV or 6 DV, respectively, to reach purities of 90% or 97% with MELs losses of 15% or 58%. While such strategy is interesting to reach the lower purity grade, the MELs losses require to attain 97% purity are prohibitive. Therefore, a sequential two-filtration diafiltrations, each with 4DV, was implemented, using a GMT-oNF-2. Such membrane has lower rejections for the small lipids, so MELs purifications will be facilitated. However, its rejection for MELs is also quite low and thus the permeate of the first diafiltration is fed to a second OSN is to recover the MELs lost. Remarkably, for GMT-oNF-2, it is possible to recovery 67.8% of MELs lost in the first filtration, achieving an overall MELs purity of 98% with losses of 11.6%. This is, so far one of the best values reported in literature for downstream processing of microbial biosurfactants. Noteworthy, as this downstream route only uses one solvent, its recycling by simple distillation will be facilitated.

Further reduction of solvent volume used can be relatively easy and significantly optimized, against solvent flux against the membrane, by increasing the concentration of crude MELs used in the OSN to values above 50 g/L (e.g. 150 g/L). Moreover, to perform only two extractions of the

fermentation broth will also reduce solvent use (MELs contents on the third extraction was residual, data not shown). However, implementation of further solvent volume reduction on the harvesting extraction step is challenge and deserve dedicated research attention.

A second interesting case study considers the presence of TAG on the end of the fermentation. This corresponds to many of the scenario reported on the literature, as large amounts of vegetable oils are used as carbon sources to attain high glycolipids biosurfactant production. To address such separation requirements, an additional operation unit was successfully developed for removal of 90% TAGs, with minimal losses of only 4.1% of MELs. In this process route, to avoid further solvent shifts, the process route for removal of other lipid derivatives (FFA, MAG and DAG) by OSN was processed in MeOH. Unfortunately, for diafiltrations in MeOH, the limitations posed by the sub-optimal performance of the membranes could not be circumvented using the two-filtration cascade system. Still, our home-made PBI 26% membrane could be used in MeOH to obtain, in a single diafiltration of 2 DV or of 6 DV, MELs purities of 90% or 97%, with losses of 15% and 32%, respectively. Note that the process here described can be further improved using membranes with rejections slightly higher MEL or lower for residual MELs, respectively, in the single diafiltration or cascade system modes.

Overall, the proposed downstream process for glycolipid biosurfactants was tailored to reduce solvent waste streams and avoid or mitigate solvent shifts. This resulted in a downstream process which is potentially more sustainable, as solvent streams, based on a single solvents facilitate their recyclability by distillation.

C.2: Techno-economic analysis of MELs bioprocess

C.2.1 Outline

In this chapter, it is reported the first economic analysis for MELs bioprocess, elucidating potential bottlenecks and pointing out future direction of research to boost MELs industrialisation, by using the software SuperPro Designer. Initially it was evaluated the cost of production for diferent factory insalled capacity (1, 10, 20, 50 and 100 m^3) aiming at a production of at least 100 tons MELs/year, where it was established that production cost decreases with increasie in factory installed capacity, but only until a certain value (20 m^3). However, to meet an annual production of 100 tons MELs/year, it was necessary to optimize downtime in the major bottleneck equipment (bioreactor), by having multiple bioreactors in sequence, where it was found that using 4 bioreactors of 20 m^3, leads to an annual production of 178 tons of MELs, with a production cost of 31.7€/$Kg_{MELs,}$ a payback time of 5.4 years, and an initial investment of 55 M€.

After identifying the best scenario, it was established that the parameters with higher impact in the final costs of the process were CO_2 emissions (36.9%), raw materials (22.7%), and labour dependencies (22.7%). The calculation on the use of different feedstocks as substrates showed that replacing glucose and culture medium by with cheese whey reduces the production costs and the payback time to 28.1€/KgMELs and 5.08 years, respectively. When pomace oil was used as hydrophobic carbon source, replacing the use of waste fried oil, MELs titres increase 6-7 folds, reaching 60-70 g/L. Consequentially, this condition corresponds to the shorter payback period here calculated (4.81 years), with a production cost of 27.2 €/KgMELs.

Overall, it was designed a model that can be adapted to different situations, allowing to save experimental time and supporting researchers in decision making.

C.2.2 Introduction

The emergence of biotechnology in 90´s, led to the intensive study of different types of microorganisms and their genetic modification, aiming the production of a wide range of bioproducts with novel functionalities (e.g penicillin), and others capable of replace the chemical analogues. Nevertheless, there exists a significant gap between the discovery of a bioproduct and the scale-up of a bioprocess for commercialization, with numerous bottlenecks along the pathway. In order to support bioprocess design, Intelligen, Inc, was founded at MIT (Massachusetts Institute of Technology) in 1991, releasing the software SuperPro Designer (SPD). This software performs energy and mass balances, facilitating design and development while also enabling economic evaluation. This evaluation provides important parameters such as the initial capital investment and payback time (the amount of time needed to recover the initial investment). To date, it remains the most widely used software by biotechnology-based companies for scaling up and controlling their processes (“Intelligen, Inc.,” 1991).

However, while this software is mainly used by established companies to simulate different scenarios, or by start-ups that want to implement a new industrial process, it can also be used at early stages of the process. Early economic assessment, using this software, allows for the identification of main bottlenecks in the process (upstream, fermentation or downstream), and supports the identification of which operations should be experimentally studied with more detail. For example, Magalhães et al (Magalhães et al., 2019), performed a techno-economic analysis of downstream process in Itaconic acid, assessing which operation (from adsorption, reactive extraction, crystallization and electrodialysis) is favourable to extract the product, and the one that should be studied in detail in laboratory scale. Overall, using this software in the early stages can save laboratory time by indicating which parts of the process need detailed study.

For the case of MELs, considering the gaps that exists in the bioprocess, when compared with RMs or SLs, it’s even more important to perform this study, which will allow to understand the economic viability of this process.

In this regard, for the first time, it was performed an economical and environmental analysis for MELs bioprocess, identifying main bottlenecks and performing a sensitive analysis to the whole process, pointing out future directions to exploit the scale-up of this process. This study offers a whole vision of MELs bioprocess, exploring different scenarios based on prices fluctuations, the

use of feedstocks over raw materials (e.g. glucose), but also the final impact of titres/productivity on different metrics (payback time, price of production).

C.2.3 Material and methods

C.2.3.1 Process simulation

A preliminary techno-economic analysis was performed for the bioprocess of MELs production from WFO and glucose. Mass and energy requirements for this process were estimated by performing a simulation with the support of the commercial software SuperPro Designer 10. For this simulation, the following conditions and specifications were used: (1) The production of MELs plant would be built in Portugal; (2) a lifetime of 15 years; (3) the construction and start-up phase would take 34 months; (4) the plant would operate for 330 days a year, 24 hours a day and (5) The depreciation was set as a straight line (5%) for a period of 10 years. The price assumed for bulk materials (raw materials, organic solvents, ect), utilities and labor are described on **Table C6**. The price of each equipment (based on the best scenario, see **section D.2.4.2**) is represented on **Table C7**.

Table C6: Raw materials, other materials, waste treatment, utilities and labour costs used for all the scenarios performed. Annual costs refer to the scenario using a reactor of 20 m3 in stagger mode (3). a) NaOH is the component used for chilled water; b) Plastic bottles used for MELs encapsulation (2kg/bottle); c) considering that ethyl acetate is recovered, it is assumed that every 100 batches, it is renewed; d) The labour price per hour was assumed based on Portugal.

Category	Components	Cost	Annual cost (k€)
Raw materials (€/kg)	Glucose	6.74	754.1
	KH2PO4	13.38	11.2
	MgSO4	0.33	0.3
	NaNO3	6.00	50.4
	Water	0.04	234.5
	Waste frying oil	0.50	7.6
	Yeast extract	2.16	6.0
Other materials	NaOH (€/kg) [a)]	0.04	63.8
	Plastic bottles (€/entities) [b)]	1.00	0.2
	Ethyl acetate (€/kg) [c)]	1.12	80.1
Waste treatment (€/kg)	Gas emissions	0.05	51.6
	Organic waste		2 249.1
Utilities	Std Power (€/kW/h)	0.09	646.5
	Steam (€/MT)	11.28	17.7
	Cooling water (€/MT)	0.05	27.7

Category	Components	Cost	Annual cost (k€)
	Chilled water (€/MT)	0.38	179.8
Labor (€/h) [d)]		23.00	1 417 205.0

Table C7: Capital equipment for the best scenario (3 x bioreactors of 20 m3 in stagger mode). The prices were determined for a process using a bioreactor of 1 m3 by using the web tool [21]. For the rest of the scales used, it price were estimated by using Williams factor (**Equation 10**). (a) others equipment include: Air filters (O_2 and CO_2), heat sterilizers and compressors.

Equipment	Dimensions	Cost (k€)
Blending tank (m3)	0.2	21.87 €
	2	87.10 €
	20	346.76 €
Seed fermenter (m3)	0.2	42.98 €
	2	168.73 €
Bioreactor (m3)	20	714.44 €
Oil storage (m3)	1.34	66.93 €
Mixer-Settler Extractor (m3)	2	440.49 €
Organic phase storage (m3)	80	708.89 €
Evaporator (m2)	900	132.75 €
Heat exchanger (m2)	33	90.51 €
MELs storage (m3)	1.7	78.09 €
Filler (Entities/h)	1554	168.96 €
Other equipment	-	411.87 €

C.2.3.2 Description of MELs bioprocess

For this economic analysis, instead of using values reported in literature, it was decided to use in-house data (more realistic scenario), using the process developed in **section B 1**. The data used for the process simulated (such as biomass, titre, purity, and CO_2 production) was obtained from the cultivation, previously reported for *M. bullatus* PYCC 5535^T in a 2-L bioreactor (New Brunswick^{TM} Bioflo^R/CelliGen^R 115), using 1.5 L of working volume. The culture medium contained, 0.3 g/L of $MgSO_4$ and KH_2PO_4, 3 g/L of $NaNO_3$, 1 g/L of Yeast extract and the

fermentation started with 40 g/L of glucose and 20g/L of WFO as carbon sources, followed by two feeds of 20 g/L of WFO at day 3 and 6, finishing at day 9. It was used a continuously agitation of 800 rpm and 1 vvm as aeration rate, and the temperature was set up at 27ºC. At the end of the cultivation, a final titre of 61 g/L of MELs were obtained, with a final purity of 87%.

In this regard, the model (see **Figure C7** for a complete visualization of whole process) was constructed based on this process. Initially, since the fermentation should start with 10% of inoculum, two seed-fermenters (SFR-101 and 102) were sequentially added for *M. bullatus* growth. In these two fermenters, the culture medium used is equally to the one previously described, and only 40 g/L of glucose are used as carbon source for *M. bullatus* growth. The metabolism of cell for biomass growth is represented by **Equation *8***, where it is assumed that after 2 days, the yeast in on exponential growth and all 70% of glucose was consumed (mimicking the results obtained in laboratory).

$$C_6H_{12}O_6 + 0.47\ NaNO_3 + 19.7\ O_2 \rightarrow 11.5\ CO_2 + 0.5\ CH_{1.98}O_{0.57}N_{0.02} + 16.65\ H_2O$$

Equation 8 : Equation of biomass ($CH_{1.98}O_{0.57}N_{0.02}$) growth using glucose ($C_6H_{12}O_2$) as carbon source, and used in Super Pro Designer® simulation. The stoichiometric coefficients are in mol.

After biomass growth, inoculum is transferred for main bioreactor (FR-101), where it will be operating for 9 days, with two feeds of WFO being added at day 3 and 6 from Oils storage (V-104). The production of MELs are represented in **Equation *9***. Finally, the fermentation broth is transferred to an extraction tank (MSX-101) where ethyl acetate is used to extract MELs in three stages. (a ratio v_{broth}/v_{EtOAc} of 1:3). The organic phase is then transferred to an evaporator (EV-101) to remove the ethyl acetate and obtain the final product. The final product obtained has a purity of 89.22% in MELs, being contaminated with residual lipids.

$$1.7\ C_6H_{12}O_6 + 0.075\ NaNO_3 + 1.88\ O_2 + 1.55\ C_{55}H_{98}O_6 \rightarrow 1.65\ CO_2 + 0.65\ CH_{1.98}O_{0.57}N_{0.02} + 0.14\ H_2O + 0.3\ C_{18}H_{34}O_2 + 2.5\ C_{30}H_{58}O_{12}$$

Equation 9 : Equation of MELs ($C_{30}H_{58}O_{12}$) production using WFO ($C_{55}H_{98}O6$) and glucose ($C_6H_{12}O_6$) as carbon sources, and used in Super Pro Designer® simulation. The stoichiometric coefficients are in mass (g).

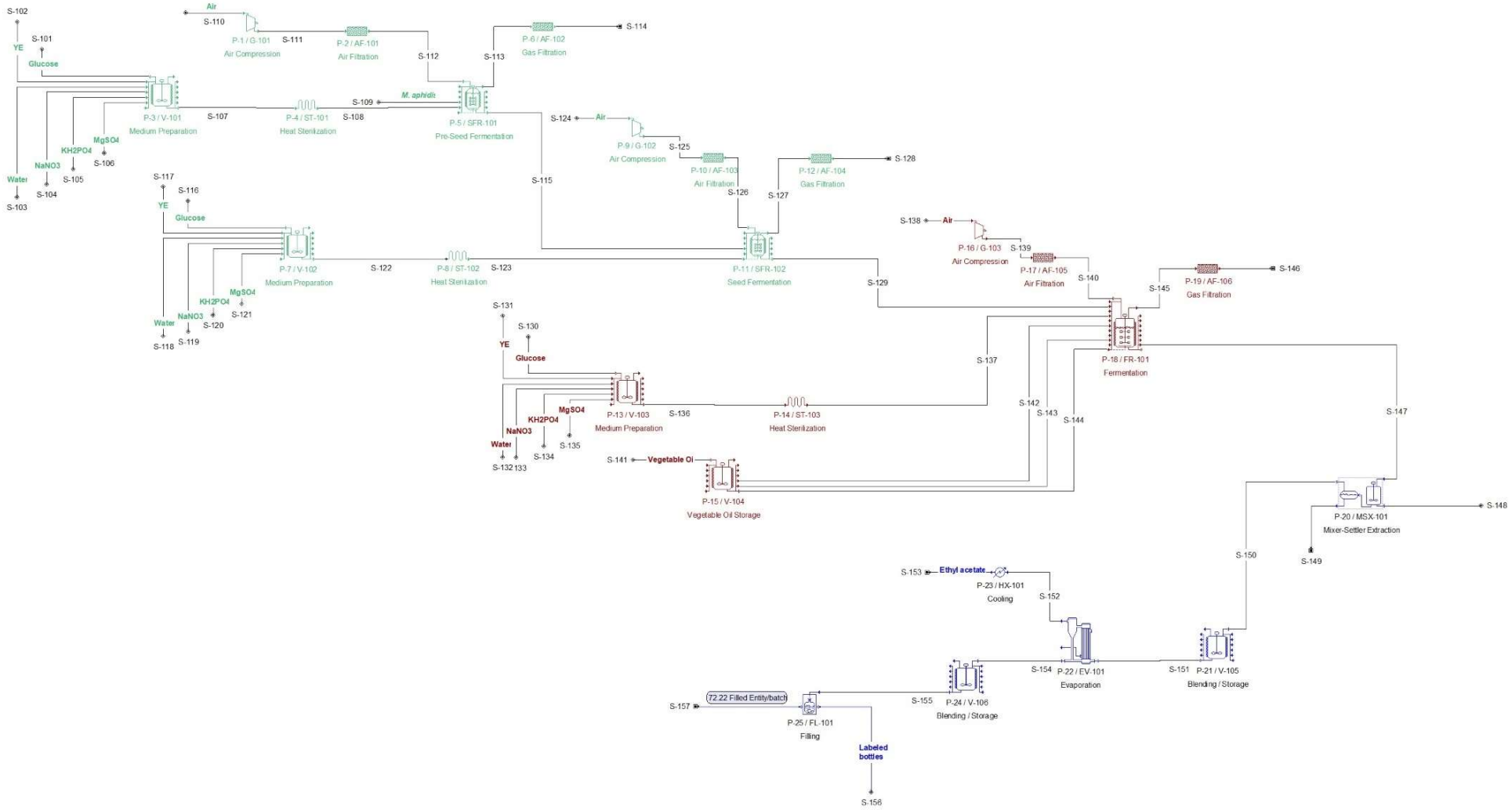


Figure C7: Scheme of the whole process, for 20 m^3 bioreactor capacity (with 0 bioreactors in stagger mode).

C.2.3.3 Economic analysis

The economical evaluation of the process was performed using SuperPro Designer 10 software, through which the total capital cost, annual production cost, and revenue generation can be estimated. The total capital cost of the MELs production plant is dependent on three different parameters: direct fixed capital, working capital, and start-up validation cost. The direct fixed capital includes equipment purchase costs, as well as other direct and indirect costs related to the construction of the plant, such as piping, insulation, and engineering, among others. The equipment purchase costs were determined through an equipment cost calculator online taking in consideration the inflation in Portugal for 2021 and considering a 1m^3 bioreactor capacity (Cost, 2003). When a scale-up of the equipment was required, William's Law (with a factor of 0.6), as represented in **Equation *10***, was used to attain the purchase price of the equipment (Peters and Peters, 1959).

$$\frac{C1}{C2} = (\frac{P2}{P1})^{0.6}$$

Equation 10 : William's Law Equation. C1 and C2 represent different capacities of an equipment with price P1 and P2, respectively.

In this study, considering the main objective was to verify the profitability of the MELs production process itself, factors such as external financing of the project and land costs were not taken into consideration. In the best scenario (using 4 bioreactors of 20 m^3 capacity in stagger mode, **section C.2.4.1)**, the contribution of the working capital and start-up validation cost to the total cost were 1.07 and 5.00 % of the direct fixed capital, respectively.

Regarding annual operating costs, raw materials usually make the most significant contribution but others costs such as waste treatment, labour, utilities, etc., are also included. For the economic assessment, the cost of raw materials and consumables was obtained from reputable suppliers of laboratory equipment, reagents, as represented in **Table C6**. About the use and treatment of ethyl acetate (EtOAc), it was assumed that that in every batch, 99% of EtOAc is recovered (53618.64 kg/batch) from the evaporator (EV-101) and after 126 batches (1 year, considering a factory capacity of 20 m^3, with 4 reactors in stagger mode) all EtOAc is treated as waste (**Table C6**), and a new batch of solvent is used. The "facility-dependent costs" correspond

to the depreciation of the fixed capital investment, equipment maintenance costs, insurance, taxes, and other general expenses. MELs production plant were designed to operate for 15 years and a straight-line method was used to calculate capital depreciation. In accordance with the local legislation, taxes were 28% of the direct fixed capital, respectively. Labour costs basically consist of the salaries of operators and engineers, to which corresponding taxes must be added. To correctly operate this plant, operators and engineers were required, thus it was considered an average value of 23 €/h for a total number of 293 employees. The cost of the quality control was estimated to be 15% of the total labor cost. The cost of waste disposal, treatment of aqueous emissions and gaseous emissions (CO_2) are represented in **Table C6**. Finally, the level of consumption of materials and energy was estimated according to the mass and energy balance calculated by the simulation software, taking in consideration the prices represented in **Table C6**.

The economic feasibility of the MELs production process was evaluated according to several indicators calculated by SuperPro Designer 10 software: gross profit, gross margin, return on investment, net present value, and payback time. Gross profit is the revenue from which the annual operating cost has been subtracted. The return on investment (ROI), evaluates the viability of the investment, according to **equation 4**, allowing to whether the investment will bring profit or not (Izaguirre et al., 2021).

$$ROI\ (\%) = \frac{Net\ Profit}{Total\ Investment} \times 100$$

Equation 11 : Calculation on ROI (%).

The net present value (NPV) measures the profitability of the production process in absolute net terms (thus, it allows one to know whether the investment will bring profits or not). A positive NPV value means that a priori, the planned investment should make a profit. The NPV can be calculated according to **Equation 12**. The payback time is the time required to recover the capital investment, is calculated by **Equation 12** (Izaguirre et al., 2021).

$$NPV = \sum_{n-1}^{T} \frac{CF_n}{(1+i)^n} - I_0$$

Equation 12 *:* Equation of NPV. T = lifetime of the investment; CFn = difference between revenues and costs in year n; I0 = initial investment; and i = discount rate.

$$Payback\ Time\ (years) = \frac{Total\ Investment}{Net\ Profit\ per\ year}$$

Equation 13: Payback time calculation.

C.2.3.4 Simulation scenarios

Initially, it was studied how the capacity of the main bioreactor influences final production, and the MELs unit cost production. After a careful analysis of which scale of operation should be applied based on economic parameters such as Net Present Value (NPV), and optimization was performed using the stagger mode function. This function allows the user to consider extra equipment to overcome timeline bottlenecks in the process and decrease each time cycle. For the best scenario (see **section C.2.4.1**) was performed an sensitive analysis to different parameters, such as: (1) reduction of carbon dioxide emissions; (2) replace of glucose in the medium by an industrial residue (like **CW**, as described in **section B.2)** and (3) modification of final titre; (4) Different number of batches/year which is related to the time of fermentation.

C.2.4 Results and discussion

Before performing any simulations or exploring different scenarios, the first objective is to determine the factory's capacity, which is entirely dependent on the market share. However, what volume of production is considered sufficient to meet industrial needs?

In 2023, the market for microbial biosurfactants (the serviceable addressable market, SAM) was valued at 800 million €, and it is impractical to plan a factory capacity to replace all microbial biosurfactants, considering that MELs don't even yet a process with a defined SOP (standard operation procedures). Therefore, considering that 20% of microbial biosurfactants are used in personal-care products, it was assumed that this factory/company would target 6% of that specific market, corresponding to a serviceable obtainable market (SOM) of €10 million, and a production capacity of more than 100 tons of MELs/year.

C.2.4.1 Scale of operations

Therefore, given the uncertainty around production scale and associated costs, the first objective was to determine the optimal factory capacity. To achieve this, an analysis was conducted on the unit production cost at various scales, alongside the required initial investment.

Analysing the correlation of the unit production cost and the factory capacity (**Figure C8** A), it is evident that production cost decreases as factory capacity increases. This reduction is particularly notable between 1m^3 (195.7 €/Kg_{MELs}) and 10 m^3 (39.9 €/Kg_{MELs}), confirming that scaling up is an effective strategy for cost reduction. Based on these results, it's clear that the factory must have a capacity of at least 10 m^3. However, it is interesting to observe that beyond 20 m^3 the production cost nearly plateaus, with a final cost of 25.5 €/Kg_{MELs} and an initial investment of €150 million for a capacity of 100 m^3. In this regard, the most favour scenario should be to have a factory capacity between 10 and 20 m^3. Comparing these two capacities, it is possible to observe that an increase in 9M in the initial investment leads to a reduction of 8.17 €/Kg_{MELs} in the production cost.

The values provided by this analysis allowed for a correlation between the two factory capacities, considering different selling prices (from 50 to 75 €/$Kg_{MELs.}$). **Figure C8** B illustrates this correlation, indicating that a factory capacity of 20 m^3 is financially advantageous. This is supported by a positive NPV value at a 7% interest rate, even with a lower selling price. A negative

NPV suggests a project will result in a net loss, while a positive NPV indicates potential profitability, justifying the investment. (Izaguirre et al., 2021).

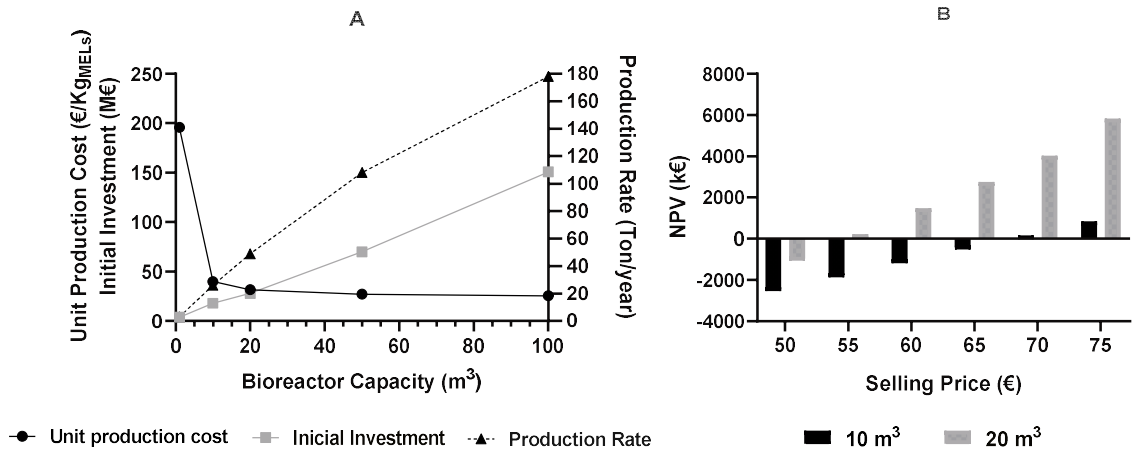


Figure C8: Comparison between different parameters for different bioreactors capacity. A) Initial investment (M€), unit production cost (€/unity) and annual production (L/year) for 1, 10, 20, 50 and 100 m3; B) NPV (k€) for different bioreactors capacity (10 and 20 m3) and the selling price ranging from 50 to 75€.

C.2.4.2 Economic analysis to different scenarios

Defining the factory capacity (20 m^3), which allows the production of 50 tons, with an operational cost of € 1.5 million/year, it was decided to study various economic parameters for different selling prices. As shown in **Table C8**, payback time is consistently above 6 years for prices ranging 50 to 75€. When correlating NPV with selling price (**Figure C9**), it is possible to determine the breakeven point (where NPV equals zero). This occurs at 54.52 €/Kg$_{MELs,}$ meaning for the current process to be profitable, the selling price must exceed this value. Therefore, a selling price of 70 €/Kg$_{MELs}$ was fixed to evaluate the following scenarios. Moreover, in **Table F9** it is represented the economic values for different factory capacities (1, 10, 20, 50 and 100 m^3), where payback time decreases with increasing capacity up to until 20 m^3, but after this scale, it starts to increase. This trend can be explained by the high initial investment required for large factory capacities, which is not sufficiently offset by the small reduction in unit product costs, as previously stated.

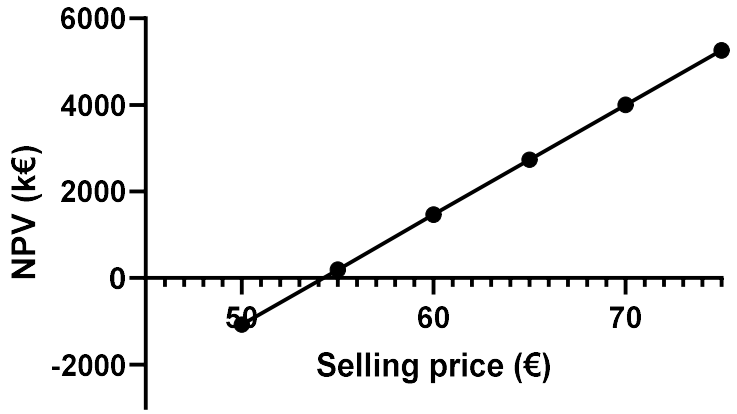


Figure C9: Correlation between the NPV (k€) and selling price (€) for a factory capacity of 20 m^3

Table C8: Comparison of process and economic parameters for MELs production process, using 20 m^3 bioreactor capacity, and varying the selling price of MELs (€/kg MELs). Values retrieved from Super Pro Designer®.

Parameters	**Selling price (€/kg $_{MELs}$)**					
	50	**55**	**60**	**65**	**70**	**75**
Investment (k€)	27 395 €					
Net Annual Operating Costs (k€)	1 568 €					
Annual Revenues (k€)	2 471 €	2 718 €	2 965 €	3 213 €	3 460 €	3 707 €
Batch Time (h)	362					
Cycle Time (h)	240					
Batches per year	32					
Production rate (Kg $_{MELs}$/batch)	1545					
Production rate (Tons $_{MELs}$/year)	49					
Net Production Cost (€/Kg $_{MELs}$)	31.7					
Gross Margin (%)	36.6	42.3	47.1	51.2	54.7	57.7

Parameters	Selling price (€/kg $_{MELs}$)					
	50	**55**	**60**	**65**	**70**	**75**
ROI (%)	11.4	12.0	12.7	13.3	14.0	14.6
Pay Back Time (years)	8.8	8.3	7.9	7.5	7.2	6.8
IRR - after taxes (%)	6.2	7.1	8.1	8.8	9.6	10.6
NPV - 7% interests (k€)	- 1 078 €	193 €	1 463 €	2 733 €	4 000 €	5 260 €

However, the annual production of MELs its only 50 tons, three times lower than the minimum previously established, resulting in a high payback time, leading to a process non-profitable. To address this issue, it was decided to optimize the process using SuperPro Designer stagger mode function. This function enables the user to choose to have extra equipment to overcome timeline bottlenecks, thereby decreasing overall cycle time. However, this function should only be applied selectively to the bottleneck equipment, which, in this case, is the bioreactor that operates for nine days.

By analysing different number of bioreactors in stagger mode (**Table C*9***), it becomes evident that different parameters (such as number of batches/year, production rate/year, cycle time and initial investment) undergo significant changes. In fact, as illustrated in **Figure C*10***, increasing the number of reactors in stagger mode reduced the cycle time nearly plateaus and increases the number of batches per year. However, beyond 3 bioreactors in stagger mode, the cycle time almost plateau, and the initial investment rises from 55 M to 64 M€, resulting in only a small increase of 7 batches per year. This explains the increase in payback time (from 5.4 to 5.7 years).

Further simulations, up to 7 bioreactors in stagger mode (data not shown), revealed that while the cycle time remains constant, the payback time continues to increase. **Figure F*21*** presents the Ghant chart of the different processes, providing a clear visualization of the effects of having multiple bioreactors. After three bioreactors in stagger mode, there are almost no differences in the number of batches per year, as the downtime is fully optimised. Therefore, it was decided to perform the following simulations with three bioreactors in stagger mode (four bioreactors of 20 m^3 in total).

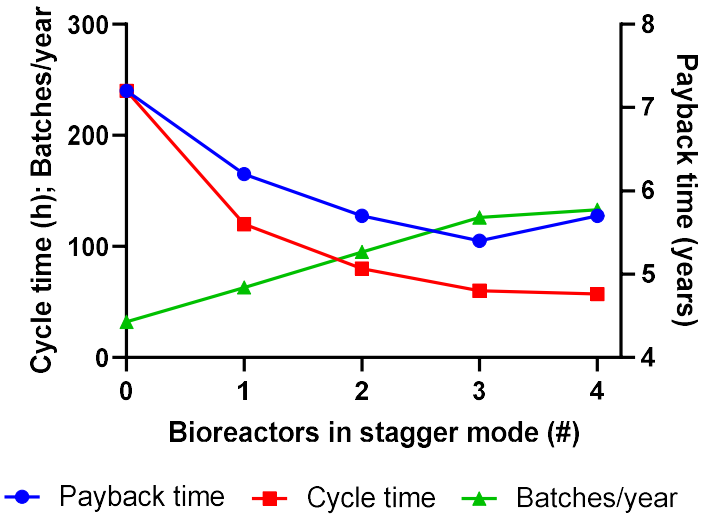


Figure C10: Relation of cycle time (h, red line with squares), payback time (years, blue line with circles) and batches/year (green line with triangles) with the number of bioreactors (20 m^3) in stagger mode (0, correspond to one bioreactor; 1, two bioreactors; 2, three bioreactors; 3, four bioreactors and 4, five bioreactors)

Table C9: Comparison of process and economic parameters for MELs production process, using 20 m^3 bioreactor, and varying the number of bioreactors in stagger mode while having a fixed production revenue per entity of 70€. Values retrieved from Super Pro Designer®.

Parameters	**Bioreactors in stagger mode (#)**				
	0	**1**	**2**	**3**	**4**
Investment (k€)	27 395	36 658	45 919	55 180	64 331
Net Annual Operating Costs (k€)	1 564	3 119	4 702	6 237	6 853
Annual Revenues (k€)	3 460 €	6 811 €	10 271 €	13 622 €	14 379 €
Batch Time (h)	362				
Cycle Time (h)	240	120	80	60	57
Batches per year	32	63	95	126	133

Parameters	Bioreactors in stagger mode (#)				
	0	1	2	3	4
Production rate (Kg $_{MELs}$/batch)	1545				
Production rate (Ton $_{MELs}$/year)	49	97	147	195	205
Net Production Cost (€/Kg MELs)	31.7	32.1	32.0	32.1	32.1
Gross Margin (%)	54.8	54.2	54.2	54.2	54.2
ROI (%)	14.0	16.2	17.7	18.6	17.7
Pay Back Time (years)	7.2	6.2	5.7	5.4	5.7
IRR - After taxes (%)	9.8	12.3	14.0	14.9	13.8
NPV - 7% interests (k€)	4 020	11 129	18 640	25 854	26 088

Using 4 bioreactors of 20 m^3 in stagger mode, a production of 195 tons of MELs is achieved annually, surpassing the 178 tons produced with a single 100 m^3 (178 tons). Comparing these two processes (**Figure F22**), it is evident that having a factory with 4 bioreactors of 20 m^3, leads to an annual cost production 1.5 higher than using one bioreactor of 100 m^3 (6.2 and 4.3 M€, respectively). However, payback time is 1.44 lower (5.4 and 7.8 years). This comparison supports a general conclusion: regardless of the factory size, it is more efficient to use smaller bioreactors in stagger mode rather than one or two larger one. This approach allows a reduction in the initial investment required, cycle time, and increases the number of batches/year. However, while stagger mode improves cycle time, it does not enhance yield per batch. Other factors, such as the type of process (fed batch, continuous) or even the regime of substrate should be considered.

After selecting the optimal process (3 reactors of 20 m^3 in stagger mode), the next objective was to analyse the allocation of process costs and identify potential bottlenecks. As shown in **Figure C11 A**, the majority of annual operating is attributed to waste treatment and disposal followed byby labour and raw material. The high waste treatment cost is due to the emission of

approximately 45 million kilograms of carbon dioxide per year. Labour costs, accounting for 22.7% of total costs, reflects an average between the wages of factory operators and the salaries of managers and supervisors required for factory operations (**Table C6**).

Raw materials constitute 23% of the total annual operating costs. Within this category (**Figure C11 B),** glucose and mineral medium/yeast extract contribute to almost 60% of the raw material costs, identifying a significant bottleneck. Replacing glucose with a possible side stream, could substantially reduce costs, as it will be discussed in section **C.2.4.3**. Other components, such as ethyl acetate, also highlight the need for a process free of organic solvents to further enhance economic viability.

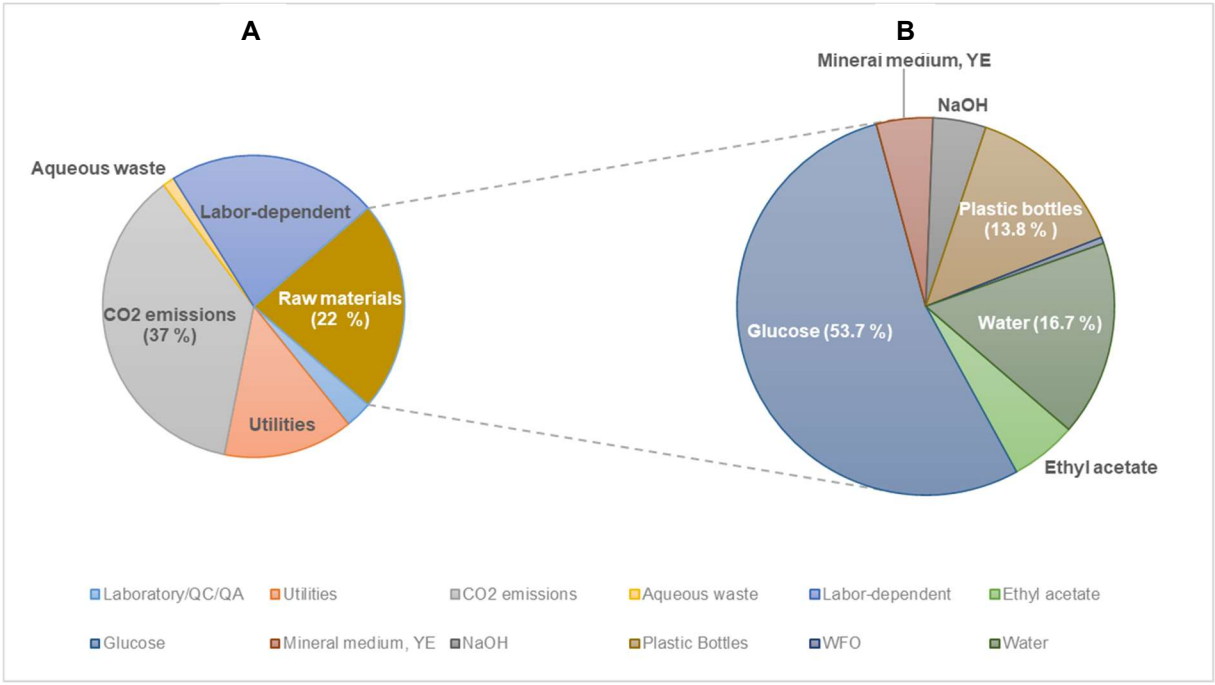


Figure C11: Analysis of costs of specific utilities for the process using 4 bioreactors of 20 m^3 (three in stagger mode). A) Description of each utility weighs on the annual operation costs; B) individual description of raw materials weighs on the annual operating cost of raw materials (22%).

C.2.4.3 Sensitive analysis to different scenarios

Based on results achieved in **section B**, where it was discovered that CW has the capacity to replace glucose and mineral medium/YE (**section B.2**) and that WFO can be replaced with pomace oil (**section B.3)**, it was decided to simulate the process using these assumptions (**Figure C*12*** and **Table F*10***). For the case of CW, as previously described, it can replace glucose and mineral medium/YE, without affecting the final productivity. In this regard, it was decided to simulate the same process, but replacing glucose and whole mineral medium with CW, and assuming a price of 0.50€/kg (value obtained in conversation had with a Portuguese company). Analyzing **Figure C*12***, it is possible to observe that the replace of culture medium and glucose lead to a reduction of 13.2 and 5.9% in annual operating costs and payback time, revealing the importance of replacing glucose, and the necessity to look for different side streams. When replacing WFO for pomace oil, it was necessary to restructure the process, since this process required 4 feeds of 20 g/L of pomace oil, instead of 3, during the 9 days of cultivation, as deeply described in **section B.3.** It is possible to observe that this simulation leads to the best payback time (4.81 years). This is due to the fact the process with pomace oil leads to higher titre (70 g/L of MELs), allowing a production rate of 1.7 ton $_{MELs}$/batch (13% higher when compared with the standard process), even though with lower batches (115 instead 124).

These simulations have demonstrated that titre and productivity (number of batches/year) significantly influence the final profit and consequently the final profit. Given that the current process (using glucose and WFO) theoretically allows a maximum titre of 80 g/L (assuming three feeds of 80 g/L of WFO),and acknowledging that final profit depends on production capacity and cost, it was decided to analyse how profit varies with a range of titres (40 to 80 g/L) and production costs (2 to 40 €).

Figure C*10* A, generated from MATLAB simulations, shows that profit increased with decreasing the production cost and increasing the titre, which was expected. However, **Figure C*10* B,** which correlates the profit with number of batches and cost of production, clearly indicated that the number of batches has a more significant impact on profit than titre. This might imply that productivity is the primary bottleneck.

This might also explain why SLs bioprocess, with productivities higher than 100 g/L/day, it is more profitable than MELs and is the most widely produced microbial biosurfactant (Gao et al., 2013). In fact, Ashby et al (Ashby et al., 2013), performed an economical analysis for SLs bioprocess, where they design a factory for an annual production of 90.7 million kg of SLs, using 8 bioreactors (stagger mode) of 20 000 m3, achieving a remarkable cost of production of 2.73€/Kg_{SLs}. However, this is an old study (2013), and the authors have used different prices for glucose (2.67 €/kg) and YE (1.14 €/kg), which are 60 and 47% lower than the values used in this study, which clear will have an impact in final profit and cost of production. Fernandes et al., (Fernandes et al., 2020) conducted an economic study, but for the RMs bioprocess, and found a production cost of 55.2€/KgRMs, which is 42.5%higher the value reported here (31.7€/Kg_{MELs}). Interesting, the authors have also observed that the carbon source, in this case glycerol, also accounts for 19.4% of total costs of the process.

It seems that MELs have already a competitive process, but there is still a long way to go, to be competitive with SLs. In **Figure C*14***, as complement to what have been discussed, it is represented how the profit varies according to different parameters. Interestingly, the parameter that have an huge impact in final profit are CO_2 emissions, where their removal leads to almost a increase in 2.5 M€ of the final profit. Interestingly, a variation of 200% in the final price of hydrophobic source almost does not impact the final profit.

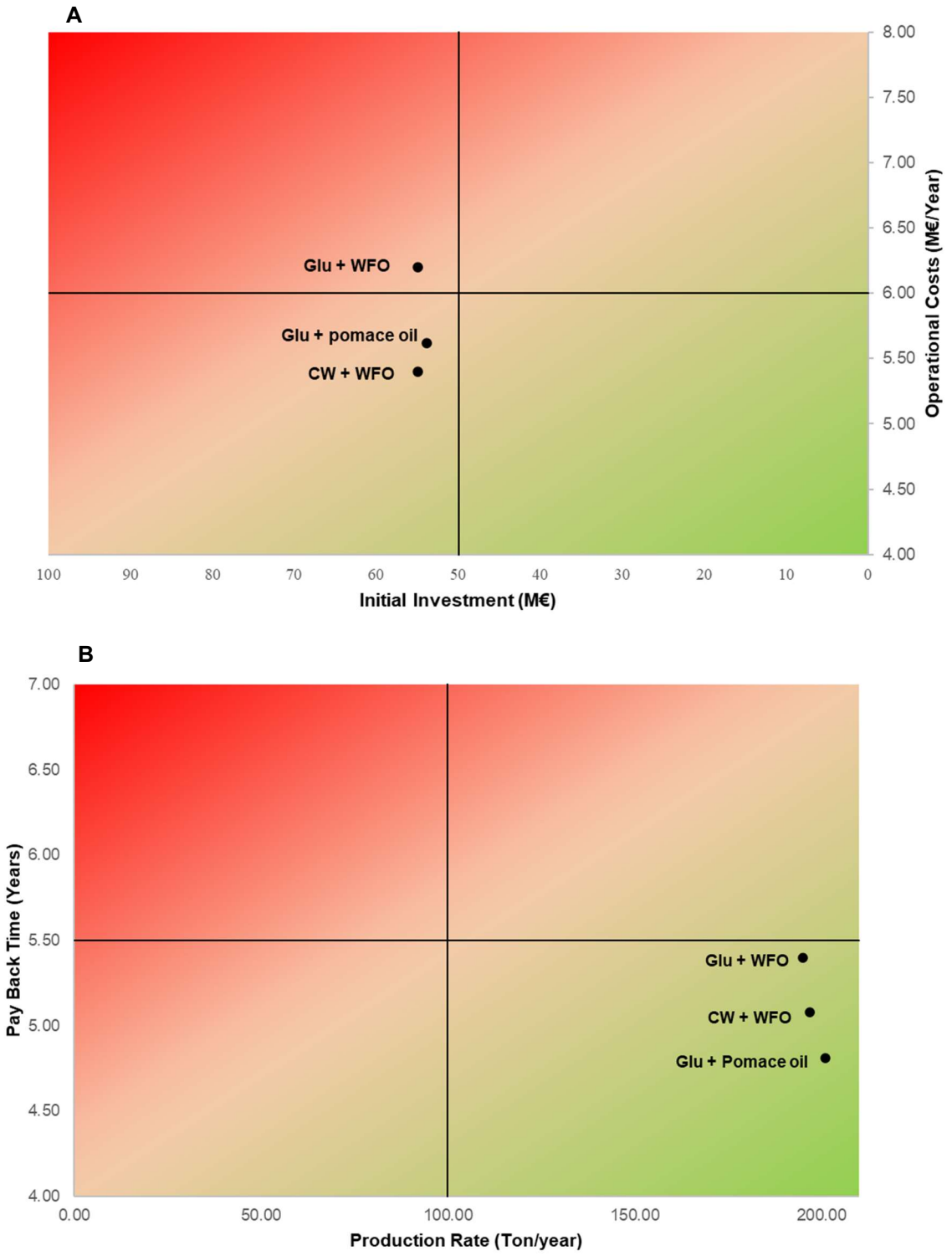


Figure C12: Comparison between different carbon sources (glucose/ waste frying oil; cheese whey/waste frying oil and glucose/pomace oil) using the four bioreactors of 20 m^3 in stagger mode. A) Operational costs (M€) vs Initial capital investment; B) Payback time (year) vs Production rate (Ton MELs/year).

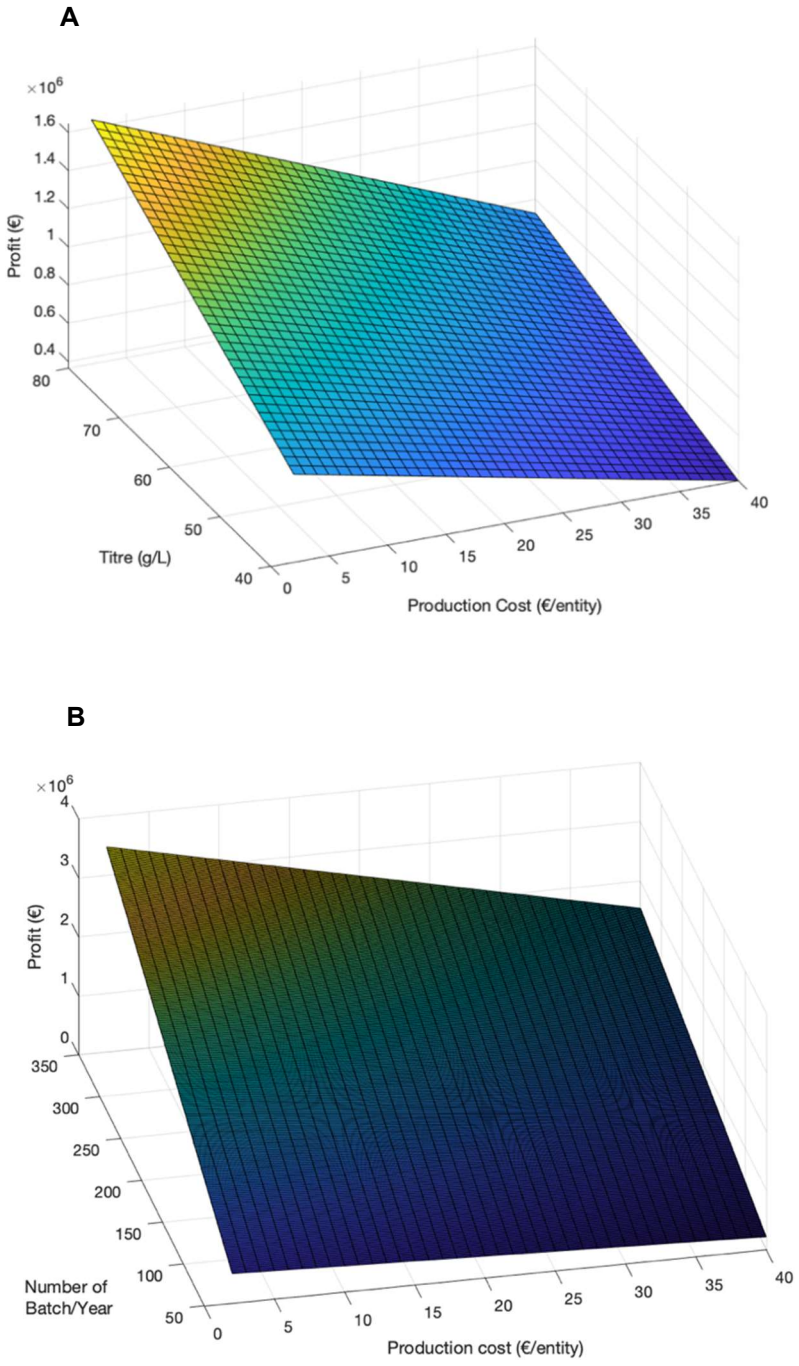


Figure C13: A) Relation between the titre (g/L) of the production process, the production cost per entity and the profit obtained; B) Relation between the number of batches per year, the production cost per entity and the profit obtained. Both graphics were obtained using in software MATLAB, using the program present in **section F.5**.

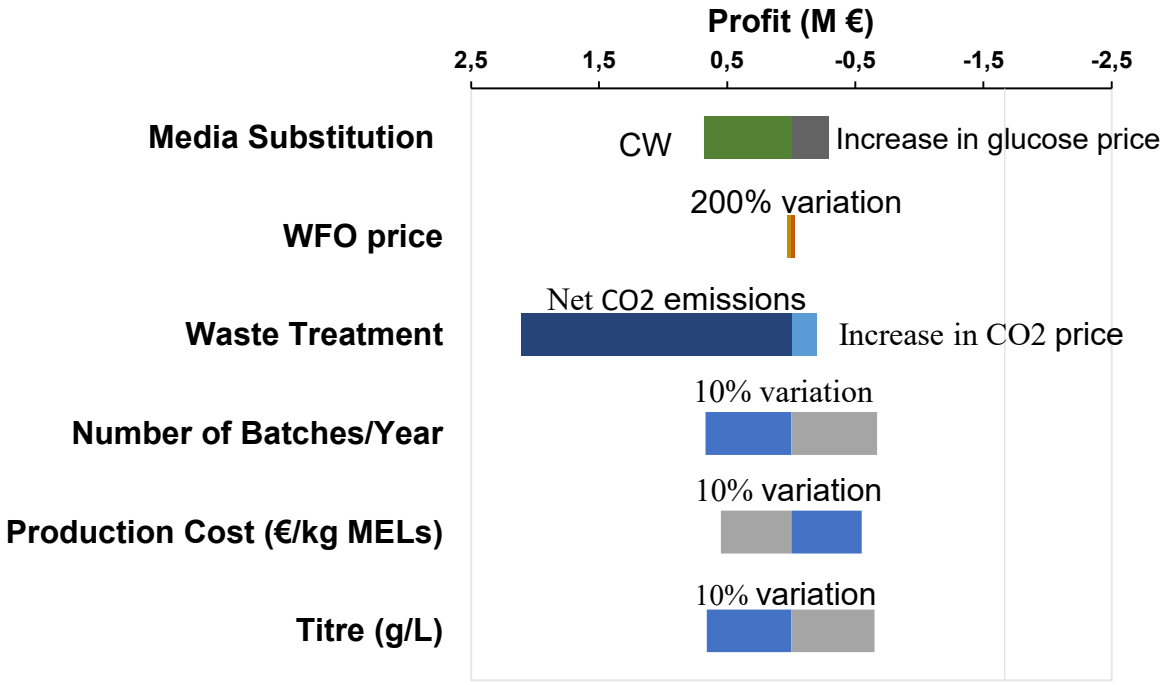


Figure C14: Sensitive analysis in final profit (M€) for different scenarios vs the actual process: 1) Media substitution: Increase of 10% in glucose price and replace of glucose by a renewable substrate (CW); 2) WFO price: 200% increase and decrease; 3) Waste treatment: Increase in 10% of emissions and the effect of having net CO_2 emissions; 4) Number of batches/year; 5) Production cost (€/kg MELs) and 6) Titre (g/L), these last three, studying the effect of increasing and decreasing in 10% variation.

C.2.5 Final remarks

For the first time, it is reported an economic analysis for MELs bioprocess, discovering potential bottlenecks. In the beginning was studied the impact in cost of production vs the increase in factory capacity (1, 10, 20, 50 and 100 m^3). Interestingly, beyond 20 m^3 the production cost nearly plateaus, with a final cost of 25.5 €/Kg_{MELs} and an initial investment of €150 million for a capacity of 100 m^3.

However, using a single 20 m^3 bioreactor is insufficient to meet the initially established annual production target of 100 tons of MELs. To address this, it was used a feature from the software (stagger mode), which allows to have extra equipment to overcome timeline bottlenecks, The optimal scenario involved the use of 4 bioreactors (20 m^3), resulting in an annual production of 178 tons of MELs, with a production cost of 31.7€/Kg_{MELs}, a payback time of 5.4 years, and an initial investment of 55 M€.

Recent discussions with different suppliers of pilot plants and bioreactors of different sizes revealed that the initial equipment cost estimates were overestimated. In fact, for a pilot plant of 10 m^3 we had received a final quotation of 700 k€. Therefore, future estimates should be adjusted to reflect a more realistic initial investment. It is important to note that this adjustment does not affect the cost of production, or the overall conclusions drawn in this chapter.

Having the scenario optimized, it was decided to simulate the replace of carbon sources by side streams of industry (CW and pomace oil). Using CW as glucose and culture medium substitute reduces the production cost and payback time decrease to 28.1€/Kg_{MELs} and 5.08 years, respectively. When waste fried oil is substituted with pomace oil (resulting in an increase of more than 10 g/L of MELs titre; from 60 to 70 g/L, respectively), the payback period is the shortest here reported (4.81 years), with a production cost of 27.2 €/$Kg_{MELs.}$

From these simulations, it can evident final productivity and replacing glucose/culture medium (particularly YE) it is essential for MELs to compete with SLs. This study provides a flexible model, that can adapt to various scenarios, saving time in the laboratory and supporting researchers in decision-making. Future simulations should include different side streams, particularly lignocellulosic residues and their pre-treatment, to access their viability. Moreover, a life-cycle

analysis should also be performed in the early stages to understand if the bioprocess will really be sustainable.

Section D: Co-cultivation of autotrophic and heterotrophic microorganisms for sustainable production of high value products

D.1 Development of a co-cultivation system of *Yarrowia lipolytica* and *Chlorella vulgaris*: Citric acid bioprocess as a case study

D.1.1 Outline

The objective of this chapter was to define the general parameters for co-cultivation between microalgae and non-conventional yeasts, to be later applied in MELs bioprocesses. This study reports, for the first time the use of co-cultivation with the yeast *Yarrowia lipolytic*a and the algae *Chlorella vulgaris*, as a stable mixed culture for the production of carboxylic acids, using citric acid bioprocess as a case study. To explore the synergistic effects of this system, different parameters were evaluated in shake-flasks, including the composition of culture medium (e.g. C-Source, N-Source), inoculum ratio of yeast/microalga and inoculation time of the two different microorganisms. When transferring the process into a photo-bubble column system (1-L) it was also evaluated the impact of different light regimes and intensities. Remarkably, the co-cultivation led to reproducible process with citric acid productivity of 0.13 ± 0.01 g/L/h, maximum titre of 30.43 ± 1.3 g/L and a final biomass of 9.75 ± 0.74 g/L. These results open future perspectives in the sustainable production of organic acids with simple systems.

D.1.2 Introduction

The European Union has established targets until 2050 to address climate change and promote a circular and low-carbon economy. By 2030, one of the objectives is to achieve a 40% reduction in greenhouse gas emissions compared to 1990 levels. Additionally, it aims for 32% of total energy consumption to be derived from renewable sources (Comisión Europea, 2018). As a result, microalgae, due to the capacity of converting CO_2 into valuable compounds (e.g biofuels, bioethanol) by using wastewaters/nutrient rich effluents (Ferreira et al., 2017), started to be envisioned as a potential addition of other microorganisms (yeasts/bacteria). Furthermore, this type of cultivation does not compete with agricultural systems for arable lands (Lopes et al., 2023).

Nevertheless, the maximum biomass achieved in these autotrophic processes can go up until 2-5 g/L, being not enough when compared with processes using yeasts/bacteria (> 100 g/L) and increasing the final costs of the process (Ruiz et al., 2022). In fact, Barros et al. (Barros et al., 2019) have shown that the production of biomass of *C. vulgaris* increases from 1.7 g/L (in 14 days of cultivation) to 174 g/L (in 8 days of cultivation), just by switching from autotrophic (using a light regime of 24:0 (light/dark) and periodic injections of CO_2 to heterotrophic process) to fed-batch mode using glucose as main carbon source). However, autotrophic processes led to the production of high added value products, such as pigments and lipids, and therefore, to tackle the bottleneck of low biomass productivity. Some studies started to suggest the co-cultivation of yeasts/bacteria with microalgae as a solution to overcome the challenges of sole microalgae process, since both can benefit from a symbiotic effect due to the exchange of O_2/CO_2, where O_2 is produced by microalgae, by consuming the CO_2 coming from yeast/bacteria activity.

The existing literature has predominantly focused on the development of co-cultivation of yeasts/microalgae to enhance biomass and intracellular lipids production. Within this field, Arora et al. (Arora et al., 2019), provides an insightful review which focuses in specific parameters tested for the optimization of the co-cultivation system (such as: culture medium; seed ratio of microalgae/yeast, light intensity, pH, ect). In this regard, remarkably, Angéla et al. (La et al., 2019), have developed a sustainable process based on a mixed co-culture of *Saccharomyces cerevisiae* and *Chlorella vulgaris* (both with a final cell concentration of 2 × 107 cells/mL, with a final biomass concentration of 0.23 g/L), showing that almost all the CO2 produced by the yeast was reutilized

by microalga within 168 h of cultivation. Also, Wang et al., (Wang et al., 2022) established a co-culture system of *C. vulgaris* and *R. toruloides*, obtaining 29.9 g/L of biomass and 70.54% intracellular lipids (~around 2-fold higher than the mono-cultivation of *R. toruloides*). Additionally, Li et al. (Liu et al., 2019), have co-cultured the same yeast used by Wang et al. with *C. pyrenoidosa* to improve wastewater treatment and used biomass for animal feeding, where they have shown that the co-cultivations leads to 86.65 % of total nitrogen and 53.51% of protein removed, respectively, contributing for a bioeconomy. Different from these studies, but also important for microalgae bioprocess, Xie et al. (Xie et al., 2013) have developed a fungi-algae system to create pellets (whole biomass) and allow a quick filtration, overcoming one of the major bottlenecks of this process, which is biomass harvesting and the costs associated.

In contrast to biomass or intracellular lipids (intracellular products), it was never attempted the use of co-cultivation for the production of extracellular products (e.g organic acids). And, among organic acids, citric acid (CA, 2-hydroxy-1,2,3-propane-tricarboxylic acid), the 2nd largest industrial fermentation process following bioethanol (Tong et al., 2023), stands as one of the most important bioproducts. This is due to its unique characteristics, such as low toxicity, odourless, colourless, and by the fact that it was recognized as GRAS (Generally recognized as safe) by FAO/WHO Expert Committee on food additives, allowing its industrial production and utilization in different fields, like food industry (as flavouring and preservative agent) and pharmaceutical applications (chelating, excipient) (Kamzolova, 2023; Liu et al., 2015; Morgunov et al., 2018). Commercially, the production of CA has been relied in large-scale submerged processes using the fungus *Aspergillus niger* (~200 g/L) (Tong et al., 2023; Zhou Ping-Ping et al., 2017). On the other hand, the non-conventional yeast *Yarrowia lipolytica* is able to produce a high level of concentration (180-200 g/L) with a productivity of 1-1.5 g/L/h (Morgunov et al., 2018) using a wide range of carbon sources (oil, glycerol, ethanol, sugars). In this regard, the process can offer an alternative, environmentally friendly and sustainable CA production in terms of substrate diversity, global warming potential and human toxicity (Becker et al., 2020).

Considering the fact *Y. lipolytica* was already successfully used in a co-cultivation system with microalgae (*C. pyrenoidosa*) for the production of biomass (Qin et al., 2019), where they show an increase of final biomass of algae and yeast together (co-cultivation; 5.8 g/L) in 48.7 % compared to mono-cultivation of yeast (mono-cultivation; 2.9 g/L) and the fact that this yeast it's a perfect

biological model for the production of citric acid (Becker et al., 2020; Förster et al., 2007; Kamzolova et al., 2022), we have made the decision to investigate the use of co-cultivation for the extended sustainable production of organic acids, using citric acid bioprocess as a case study.

In this section, it is reported for the first time, a co-cultivation system of *Y. lipolytica* and *C. vulgaris* as a stable and reproducible mixed culture for the production of organic acids, such as citric acid (CA) as a model target compound. The main objective of this work was to show that a co-cultivation of algae and yeasts leads to a higher productivity of CA formation compared to the mono-cultivation of yeast *Y. lipolytica*.

Overall symbiotic effects of algae and yeast components in co-cultivation should lead to reduce the amount of oxygen/external air required to produce these bioproducts by utilising the oxygen supplied by *C. vulgaris* and reducing the complexity of the bioreactor system used (photobioreactors, instead of common stirred tank bioreactors). At the same time, it was possible to define important parameters (medium selection, appropriate carbon source and ratio yeast/microalgae, light intensity and time delay of co-cultivation process), which are crucial for the development of the co-cultivation process and the transfer to any type of co-cultivation system. These parameters will be used when developing a co-cultivation process for MELs production (**section D.3)**.

D.1.3 Materials and methods

D.1.3.1 Cultivation conditions

D.1.3.1.1 Yeast and microalgae maintenance

Yarrowia lipolytica H181 (DSM 7806) was obtained from Germany collection of microorganisms and cell culture (DSMZ). Strains were plated in YPD Agar (yeast extract 10 g/L, peptone 20 g/L, D-glucose 20 g/L and agar 20 g/L) and incubated for 2 days at 30 °C. Stock cultures were prepared by propagation of yeast cells in Behrens medium (0.7 g/L KH_2PO_4, 0.35 g/L $MgSO_4.7H_2O$, 3 g/L NH_4Cl, 1 mg/L thiamine hydrochloride, 3.5 mg/L $FeSO_4.7H_2O$, 0.13 g/L $CaCL_2.2H_2O$, 0.1 g/L NaCl and 1 mL/1000 mL of trace elements Behrens), supplemented with 20 g/L of glycerol, at 130 rpm and 30ºC and after 2 days, they were stored in 20% (v/v) glycerol aliquots, at 26 °C (Behrens et al., 1987, 1978).

Chlorella vulgaris SAG 211-11b, *Dunaliella tertiolecta* SAG 13.96, *Scenedesmus rubescens* SAG 5.95, *Dunaliella salina* SAG 19/3 and *Dunaliella acidophila* SAG 2045 were obtained from culture collection of algae at Göttingen university. These strains were maintained in modified Šetlik medium (0.34 g/L KH_2PO_4, 1 g/L $MgSO_4.7H_2O$, 1.5 g/L NH_4Cl, 2.02 g/L KNO_3, 1 mg/L thiamine hydrochloride, 6.3 mg/L $FeSO_4.7H_2O$ and 10 mL/1000 mL of trace elements Šetlik). The cultures started by adding 10% ($v_{inoculum}/v_{culture\ medium}$) of stock culture to an Erlenmeyer shake flask with chicanes and 1/5 working volume (100 mL) and incubated at 26ºC with an agitation of 130 rpm, under an irradiance of 30 $\mu E/m^2/s^1$ Photosynthetic Photon Flux Density (PPFD), with a light regime of 12:12h (light/dark). The cultures were renewed with fresh culture medium every two weeks.

Composition of trace elements for Behrens and Šetlik medium can be found in supplementary data **(Table F*11*)**.

D.1.3.1.2 Yeast and microalgae pre-cultivation

The pre-inoculum preparation of yeast for all experiments performed in shake flasks **(section D.1.3.1.3)** and bioreactors (**section D.1.3.2)** started by transferring colonies of colonies of *Y. lipolytica* to an Erlenmeyer flask with chicanes and 1/5 working volume (100 mL) of modified Šetlik or Behrens medium (depending on the medium selected for main cultivation), using 20 g/L of

glycerol as carbon source, during 48 h at 26ºC and 130 rpm. The final cell number achieved was 1.38E+8 ± 4.48E+7 cell/mL.

The pre-inoculum of microalgae started by transferring 10% ($v_{inoculum}/v_{culture\ medium}$) of stock solution to an Erlenmeyer shake flask with 4 baffles and 1/5 working volume (100 mL) containing modified Šetlik medium as previously described. The cultivation was carried out for 10 days, under an agitation of 130 rpm, with an irradiance of 30 $\mu E/m^2/s^1$ PPFD and a light regime (light/dark) of 12:12h. The final cell number achieved was 5.94E+7 ± 3.13E+6 cell/mL (for *C. vulgaris* 212-2b).

D.1.3.1.3 Yeast and microalgae cultivation in shake flask.

All the experiments described on this section, were only performed in shake flask. In this regard, initially it was studied the effect of CA production on Šetlik and Behrens medium, using *Y. lipolytica* H181. After 2 days of pre-culture cultivation (as described in **section D.1.3.1.2**), 10% yeast ($v_{inoculum}/v_{culture\ medium}$) was added to an Erlenmeyer shake flask with 4 baffles and 1/5 of volume (100 mL). Medium was supplemented with 20 g/L of pure glycerol (> 99.5% glycerol content), followed by 5 feedings of each 20 g/L glycerol (40, 96, 168 and 216 h) during 240 h cultivation time. Afterwards, all conditions tested in shake flasks used modified Šetlik medium, supplemented with 50 g/L of glycerol in the beginning and followed by feedings of each 20 g/L of glycerol when the concentration of glycerol was below 20 g/L, during 240 h (10 days) cultivation, at 130 rpm and 26ºC. In another set of experiments, using the same conditions descried above, it was evaluated the use of different carbon sources: glucose, glycerol and two fractions of crude glycerol (P105 and RO70104), supplied by the company Glaconchemie GmbH, Germany. The composition of these two fractions can be found in **Table F*12***.

For the co-cultivation experiments, initially different inoculum ratios ($v_{inoculum}/v_{culture\ medium}$) of *Y. lipolytica* and *C. vulgaris* were tested with 100 mL working volume in shake flask with 4 baffles: 1) **10%Y - 0%A (Control):** 10% of yeast (10 mL) was added to the shake flask; 2) **0.01%Y - 0%A (control):** 0.01 % (0.01 mL) of yeast was added to the shake flask; 3) **0%Y - 10%A (control):** 10% (10 mL) of algae was added to the shake flask and 4) **0.01%Y - 10%A :** 0.01% (0.01 ml) of yeast and 10% (10 mL) of microalgae were added to the shake flask. All conditions started with 50 g/L of glycerol, and feedings of each 20 g/L of glycerol were added when the concentration was below than 10 g/L, during 240 h.

The next experiment involved the study of time decoupling of the inoculation of the yeast and microalgae cultures (0, 2, 5, 7 and 10 days of delay). From this point, the % of yeast inoculum used in co-cultivation was always 0.01% $v_{inoculum}/v_{culture\ medium}$. In the moment of yeast addition, it was also added NH_4Cl (1.5 g/L), thiamine hydrochloride (1mg/L), $FeSO_4.7H_2O$ (6.3mg/L), trace elements (1 mL) and 50 g/L of glycerol. All cultures were incubated at 26ºC, with an agitation of 130 rpm, under an irradiance of 30 $\mu E/m^2/s^1$ PPFD and a light regime of 12:12h (light/dark). All cultivations were performed in two biological replicates.

D.1.3.2 Photobioreactor conditions

The experiments were performed in a multi-photobioreactor system with four culture vessels in bubble column design (xCUBIO, bbi biotech, Germany), using 1 L working volume with modified Šetlik medium. The temperature was kept at 26ºC and mixing was carried out using a sparger with compressed air at 2 vvm and a magnetic stir bar of 120 rpm. The pH was controlled at 6.5, using a solution of 3% NaOH (0.774 mol/L). Dissolved oxygen (DO) was monitored continuously. Cultures were irradiated with an intensity of 50% of light (corresponding to 500 $\mu E/m^2/s^1$ PPFD) with a light regime of 12:12 or 16:8 (light/dark), depending on the condition used as listed below.

Initially, it was studied mono-cultivations of *Y. lipolytica* at different vvm (0.2, 0.5, 1,0, 1.5 and 2 vvm) starting with an inoculum of 0.01 % of yeast ($v_{inoculum}/v_{cultured\ medium}$) and supplemented with 50 g/L of glycerol, during 240 h. Except the condition using 0.2 vvm, all others required one feeding of 20 g/L glycerol at day 6, (to avoid a concentration below than 10 g/L). The light regime used was 12:12 (light/dark).

Mono-cultivations of *C. vulgaris* (15 days (360 h)) were also performed in the multi-photobioreactor system. Firstly, it was varied the type of nitrogen source (only NH_4Cl, KNO_3 or both together) using the same approach. Then, using only KNO_3 as nitrogen source and the same light regime (12:12, light/dark), it was tested the use of 50% of light intensity for 5 days, and in the rest 10 days, 75% (800 $\mu E/m^2/s^1$ PPFD) of light was chosen (**Figure F28**).

Co-cultivation of algae and yeast was carried out with time decoupling of the algae and yeast inoculation, starting with 10% (v/v) of *C. vulgaris*, for 5 days. After 5 days, *Y. lipolytica* was added

to the bioreactor (0.01% $v_{inoculum}/v_{culture\ medium}$), and supplemented with NH_4Cl, thiamine hydrocholoride, $FeSO_4.7H_2O$, trace elements and 50 g/L of glycerol. The cultures were irradiated with 50% of light intensity, where it was tested different light regimes: 12:12 and 16:8 (light/dark). Similar to these conditions, it was also tested the use of 20% algae inoculum, instead of 10%. For this specific case, the co-cultivation started after 3 days of mono-cultivation of *C. vulgaris*, since it is achieved the same cell number as observed for 10% with 5 days of cultivation. Through all co-cultivation time, feeds of glycerol were fed to the bioreactor (20g/L) to avoid a concentration below of 10g/L. All these conditions previously described were performed in biological duplicates.

In another set of experiments, using a light regime of 16:8 (light/dark), it was varied the percentage of inoculum microalgae used (10 and 20% $v_{inoculum}/v_{cultured\ medium}$), under conditions of replacing glycerol (pharma quality) by crude glycerol (Fraction P105). However, no replicates were performed.

D.1.3.3 Parameters determination

For all experiments performed, as described in figures and tables, different parameters for cell and product formation were determined (titre, yield, final ratio microalgae/total cell number), with especially emphasis for two types of productivity: 1) CA Productivity for the phase of co-cultivation algae and yeast (**Equation *14***), and 2) overall CA productivity (**Equation *15***), considering the total time of cultivation (phase of mono-cultivation algae and co-cultivation), respectively.

Furthermore, once per day of cultivation time, yeast and microalgae cells count were determined with a haemocytometer (Thomas, LW scientific) using the microscope (Axiostar plus, Zeiss). **Equation *16*** describes how cell number/mL is calculated. In the end of each cultivation, final biomass concentration of *Y. lipolytica* and *C. vulgaris* was measured with the aid of a MA40 moisture meter (Sartorius) at 105 °C, after filtering 10 to 40 ml of culture broth through a membrane filter (cellulose acetate, 0.45 µm; Sartorius Stedim Biotech S.A.). Note that every sample, after being filtrated, was flushed with water two times.

$$Productivity\ co-cult = \frac{Titre\ of\ CA}{Time\ of\ co-cultivation}$$

Equation 14: Citric acid productivity for the phase of co-cultivation.

$$Overall\ productivity = \frac{Titre\ of\ CA}{Total\ time\ of\ cultivation}$$

Equation 15: Overall citric acid productivity.

$$\frac{Cell\ number}{mL} = \frac{\left(\frac{Cell\ count}{2}\right) x\ Df\ x\ 1000}{4 * 0004}$$

Equation 16: Determination of cell number.

D.1.3.4 Determination of volumetric mass transference coefficient for oxygen (kLa)

To determine the volumetric mass transference coefficient for oxygen the K_La of the bubble column (**Equation *1***), 1 L working volume with modified Šetlik medium was used. K_La was measured at different aeration rates (0.2, 0.5, 1, 1.5 and 2 vmm). The temperature was kept at 26ºC, using an agitation of 120 rpm, provided by a stirrer magnet. The method used to determine the coefficient was dynamic method, as described by Linek et al. (Linek et al., 1993).

D.1.3.5 Determination of substrates, organic acids, anions and cations concentration

Determination of glycerol and glucose were performed using the enzymatic test kit (R-Biopharm, Darmstadt, Germany), where the protocols can be found online. Anions (CA, nitrate, phosphate) and cations (ammonium, sodium and potassium) were analysed using the ICS-5000+DP ion chromatography system (Thermo Fisher Scientific; Germany). For anions: Column IonPac AS 19 with 2 mm in diameter (precolumn: IonPac AG 19), using potassium hydroxide (KOH) as eluent (0–5 min isocratic at 10 mM, 5–15 min linear to 40 mM, 15–28 min isocratic at 60 mM, 28–40 min isocratic at 10 mM) at a flow rate of 0.25 mL/min and 20 °C, with a sample injection volume of 5

µl. For cations: Column IonPac CS 12 with 2 mm in diameter (precolumn: IonPac CSG12A), using methane sulfonic acid (MSA) as eluent (0–12 min isocratic at 5 mM, 12–27.5 min linear to 20 mM, 27.5–30 min isocratic at 5 mM) at a flow rate of 0.25 mL/min and 20 °C with a sample injection volume of 5 µL. Both anions and cations were quantified with the software Chromeleon 7.2.4 (Thermo Fisher Scientific, Germany) using calibration curves.

D.1.3.6 Statistical analysis

Statistics were performed for comparing CA productivities and cell number/mL obtained in different conditions (**Figure D3** and **Figure D4; Figure F25 Figure F*26*)**, using Graph-pad, by analysis of variance (one-way or two-way ANOVA) and p-values of the differences between groups were corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p \leq 0.05$.

D.1.4 Results and discussion

In the development of a co-cultivation system with microalgae and yeasts various parameters need to be considered due to the different requirements of the microorganism. Certain ones take priority over others (strain selection, C-source for heterotrophic yeast), including: 1) Definition of the culture medium and selection of a promising N-source for microbial growth, 2) Inoculum ratio of yeast/microalgae and timing of algae inoculation and 3) Determining appropriate light regime and intensity. In this regard, during the following sections the objective is to develop a co-cultivation system that enables CA production, which can subsequently be optimised and used as a reference for the production of other organics acids.

D.1.4.1 Selection of algae strain and hetetrotrophic C-source for the yeast.

The algal strain *C. vulgaris* was selected from a tested group of lipid-accumulating algae (*D. salina, D. acidophila, S. rubescens, D. tertiolecta, and C. vulgaris*). However, the focus was not on lipid formation for now, but on finding a robust and easy-to-cultivate algae strain for co-cultivation with yeast. These experiments were carried out with combining 10% of inoculum of microalgae and *Y. lipolytica* each, using glucose as carbon source, during 186 h. Among all strains, *C. vulgaris* 212-2b was chosen as the candidate to develop a co-cultivation system (**Figure D1**), due to the highest cell number/mL achieved ($5x10^5$ cell number/mL) in comparison with the other microalgae strains (< 40%). This explanation can be associated to the resistance of *C. vulgaris* to acidic pH (3-4) (Savvidou et al., 2021), since in the first 48 hours of co-cultivation the pH tends to drop to values of < 3, even though if the pH was adjusted to 6 every day. However, the final ratio of *C. vulgaris* 212-2b (microalgae $_{\text{cell number/mL}}$/total cell $_{\text{number/mL}}$) was 0.20%, an extremely negligible value. This result can be attributed to the contrasting growth of *Y. lipolytica* with a rate of 5.76 per day (Workman et al., 2013) and *C. vulgaris* (without supplementation of external CO_2) with a rate of 0.15 per day (Li et al., 2021). This difference in growth rates, with *Y. lipolytica* growing 38-times faster, leads to an overpopulation of the yeast and can influence the light irradiance and intensity reaching the *C. vulgaris* cells, reducing their photosynthetic activity and had to be optimised in the following steps.

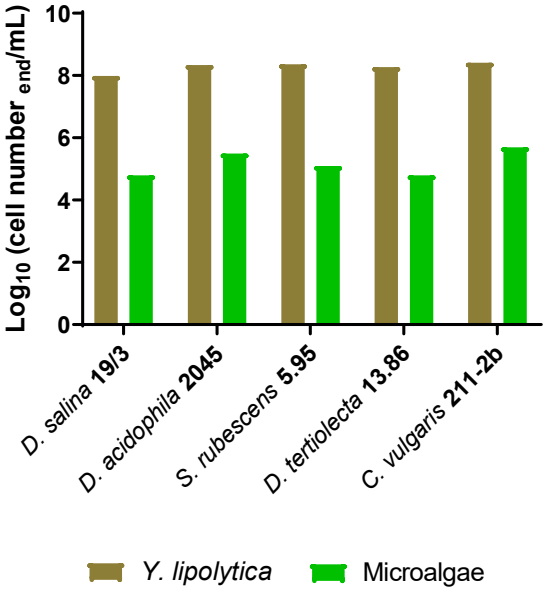


Figure D1: Co-cultivation of *Yarrowia lipolytica* H181 (brown bars) with different microalgae strains (*Dunaliella salina* 19/3; *Dunaliella acidophila* 2045; *Scenedesmus rubescens* 5.95; *Dunaliella tertiolecta* 13.86 and *Chlorella vulgaris* 211-2b), where it is represented the final cell number/mL. Co-cultivation was carried out for 184 h, starting with 50 g/L of glucose. The temperature was kept at 26ºC, using an agitation of 130 rpm, and a light regime (light/dark) of 12:12, with an intensity of 60 µmol/(s·m²). pH was manually controlled at 6.5 every day, using 27 or 25% of NaOH and H_2SO_4, respectively.

Before starting any developments in a co-cultivation system, it is still necessary to select an efficient carbon source to use. As previously described *Y. lipolytica* can metabolize a wide range of substrates, including vegetables oils/side streams enriched in lipids, alcohols (ethanol) and different sugars (glucose, glycerol). However, lipidic substrates were excluded, as these are insoluble in water and can hinder the passage of light in the culture medium (which reduces the photosynthetic activity of microalgae) and the use of alcohols, which in excess can be toxic to microalgae cells. In this regard, the use of different soluble carbon sources (glucose, glycerol, and crude glycerol) was evaluated in mono-cultivations of *Y. lipolytica*. **Table F*13*** represents the final titres/productivities, which indicate that there are no significant differences in overall productivity among all sugar types used. Recently, Becker et al. (Becker et al., 2020), performed a life-cycle analysis for the CA production with *Y. lipolytica* from different carbon sources, where they have shown that the use of crude glycerol as carbon source leads to a higher environmental

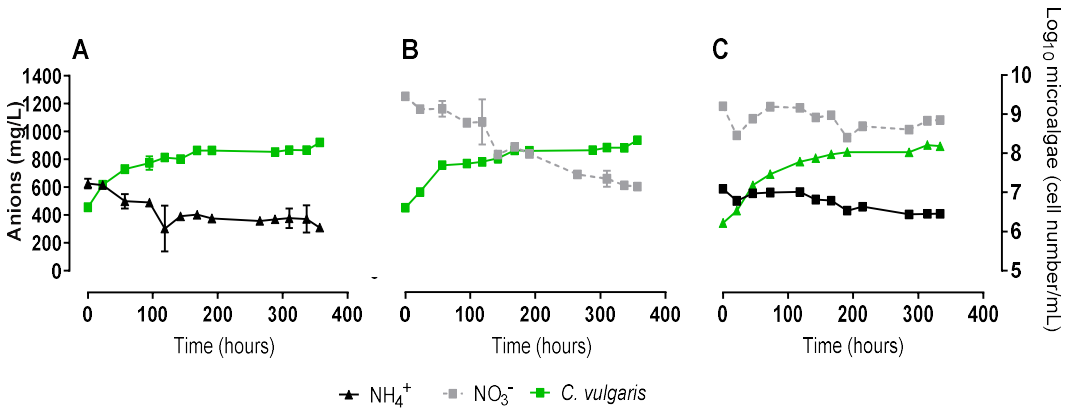
performance, when compared with glucose. Furthermore, crude glycerol can be used in non-sterile processes (more difficult to be consumed by other microorganisms), so glycerol, and later crude glycerol was selected as the main carbon source for use in co-cultivation systems.

D.1.4.2 Medium design and N-source selection.

It is well known that the production and extracellular accumulation of CA by yeast *Y. lipolytica* is induced by growth-limiting conditions, preferably as a limitation of nitrogen source (Cavallo et al., 2017; Kamzolova, 2023; Kamzolova et al., 2022; McKay et al., 1994)Click or tap here to enter text.. This yeast is only able to utilize inorganic N-sources in the form of ammonium ions such as NH_4Cl and $(NH_4)_2SO_4$ but not as nitrate ions (Heard, 1999; Kamzolova, 2023). On the other hand, green algae, such as *Chlorella vullgaris,* are able to utilize ammonium as well as nitrate ions as a source of nitrogen (De Lourdes et al., 2017; Pozzobon et al., 2021). This information was used for the design and composition and of the nutrient medium for co-cultivation. Preferably, the medium should provide NO_3^- ions for the growth of *C. vulgaris* and NH_4^+ ions for the limited growth of *Y. lipolytica* with depletion of the latter leading to accumulation of CA.

In the literature there are plenty of culture media used for the production of CA and also for the growth of different microalgae strains. For this specific study, Behrens medium was used, which is commonly used several times for the production of CA with yeast *Y. lipolytica* (Behrens et al., 1987, 1978; Förster et al., 2007). This medium was developed for ammonium nitrogen limited induction of citrate formation by *Y. lipolytica*. A modified Šetlik medium was used for the growth of microalgae, previously used for the growth of *C. vulgaris* (Šetlík et al., 1972). Therefore, the first experiments performed were to test the influence of these two-culture media (Behrens and modified Šetlik medium) on growth and product formation (CA) with *Y. lipolytica*. In **Figure F25** it is possible to observe that in terms of yeast cultivation with Šetlik medium the production of CA leads to 30% of decrease (46.6 ± 2.5 and 31.1 ± 1.2 g/L, for Behrens and Šetlik medium, respectively). Nevertheless, knowing that *C. vulgaris* shows a poor growth in Behrens medium (data not shown), from this point on, all experiments were performed with modified Šetlik medium.

So far, it was already shown the suitable algae strain, culture medium and carbon source in co-cultivation with *C. vulgaris* and *Y. lipolytica* in shaking flasks. Still, there is one more parameter that can be optimized prior to the co-cultivation, related with the nitrogen source. While *Y. lipolytica* is only able to metabolise ammonium derivatives, *C. vulgaris* can consume both, where ammonium is rapidly uptake (GS/GOGAT cycle) and nitrate needs first to be converted in ammonium (Pozzobon et al., 2021). So, the impact of growing *C. vulgaris* with NH_4Cl, KNO_3 or both together was studied after transferring the process in a photo bioreactor system. In **Figure D2** it is possible to observe after 15 days, that the use of separated nitrogen sources leads to higher consumption of NO_3^- and NH_4^+ and double the final biomass compared to the cultivation with both nitrogen sources together. These results have shown that the growth of *C. vulgaris* increases when one single nitrogen source is used. Since *Y. lipolytica* can only consume NH_4^+ derivatives, this will allow to cultivate *C. vulgaris* in the presence of $NaNO_3$ and only add NH_4Cl in the moment of yeast addition.



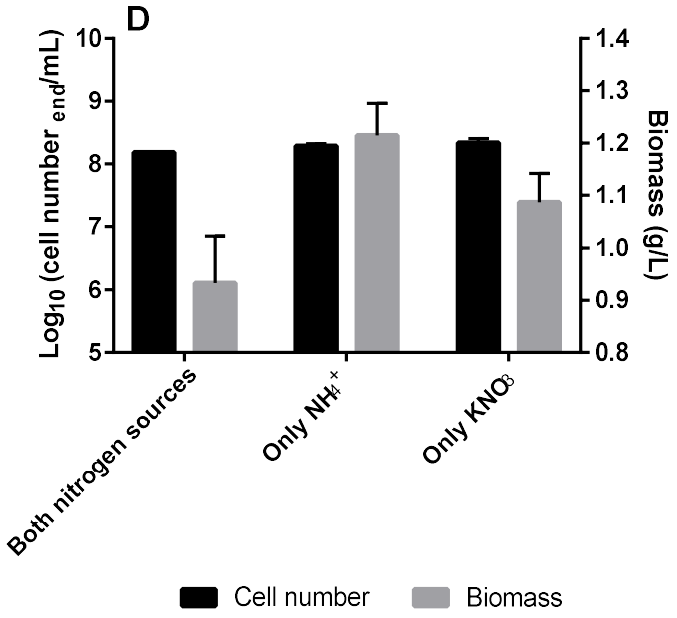


Figure D2: Cultivation of *C. vulgaris* 212-2b, varying the nitrogen source. A) Using only NH_4Cl (1.5 g/L); B) Using only KNO_3 (2.02 g/L); C) using both nitrogen sources and D) final biomass for each condition. Experiment was performed in bioreactors during 15 (A, B) and 14 (C) days, at 26ºC, using 2 vvm and 130 rpm (provided by a magnet). It was used a light regime (light/dark) of 12:12, with an intensity of 500 µmol/(s·m²). pH was manually controlled at 6.5 every day, using 27 or 25% of NaOH and H_2SO_4, respectively.

D.1.4.3 Inoculum ratio of yeast/microalgae and timing of inoculation

To allow a faster growth of *C. vulgaris* under conditions of co-cultivation, one of the ideas was to reduce the percentage of yeast culture at the beginning of cultivation. In this regard a parallel experiment was performed (**Figure F*26*** and **Table F*14***), using 0.01% of *Y. lipolytica* in mono- and co-cultivation, instead of 10%. Interestingly, the decrease in inoculum yeast did not affect final productivity (0.121 ± 0.004 g/L/h) when compared with the condition using 10% of yeast inoculum (10Y%-0%A; 0.130 ± 0.005 g/L/h). Also, the decrease in inoculum of *Y. lipolytica* from 10 to 0.01% allowed to increase the final ratio of *C. vulgaris* (microalgae $_{\text{cell number/mL}}$/total $_{\text{cell number/mL}}$) from

0.20% to 4.56 ± 0.2%. However, the final cell number (2.81E+7 ± 3.15E+6 cell/mL) is 83% lower than the one observed in mono-cultivation of *C. vulgaris* (0%Y-10%A; 1.60E+8 ± 4E+6), still revealing some stress provoked to microalgae cells (low light intensity reached to cells). In literature, Chioke et al. (Chioke, 2021),in order to develop a co-cultivation system using *C. lewinni*, have tested different ratios microalgae/yeast (30:1, 40:1, 60:1, 80:1 and 100:1), finding that the best ratio is 30:1, which is similar to the one here used (20:1).

Nevertheless, even decreasing the percentage of yeast added to the co-cultivation system, the growth observed for *C. vulgaris* it is still very low, when compared with *Y. lipolytica*, meaning the yeast is still growing faster than the microalgae, leading to a overpopulation of microalgae cells, and consequently light obstruction, which potentially inhibits photosynthetic activity. In this regard and in order to give *C. vulgaris* a head start for growth, a decision was made to delay the addition of *Y. lipolytica,* allowing *C. vulgaris* to grow independently for different specific days (0, 2, 5, 7 and 10) using only KNO_3 as nitrogen source, as previously discussed (**Section D.1.4.2**). After these days, 0.01% ($V_{inoculum}/V_{culture\ medium}$) of *Y. lipolytica* inoculum was added, followed by nutrients (see **section D.1.3.1.3**) and glycerol (50 g/L), with feeds of 20 g/L in specific days to avoid a concentration bellow than 10 g/L. The co-cultivation was carried out for 10 days.

For these experiments, two types of productivity were determined for evaluation and discussion: 1) Productivity only during the time co-cultivation, considering 10 days after inoculation of the yeast and 2) Overall productivity, considering the total time of cultivation from the beginning; means total time of mono-cultivation algae and co-cultivation of algae and yeast. In **Figure D3,** when the co-cultivation is delayed until the 5^{th} day, there are no differences in both types of CA productivity. However, as the mono-cultivation of *C. vulgaris* is extended to 7 and 10 days, the CA productivity in co-cultivation increases up to 81%, without statistically differences among them. Moreover, analysing the values obtained for the ratio of microalgae over total cell number, is it evident that after 5 days, the values exceed 7%, with special focus for 7 days, where they reach almost 10%.

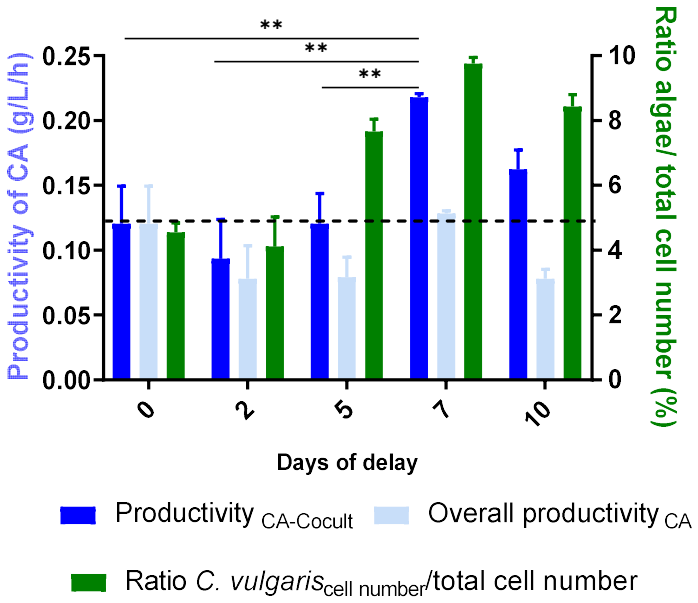


Figure D3: De-coupling the co-cultivation process, where for specific days (0, 2, 5, 7 and 10), it was performed a mono-cultivation of *C. vulgaris* using KNO_3 as nitrogen source. Afterwards, co-cultivation started with the addition of the yeast (0.01% $V_{inoculum}/V_{culture\ medium}$), NH_4Cl (1.5 g/L), thiamine (1 mg/L), $FeSO_4.7H_2O$ (6.3 mg/L), glycerol (50 g/L) and trace elements (1mL). Co-cultivation was carried out for 240 h (10 days), followed by feeds of glycerol (20 g/L), to avoid a concentration below than 10 g/L. The temperature was kept at 26ºC, using an agitation of 130 rpm, and a light regime (light/dark) of 12:12, with an intensity of 60 µmol/(s·m²). pH was manually controlled at 6.5 every day, using 25 and 27% of H2SO4 and NaOH, respectively. Productivity $_{CA-Cocult}$, it is calculated based only on the time after yeast addition (dark blue bar), whereas overall productivity also considers the time of *C. vulgaris* mono-cultivation (light blue bar). It was used a light regime (light/dark) of 12:12 h, with an intensity of 60 *µmol*/(s·m²). * $p \leq 0.042$; ** $p \leq 0.002$; *** $p \leq 0.0006$.

These findings suggest that the delaying the addition of yeast *Y. lipolytica* in the co-cultivation process plays a crucial role in promoting enhanced microalgae growth and consequently, increasing the production of oxygen by *C. vulgaris*. Interesting, when the mono-cultivation of *C. vulgaris* is extended to 7 days, the initial ratio algae/yeast was 800:1, 13-fold higher than the optimized value used by Chioke et al. (Chioke, 2021).Therefore, next experiments of co-cultivation will start with an initial mono-cultivation of *C. vulgaris* for specific days, allowing a faster growth.

Based on these results, it was discovered that to achieve a good interaction between *Y. lipolytica* and *C. vulgaris*, it is necessary to decouple the co-cultivation process, initially starting with mono-cultivation of *C. vulgaris* for specific days, allowing to achieve more biomass of microalgae, before co-cultivation starts. Afterwards, the percentage of *Y. lipolytica* inoculum used to start co-cultivation will be 0.01% instead of 10%.

D.1.4.4 Transfer of algae-yest co-cultivation to a photobioreactor system

The results at shaking flask scale have provided a platform for a successful co-cultivation of *Y. lipolytica* and *C. vulgaris.* However, prior to transferring into photo-bioreactors, it is necessary to find the appropriate conditions for the yeast-based production of CA in photobioreactor system, and to realise how light intensity will affect the growth of *C. vulgaris*, when cultivated under conditions of mono-cultivation.

D.1.4.4.1 Characterization of photobioreactor at mono-cultivation of at mono-cultivation of *Y. lipolytica* and *C. vulgaris*

Initially, the production of CA was carried out using a mono-cultivation of *Y. lipolytica* in the photobioreactor as a bubble column system with different airflows (0.2, 0.5, 1, 1.5 and 2 vvm) (**Figure F27** and **Table D1**). As predicted, there is only CA production for conditions with a an air-flow higher than 1 vvm. If using 2 vvm, an overall productivity of 0.07 ± 0.01 g/L/h is achieved, a value 93% lower (1-1.5 g/L/h in a standard stirring bioreactor) than reported by Morgunov et al. (Morgunov et al., 2018). These results are explained by the low oxygen gas diffusion observed in bubble columns (2 vvm, kL_a 50 h^{-1}), as shown in **Figure F*23*.**

Parallel to this experiment, a mono cultivation of *C. vulgaris* was performed, in which 2 vvm were used and two different light intensities were tested: 1) Keeping at 50% (**Figure F*28* A**) and 2) Switching from 50% to 75% after 5 days (**Figure F*28* B**). The main objective was to promote a faster growth of the microalgae by increasing the light intensity. However, no differences were observed in the cell number, nor in the biomass values (1.09 ± 0.01 and 1.05 ± 0.02 g/L,

respectively). The intensity of light with different vvm was also quantified, and can be seen in **Figure F*24***.

Table D1: Citric acid maximum titre (g/L); Productivity in co-cultivation (g/L/h) and overall productivity (g/L/h); final biomass (g/L) cell number for different conditions (the % of yeast added was 0.01% $v_{inoculum}/v_{culture\ medium}$): Mono-cultivation of *Y. lipolytica* in bioreactors, using different vvm (0.2; 0.5; 1; 1.5 and 2); co-cultivation of *Y. lipolytica* H181 and *C. vulgaris* 212-b, using different light regimes (12:12 or 16:8, light/dark), different carbon sources (pure vs crude glycerol) and different % of C. vulgaris inoculum (10 vs 20%).

Experiment	Condition	Total time (h)	Time of yeast addition (h)	Titre max (g/L)	Productivity $_{co\text{-}cultivation}$ (g/L/h)	Overall productivity (g/L/h)	Final Biomass (g/L)
Different vvm (mono-cultivation of *Y. lipolytica*)	0.2	248	0	0 ± 0	-	0 ± 0	1.47 ± 0.23
	0.5	248		0.07 ± 0.03	-	0 ± 0	2.54 ± 0.21
	1	237.5		0.17 ± 0.02	-	0 ± 0	11.34 ± 0.93
	1.5	237.5		11.46 ± 2.09	-	0.05 ± 0.01	12.6 ± 0.76
	2	241.17		17.36 ± 2.78	-	0.07 ± 0.01	10.63 ± 3.31
Co-cultivation using pure glycerol (2 vvm)	12:12 - with 10%	359	124	22.38 ± 1.72	0.1 ± 0.01	0.06 ± 0	12.84 ± 0.94
	16:8 - with 10%	355	121	30.43 ± 1.3	0.13 ± 0.01	0.09 ± 0	9.75 ± 0.74
	16:8 - with 20%	365	69	30.19 ± 0.88	0.11 ± 0	0.09 ± 0	10.7 ± 0.5
Co-cultivation using crude glycerol (2 vvm)	16:8 - with 10%	359	118	19.67	0.08	0.05	14.36
	16:8 - with 20%	334	68	25.75	0.1	0.07	14.71

D.1.4.4.2 Influence of light/dark regime on co-cultivation process

Afterwards, co-cultivation was started in the bubble column photobioreactor system with the de-coupling of the co-cultivation process and using 0.01% of *Y. lipolytica* inoculum, as previously discussed in **section D.1.4.2**. However, here *C. vulgaris* has grown solo for 5 days rather than 7 (**Figure D3**), as the cell number/mL is constant from day 5 onwards, as was observed in mono-cultivation of *C. vulgaris* (**Figure F*28* A).**

Therefore, two alternative light/dark regimes (12:12 and 16:8, (light/dark)) were performed with a decoupled co-cultivation process for 5 days. The condition using co-cultivation and a light/dark of 12:12 (light/dark), leads to an increase in CA-productivity$_{co\text{-}cult}$ of 42.9 % (0.1 ± 0.01 g/L/h) (**Figure D4**) compared to mono-cultivation of *Y. lipolytica* in bubble columns (0.07 ± 0.01g/L/h) (**Figure F*29* A, B**). However, when considering the overall productivity for both conditions, there are no differences in the profile of CA production. Remarkably, when using a light regime of 16:8h (light/dark), the productivity$_{co\text{-}cult}$ increased to 85.7% compared with mono-cultivation of *Y. lipolytica* in bubble coloumns and 30% compared with the process using a light regime of 12:12 h, respectively. These results can be explained by the higher photosynthesis activity when the number of hours of light cycles increases from 12 to 16 h, which will lead to more production of oxygen by *C. vulgaris.* Since the production of CA dependents on the oxygen availability (**Figure F27**), any increasement in oxygen will be consumed by *Y. lipolytica.* These results show a possible interaction between both microorganisms, and the potential of using this system for the production of CA or others possible organic acids in simple photobioreactor systems. Nevertheless, the best CA-productivity$_{co\text{-}cult}$ achieved in bubble colums (0.13 ± 0.01 g/L/h using 16:8 (light/dark) light regime) is 40.3% lower than the highest value obtained in shake flasks (0.22 ± 0.02 g/L/h, **Figure D3)**, reflecting the low gas transference observed in this type of bioreactors, as previously discussed.

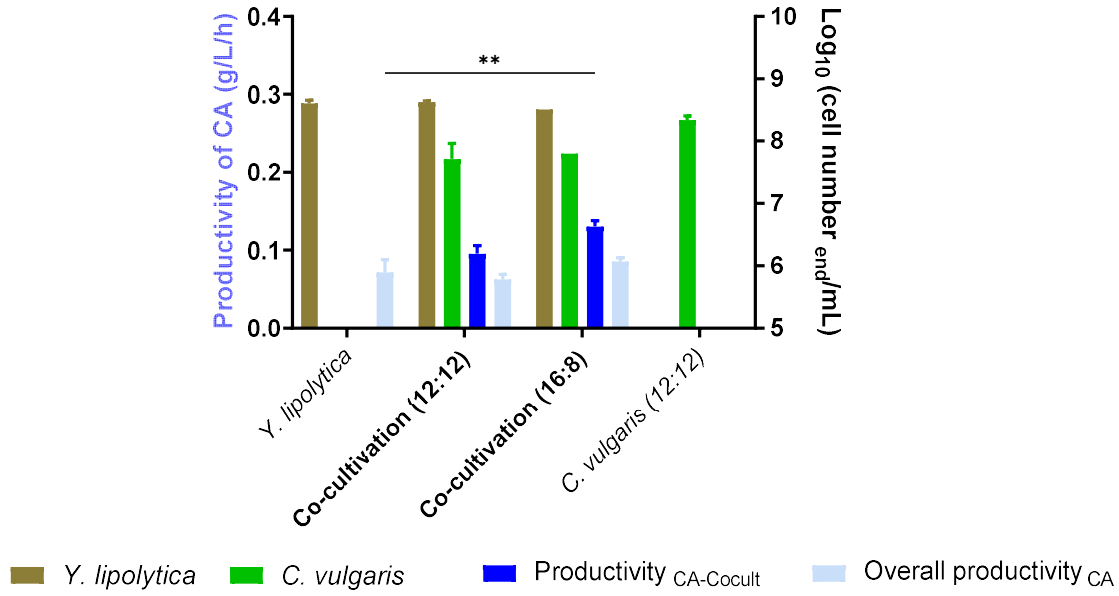


Figure D4: Production of citric acid, using different light regimes (12:12 and 16:8) and compared with mono-cultivations of *C. vulgaris* 212-2b and *Y. lipolytica* H181. Mono-cultivation of C. vulgaris 212-2b was carried out during 5 days, and afterwards, co-cultivation started with the addition of the yeast (0.01% $V_{inoculum}/V_{culture\ medium}$), NH_4Cl (1.5 g/L), thiamine (1 mg/L), $FeSO_4.7H_2O$ (6.3 mg/L), glycerol (50 g/L) and trace elements (1mL) were added into the bioreactor. Co-cultivation was carried out for 240 h (10 days), followed by feeds of glycerol (20 g/L), to avoid a concentration below 10 g/L. The temperature was kept at 26ºC, using an agitation of 120 rpm (provided by an stirrer magnet) and an air flow rate of 2 vvm. pH was automatically controlled at 6.5 using a 3% of NaOH. It was used light intensity of 500 µmol/(s·m²). For each condition it is shown final yeast (brown bar) and microalgae (green bar) cell number, as well Productivity $_{CA\text{-}Cocult}$ (dark blue bar) and overall productivity. It was used a light intensity of 500 *µmol*/(s·m²). * p ≤ 0.042; ** p ≤ 0.002; *** p ≤ 0.0006.

In order to even promote more interaction between *C. vulgaris* and *Y. lipolytica*, it was decided to increase the % of *C. vulgaris* inoculum, from 10% (**Figure D5 A, B**) to 20% (**Figure D5 C, D).** For this experiment the optimised conditions were used, starting with 2 vvm and using a light regime of 16:8 h (light/dark), however while for 10% the co-cultivation started after 5 days of mono-cultivation of *C. vulgaris*, for 20% it started after 3 days, since it reached the same cell number (8.75E+7) as the condition using 10 % on day 5 (6.41E+7 cell number/mL). By comparing the patterns of CA production, particularly in the last two data samples highlighted with red traced

lines ((**Figure D5 C,D**), it seems that the productivity of condition using 20% (0.21 ± 0.07 g/L/h) its 70% higher than the condition using only 10% (0.12 ± 0.05 g/L/h). This might indicates a potential for increase in CA production under 20% condition. However, there are no differences in productivity$_{co\text{-}cult}$ and overall productivity, and more data are needed in future.

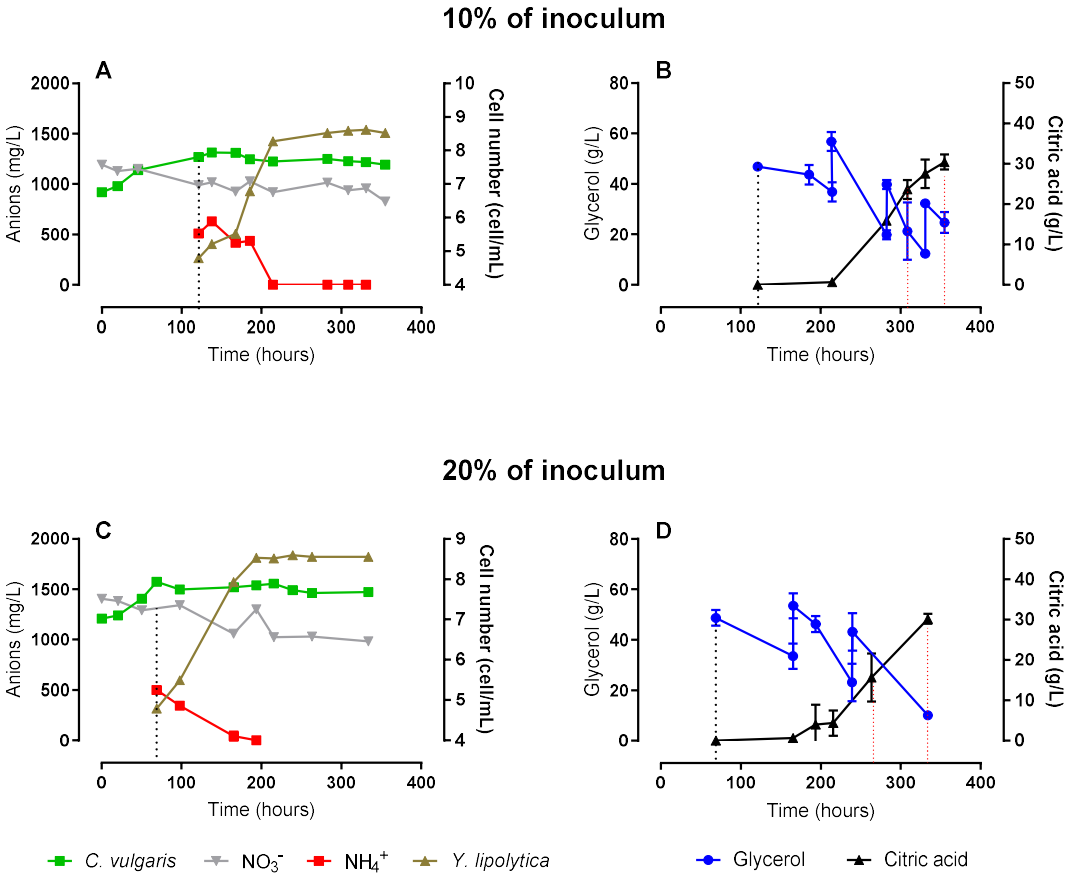


Figure D5: Citric acid production was carried out through co-cultivation of *Y. lipolytica* H181 and *C. vulgaris* 212-2b, using pure glycerol as carbon source. It was tested the effect of varying the initial % of *C. vulgaris* inoculum, 10% (A, B) and 20% (C, D). The co-cultivation process began by adding 0.01% (0.01% $V_{inoculum}/V_{culture\ medium}$) along with NH_4Cl (1.5 g/L), thiamine (1 mg/L), $FeSO_4.7H_2O$ (6.3 mg/L) and crude glycerol (50 g/L). Co-cultivation for the 10% inoculum started on the 5th day, while for the 20% inoculum, it started on the 3rd day. The duration of cultivation was 354 hours and 334 hours, respectively. Glycerol feed of 20 g/L was added at 213, 282, and 330 hours for the 10% inoculum, and at 165 and 239 hours for the 20% inoculum. The temperature was maintained at 26ºC with an air-flow rate of 2 vvm and agitation at 120 rpm using a magnet. The pH was automatically controlled at 6.5 with 3% NaOH solution. The light regime was set at

12:12 (light/dark) with an intensity of 500 µmol/(s·m²). Ion NH_4^+ (squares), ions NO_3^- (inverted triangles), (inverted triangles), yeast (hexagons) and algae cell number (hexagons) and (A, C); Glycerol consumption (circles) and citric acid production (triangles) (B, D). Arrow dashed line indicates the beginning of co-cultivation (addition of yeast to the bioreactor). Standard deviations values lower than 1 g/L are not represented.

D.1.4.4.3 The use of crude-glycerol as carbon source in co-cultivation system

In the end, to achieve a more sustainable process, it was decided to replace pure glycerol by crude glycerol, while maintaining optimised conditions, same light/dark (16:8 h) and comparing also the effect of starting with 10% (**Figure F*30* A, B**) and 20% (**Figure F*30* C, D**) of *C. vulgaris* inoculum. Interesting was this time, that there is a clear difference between these two conditions. When using 20% of *C. vulgaris* the CA-productivity$_{co\text{-}cult}$ is 25% higher (0.1 g/L/h) when using 10% of inoculum (0.08 g/L/h). However, the values obtained here are 23% lower than the best values achieved with pure glycerol with 10% of *C. vulgaris* inoculum (0.13 ± 0.01 g/L/h).

This might be explained by some inhibitors presented in crude glycerol (where the purity its around 80%, for both fractions; see **Table F*12***). Nevertheless, with crude glycerol the final biomass achieved is 14 g/L, which is the highest obtained in this study (35% higher when compared with mono-cultivation of *Y. lipolytica* in photobioreactors; 10.63 g/L), and open new perspectives for the use of this system for animal protein production, since both microorganisms used in this study (*C. vulgaris* and *Y. lipolytica*) are already recognized as safe (GRAS) by regulatory agencies (U.S Food and drug administration and the European Food Safety Authority), which allows their use in food alternative products.

Overall, the parameters here defined and selected (culture medium; percentage of yeast inoculum; time of yeast delay; light regime) improved, for the same system (photobioreactor), the final productivity of CA when compared with mono-cultivation of *Y. lipolytica.* Consequently, these parameters can further be used for other types of co-cultivation (especially when its used two microorganisms with different growth rates, which is always the case of yeasts/bacteria when co-cultivated with microalgae), with focus in producing extracellular products.

Unfortunately, due to technical errors, it was not possible to determine CO2 emissions. However, when comparing the patterns of both experiments, it appears that co-cultivations lead to a reduction of CO2, as it was expected. In the future, it is essential to repeat the best condition obtained herein obtained, and compare with mono-cultivation of yeast, to accurately measure CO2 emissions.

Nevertheless, it's clear that the co-cultivation here reported can't compete with the actual process already established for the production of CA (where the productivity ranges from 1-1.5 g/L/h, 80% higher than the best value reported here for co-cultivation). Moreover, the results obtained when using crude glycerol are interesting in the context of a biorefinery, where more than one product with high value product can be achieved (CA and biomass for food application). However more studies are required for nutritional analysis of biomass after co-cultivation.

D.1.5 Final remarks

Co-cultivation systems of microalgae (autotrophic) and other microorganism (bacteria/yeasts) have been widely used in literature since it can take advantages of the symbiotic nature of these microorganisms to both metabolically uptake and produce gaseous CO_2 and O_2, whilst promoting the formation of products with economic interest. In this study, for the first time a co-cultivation with *Y. lipolytica* and *C. vulgaris* for the production of organic acids was developed, using CA bioprocess as case study.

Initially, some parameters were explored in shake flasks to improve the co-cultivation systems, such as: 1) selection of the appropriate culture medium (modified Šetlik) and selection of appropriate N-source ($NaNO_3$), where it was shown that the use of a single nitrogen source increases the biomass of *C. vulgaris*, allowing NH_4Cl to be consumed only on the moment of yeast addition; 2) Inoculum ratio of yeast/microalgae due to the differences in growth rates, where it is shown that CA productivity is not affected when decreasing from 10 to 0.01 % of *Y. lipolytica* inoculum (0.130 ± 0.005 and 0.121 ± 0.004 g/L/h) and time of inoculation, where it was proved that delaying addition of yeast for 7 days, can increase CA productivity to 87% when compared with co-cultivation where yeast and microalgae were added at the same time (day 0).

Having defined these parameters, the process was transferred to a photobioreactor as a bubble column system, where it was tested the influence of light regime (12:12 and 16:8h (light/dark)) and light intensities and compared with mono-cultivation of *Y. lipolytica* in the same system. Remarkably, when CA was produced in co-cultivations using a light regime of 16:8 (light/dark) the CA-productivity$_{co\text{-}cult}$ increased to 85.7% (0.13 ± 0.01 g/L/h), when compared with mono-cultivation of *Y. lipolytica* (0.07 ± 0.01 g/L/h). Considering that CA production is significantly impacted by the absence of oxygen, these results suggest a potential synergistic interaction both microorganisms, with microalgae serving as an oxygen producer. Those are promising results on further bioprocess development of organic acids in terms of an efficient gas exchange between algae and yeast, and will serve as basis for the development of a co-cultivation process for the production of MELs (**section D.3**). Nevertheless, in the future co-cultivation for production of organic acids, system could be carried out to others type of bioreactors, such as flat panels photobioreactors, or raceway ponds, which have clear different gas transference and light

intensity due to different geometries. Furthermore, considering this specific process, using *Y. lipolytica,* more studies should be conducted with this system and the use of crude glycerol as carbon source since the value achieved for biomass was high (14 g/L) and the content of biomass can be suitable for animal protein replacement or even as an alternative for food substitute more studies should be conducted with this system and the use of crude glycerol as carbon source since the value achieved for biomass was high (14 g/L) and the content of biomass can be suitable for animal protein replacement or even as an alternative for food substitute.

D.2 Two sequential microorganism culture approach: Production of MELs using lipids as carbon source produced from microalgae

D.2.1 Outline

The production of MELs using different agro-industrial residues, such as cheese-whey (**section B.2**) and pomace oil (**Section B.3**) was evaluated in previous sections. Therefore, altought the aim at the development of a co-cultivation process for MELs production with lower CO2 emissions, using microalgae and non-conventional yeasts, the cultivation of oleaginous microalgae was explored under the perspective of microbial oil production, and consequent use as feedstock for MELs production. The bio-oil was extracted from *Neochloris oleoabundans,* and evaluated for its use as sole carbon source, or in a co-substrate strategy, using as additional carbon source D-glucose as additional carbon source. Both *Moesziomyces antarcticus* and *M. bullatus* were able to grow and produce MELs using algae-derived's bio-oils as carbon source. Using a medium containing 40 g/L of D-glucose and 20 g/L of bio-oils as carbon sources, *Moesziomyces antarcticus* and *M. bullatus* produced 12.47 ± 0.28 and 5.72 ± 2.32 g/L of MELs, respectively. Interestingly, there are no significant differences in productivity when using oils from microalgae or vegetable oils as carbon sources. The MELs productivities achieved were 1.78 ± 0.04 and 1.99 ± 0.12 g/L/day, respectively, for *M. antarcticus* fed with algae-derived's bio-oils or vegetable oils. These results open new perspectives for the production of MELs in co-cultivation with oleaginous microalgae.

D.2.2 Introduction

In the previous sections, MELs bioprocess have been studied and developed, using a basic condition (see **section B1**), which involves the use of a co-substrate strategy (hydrophilic and hydrophobic carbon sources), allowing to reduce the dependence on the use of high quantities of hydrophobic carbon sources, while keeping high titres and MELs purities (**Figure B7**). Furthermore, the use of vegetable oils (hydrophobic carbon source) represent a threat for food availability and prices, requiring requires a large area of arable land for their production (Anto et al., 2020).

To answer to this call, some studies attempted the replacement of SBO, for WFO, showing minimal impact on MELs productivity (Niu et al., 2019), and also shown in previous sections (**Section B1, B2**). Nevertheless, the use of WFO as substrate for fermentations, depending on the source, previous intensity of use, and consequently level of oxidation and presence of inhibitory species can lead to different yeast cell growths and glycolipids productivity. Additionally, even in the context of this thesis (**Section B3**) it was tested the use of pomace oil as a potential replacer of WFO, although this is a seasonal substrate, and a potentially stockout can happen if MELs bioprocess only relies on this type of substrate. Furthermore, for some applications, to produce pharmaceuticals, cosmetics and food formulations, the use of more pure substrates is required.

In this regard, in this section, with the aim to search for alternative sustainable hydrophobic substrates, it is suggested the use of lipids derived from microalgae. In particular, the use of the oleaginous microalgae *Neochloris oleoabundans* came out as a promising candidate to produce such substrates, due to its capacity to accumulate high contents of intracellular lipids. Indeed, *N. oleoabundans* has been reported to accumulate up to 56 % of biomass in lipid content for cultivations carried out without CO2 supplementation (Gouveia et al., 2009). This microalgae also has been reported to be cultivated using industrial effluents, such as brewery effluents (A. Ferreira et al., 2019), which is interesting from the perspective to use residues as nitrogen sources. **Figure D6** describes the strategy assessed in the current section, where, for the first time, the production of MELs using oils from oleaginous microalgae was investigated.

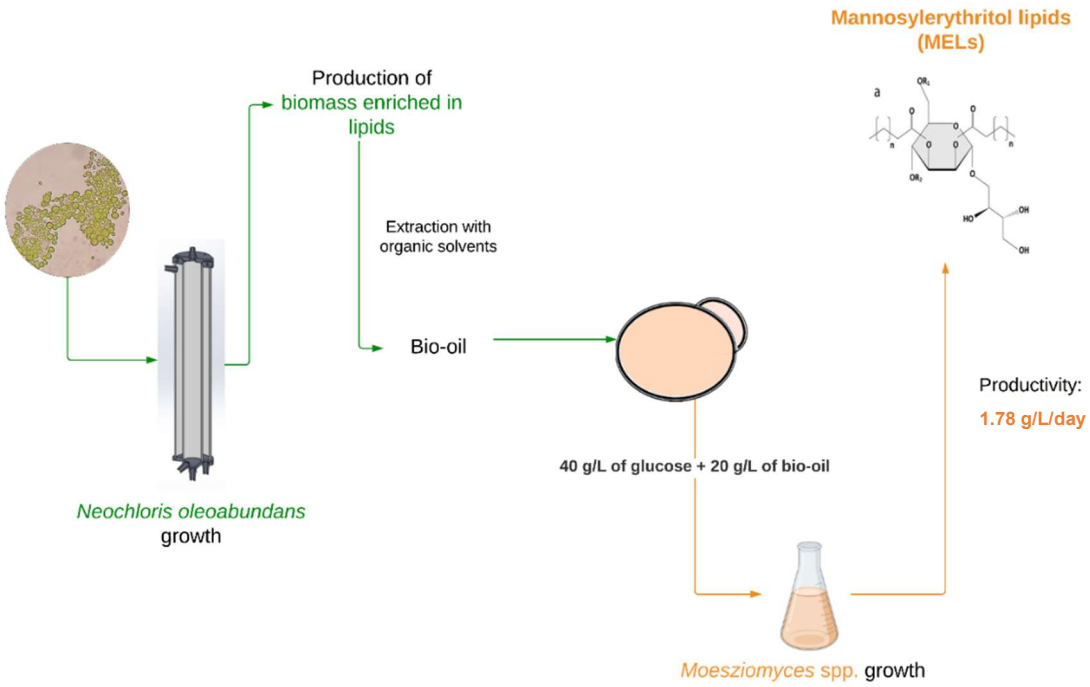


Figure D6: Schematic overview of MELs production from algae-derived bio-oils, produced by *Neochloris oleoabundans*.

D.2.3 Materials and methods

D.2.3.1 Microalgae cultivation

The microalgae *Neochloris oleoabundans* #1185, obtained from the UTEX culture collection of the University of Texas, Austin, TX, USA, was used in this work. The stock culture was maintained with indirect sunlight in an Erlenmeyer shake flask, placed on the laboratory bench, and filled with 1/5 of working volume, corresponding to 50 mL, of Bristol medium. This medium comprises 0.25 g/L of $NaNO_3$, 0.175 g/L of KH_2PO_4, 0.075 g/L of K_2HPO_4; 0.075 g/L of $MgSO_4 . 7H_2O$; 0.075 g/L of $MgSO_4$, 0.060 g/L of Fe-EDTA, 0.075 g/L of $CaCl_2$, 0.025 g/L of NaCl, and 1mL/L of trace elements, i.e. 2.860 g/L of H_3BO_3, 2.030 g/L of $MnSO_4 . 4H_2O$, 0.220 g/L of $ZnSO_4$, 0.090 g/L of $CoSO_4 . 7H_2O$, 0.060 g/L of $Na_2MO_4 . 2H_2O$, and 0.050 g/L of $CuSO_4$. The stock cultures were renewed every two weeks. The bioreactors were started by adding 10% ($V_{stock}/V_{culture\ medium}$) of stock culture as inoculum to an Erlenmeyer shake flask with 1/5 of working volume, corresponding to 50 mL volume. The inoculated Erlenmeyer shake flask were incubated at 26 °C with an agitation of 130 rpm, with a light intensity of 80 $\mu E/m^2/s$ photosynthetic photon flux density (PPFD), with a light regime of 16:8 (light/dark). The cultures were renewed with fresh culture medium every two weeks.

Neochloris oleoabundans was grown in two different bioreactors. Firstly, *N. oleoabundans* grown in two home-made air bubble column bioreactors of 500 mL. Those experiments were carried out for characterization of the biomass, cell number, nitrate consumption and lipid formation over the cultivation period. Those microalgae cultivations were carried in duplicates, at 27 °C, with continuous agitation provided by bubbling filtered air set at a value of 1 vvm for 15 days under an illumination regime set to have 16:8 h of light/dark with a light intensity of 150 $\mu E/m^2/s$ PPFD on a light period. Then, for increase of biomass and to have more lipids to use on experiences, the microalgae were grown in larger 1 L glass bubble column bioreactors, where continuous agitation was achieved by bubbling filtered air at 0.9 VVM in Bristol medium at temperature of 30 °C. While the nitrogen source was present on the medium, the CO_2 was supplemented to obtain a biomass content on the range of 2 g/L. Once the microalgae reached this level of biomass, the culture was carried out for five additional days, but under nitrogen starvation and without supplementation of CO_2. A final lipid content of 56% (DW) was achieved under such conditions. This first system was

continuously illuminated by using six fluorescent lamps (Philips TL-DM 36W/54-765) with a light intensity of 150 μE/m^2/s PPFD.

D.2.3.2 Yeast strains, substrate, and cultivation conditions

Moesziomyces antarcticus PYCC 5048^T and *Moesziomyces Bullatus* PYCC 5535^T were obtained from the Portuguese Yeast Culture Collection (PYCC), Centro de Recursos Microbiológicos, Research Unit on Applied Molecular Biosciences at NOVA School of Science and Technology (CREM, UCBIO, FCT NOVA), Caparica, Portugal. Strains were plated in YM Agar (3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, 10 g/L of D-glucose and 20 g/L of agar) and incubated for 3 days at 30 °C. Stock cultures were prepared by propagation of yeast cells in the liquid media, with similar composition to the one described below for use in inoculum preparation, after which were stored in 20% (v/v) glycerol aliquots, at -70 °C. An inoculum was prepared by transferring the stocks cultures of *M. antarcticus* and *M. Bullatus* into an Erlenmeyer flask with 1/5 working volume, corresponding to a volume of 50 mL of medium. Such medium contains 0.3 g/L of $MgSO_4$, 3 g/L of $NaNO_3$, 0.3 g/L of KH_2PO_4, 1 g/L of yeast extract (YE), 40 g/L of D-glucose. These cell cultures were incubated at 27 °C and kept at 250 rpm for 48 h. Then 2.5 ml of this inoculum was added, corresponding to a ratio of 10% (v/v) of inoculum to culture volume, into an Erlenmeyer flask with 1/5 working volume, i.e. a 25 mL of cultivation medium. This medium was used for *Moesziomyces* media, aforementioned before, using as carbon source 40 g/L of D-glucose and 20 g/L of a hydrophobic source, which according with the specific experimental condition was a different type of oils: (i) waste frying oils (WFO); or (ii) oils from *N. oleoabundans* (algae-derived bio-oils). As a control, a fermentation was also carried out, using 60 g/L of D-glucose as the only main carbon source, with the rest of mineral salts, nitrate and YE described above. All cultures, using oils were carried out in biological duplicates and incubated at 27 °C and kept at 250 rpm for 11 days.

D.2.3.3 Growth and biomass determination

Yeast and microalgae growth was estimated by measuring the cell dry weight (CDW), periodically, over the fermentation period. CDW was determined collecting 1 mL of culture broth, which it was centrifugation at 10 000 x rpm for 6 min. The supernatant was discharged, and the cell pellet washed with deionized water (twice) and drying at 60 °C for 48 h. Additionally, for

microalgae cultivation, the concentration of cells per mL of culture was also quantified by counting the cells every day using a hemacytometer (Thomas, LW scientific) and a microscope (Axiostar plus, Zeiss). Briefly, a sample of cell cultivation broth was collected and diluted with an appropriate dilution factor (Df), then 15 µL of the resulting solution was added to the hemacytometer and cells on the 4 chambers were counted in duplicated and averaged. The cell density was estimated considering that each chamber has a volume of 10^{-4} mL,as represented in **Equation *17***.

$$\frac{Cell\ number}{mL} = \frac{\left(\frac{Cell\ count}{4}\right)}{10^{-4}\ ml} \times Df$$

Equation 17 : Determination of cell number/mL

D.2.3.4 Extraction of oils from microalgae

The extraction of bio-oils was carried out following the Bligh Dyer method, adapted by Araujo et al (Araujo et al., 2013). *Neochloris oleoabundans* biomass was spray-dried (Christ Alpha 1-2 L0 plus) and an extraction was performed using methanol, chloroform, and water at volume ratio of 5:3:1. The mixture was subjected to ultrasounds (Emmi-H30) for 40 min. After that, the biomass was separated by filtration. A solution of KCl at a concentration of 0.88% w/v was added to the liquid fraction in a peer-shaped separating funnel, allowed to settle for 24 h. The bottom phase is recovered and evaporated in a rotary evaporator (Bucher) at 40 °C using 400 mbar, obtaining bio-oils. After evaporating, bio-oil was dissolved in hexane/ethyl acetate at a ratio 1:1 v/v and, again the solvent evaporated to remove traces of chloroform, and the bio-oil recovered for further use.

D.2.3.5 MELs, residual lipids and substrate quantification

During the fermentations, 1 mL of the culture broth samples was periodically taken and freeze-dried. The fatty acid content of the biological samples was determined by Gas Chromatography (GC) with a Flame Ionization Detector (FID), after to be transformed to methyl esters by methanolysis as described by Welz et al (Welz et al., 1990). MELs were quantified through the amount of methyl esters comprising fatty acid chains with 8, 10 and 12 carbons long, as previously described (N. T. Faria et al., 2014).

The quantification of D-glucose and nitrate was performed using high-performance liquid chromatography (HPLC). Culture broth samples were centrifuged at 10,000 rpm for 6 min, the supernatants were filtered through a 0.22 μm-pore size-filter and injected into the HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany) for D-glucose quantification and a UV-VIS detector (L-2420 VWR Hitachi, Darmstadt, Germany) for sodium nitrate quantification. An Rezex ROA Organic Acid H+ column (300 mm× 7.8 mm, Phenomenex, Torrance, CA, USA) was fitted on the HPLC and operated at 65°C. Sulfuric acid (5 mM) was used as mobile phase at 0.5 ml/min. he quantification of D-glucose and sodium nitrate was performed using HPLC.

D.2.3.6 Thin Layer Chromatography (TLC)

TLC was performed only for qualitative evaluation of the type of MELs produced. 3 ml of culture broth samples was extracted with twice the volumes of ethyl acetate. The organic and aqueous phases were separated, the organic solvent evaporated and the crude MELs re-dissolved in methanol. An aliquot of 0.5-1.0 mg (dw) of each sample to be assessed was placed on a different lane of an aluminium TLC sheets pre-coated with a silica gel 60 layer (Macherey-Nagel Alugram Xtra SIL G/UV254). The standard solvent system used for elution of the different MELs congeners was a mixture of chloroform, methanol and water in a ratio of 6.5, 1.5 and 0.2. After elution, a solution of α-naphthol in sulfuric acid, comprised by 1.5 g of naphthol, 51 ml of ethanol, 4 ml of water and 6.5 ml of sulfuric acid was sprayed on the TLC sheet, which was heated over 100 °C for 1 min, allowing visualization of the position in the of the different eluted compounds. MELs produced by *M. bullatus* PYCC 5535T over a 14 days cultivation, where SBO was the main carbon source, was used as reference for TLC. Such MELs was obtained by extraction of 250 ml of the culture broth with ethyl-acetate using the volume ratio of 1:2. The solvent of the organic phase was vacuum evaporated and the crude MELs washed with a mixture of n-hexane, methanol and water at a ratio of 1:6:3 to remove residual oils and free fatty acids (top phase). The aqueous phase (bottom phase) was washed twice with 100 ml n-hexane. Methanol and water were removed, respectively, by vacuum evaporation and freeze-drying, obtaining around 1 g of purified MELs mixture. Around 0.2 g of such MELs mixture was dissolved in chloroform and added in a silica gel chromatograph column, which was eluted with a mixture of chloroform and acetone (7:3, v/v) to isolate MEL-A from the other congeners. The solvent of the collected eluted fractions was

evaporated and their content submitted to TLC to identify the fractions containing purified MEL-A.

D.2.3.7 Statistical analysis

Statistics were performed, using Graph-pad software by analysis of variance (two-way ANOVA) and estimations of p-values to evaluate the statistical significance of the differences between groups were corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p < 0.05$.

D.2.4 Results and discussion

D.2.4.1 *Neochloris oleoabundans* growth and lipids production

Neochloris oleoabundans was initially grown in an air-lift bioreactor, during 15 days, without supplementation of CO_2. Cell growth was monitored measuring the cell dry-weight (biomass) and counting the cell number (**Figure D8 A)** during culturing time. The nitrate consumption and production of bio-oil along the culture was also quantified (**Figure D8 B)**. The cell number and biomass grow continuously over the 15 day of culturing, with a cell number growth deacceleration, while biomass continuously grow until 2.25 ± 0.25 g/L, after 10 days of cultivation. These data suggest that after day 10, the cells became larger and/or heavier. The production of lipids and/or carotenoids in microalgae strains is triggered by stress conditions, such as nutrient limitation or exposure to some physical factor (e.g. oxidative damage caused by light intensity, salt stress among others) (Sun et al., 2018). In this study, the accumulation of intracellular lipids in *N. oleoabundans* was stimulated by the limitation of nitrogen source (sodium nitrate). Usually, as soon as the nitrogen source is consumed, the production of lipids by microalgae starts. However, in our experience, it is possible to observe a hiatus between sodium nitrate virtually complete depletion at day 5 of cultivation and the kick-off of intracellular lipids production, that starts at day 10. This can be explained by a metabolic delay associated with the conversion of NO_3^- ion, after entering the cell, into NO_2^- by nitrate reductase, and follow up conversion of NO_2^-, after entering the chloroplast into NH_4^+ by the nitrite reductase, as reviewed by Salbitani et al. (Salbitani and Carfagna, 2021). The production of lipids is noticed by a change of colour of culture broth (**Figure 3)**, which after 10 days, changes from a green (**Figure 3 A, B)** to a yellow/orange colour (**Figure 3 C)**.

After 15 days of cultivation, a lipid cell content of 0.731 ± 0.259 $g_{bio\text{-}oil}/g_{biomass}$ was achieved, which is quite impressive, comparing to the result obtained by Li et al. (Li et al., 2008). However, in this study the authors achieved biomass and lipid productivities at values of 0.61 g/L/day and of 0.43 g/L/day, respectively, while in this study the values obtained were 0.15 ± 0.016 g/L/day and 0.10 ± 0.026 g/L/day, respectively. The differences on productivities can be explained by the fact, Li et al., used 5% of enriched CO_2 on the reported *N. oleoabundans* cultures, where the photosynthesis process reported here relies only on atmospheric CO_2, which most probably delays and limits

biomass and lipids production. Li et al. (Li et al., 2008) also investigates the effect of the different nitrogen sources on the microalgae cultivation parameters, and concludes that the one that leads to higher lipid productivity was sodium nitrate when used at an optimal concentration of 0.84 g/L of sodium nitrate. Indeed, in the current study the sodium nitrate concentration was 3.4-fold lower than such optimal value, which can lead to lower biomass production and therefore to lower lipids productivity. These observations imply that the microalgae cultivation in the current study were still performed under sub-optimal conditions, and more studies are required to maximize lipids production by *N. oleoabundans.*

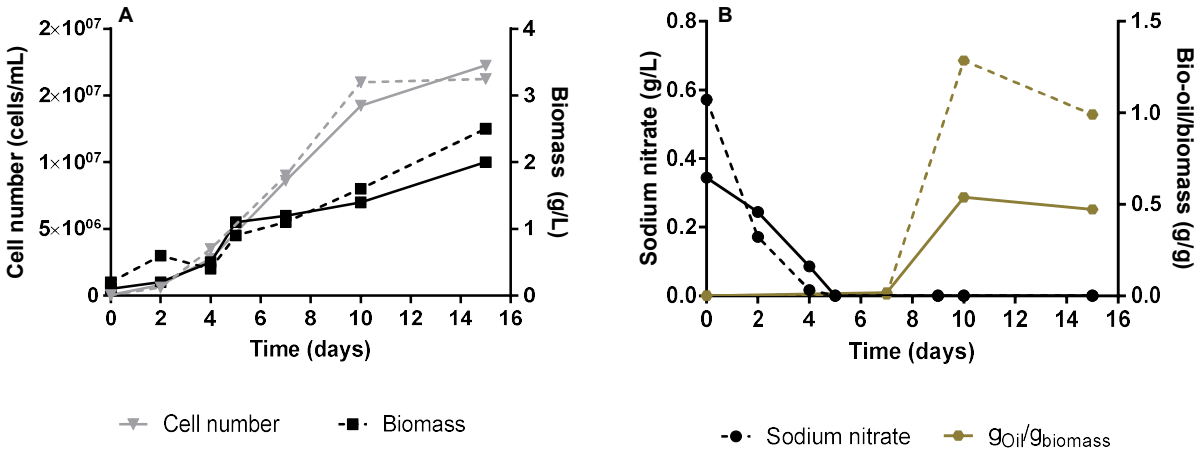


Figure D8: Cultivation of *Neochloris oleoabundans* #1185 in air-lift bioreactors for 15 days. Cells number (inverted triangles) and biomass growth (squares) (A); Sodium nitrate consumption (circles) and production of bio-oil per grama of biomass (B). Dashed and filled line corresponds to biological duplicates.

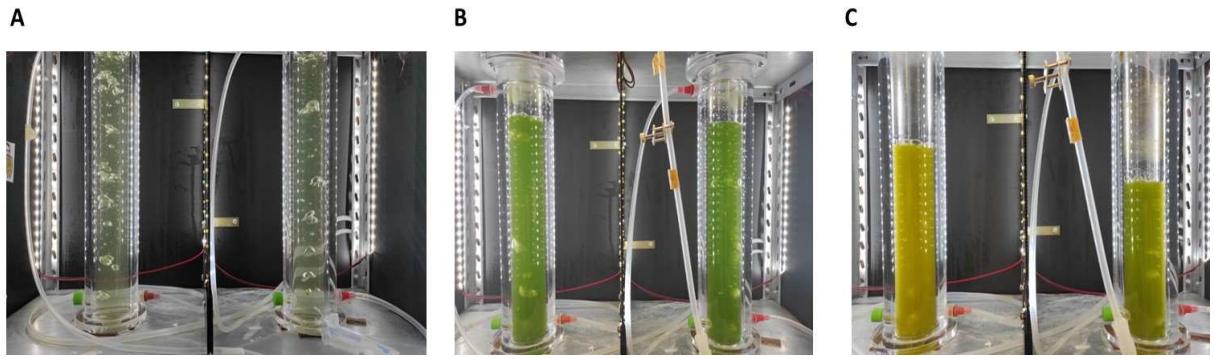


Figure D7: Images of *Neochloris oleoabundans* #1185 cultivation in air-lifts bioreactors at day 0 (A), 5 (B) and 15 (C) of fermentation.

D.2.4.2 Production of MELs using algae-derived bio-oils

After studying a production of lipids by *N. oleoabundans*, the capability of *Moesziomyces spp.* to produce MELs using the lipids (algae-derived bio-oils) produced by this microalgae was assessed. To obtain a higher amount of biomass and lipids additional, *N. oleoabundans* cultivations were performed on larger bioreactors of 1 L in the conditions described in **section D.2.3.1**. The produced intracellular lipids were extracted from spray-dried biomass, as described in **section D.2.3.4**, and the algae-derived bio-oils were obtained, as represented in **Figure D6**. The algae-derived bio-oils characterization (size of fatty acid chain) can be found in **Table D2**, with a profile similar to the one reported by Gouveia et al. (Gouveia et al., 2009). Interestingly, when comparing algae-derived bio-oils and WFO fatty chain profile, one can observe that the former have more C16 chains, while WFO only have C18:0 fatty acid chain, which is in agreement with the reported values for acidic values, being 3.3 fold-higher for algae-derived bio-oils, at a value of 15.3 mgKOH (L and J, 2016), than the ones reported for WFO , at a value of 4.67 mgKOH (Robles Arévalo, 2015).

Table D2: Size of fatty acid chain (%) for algae-derived bio-oils and waste frying oils (WFO).

Fatty acid chain (%)	Algae-derived bio-oils	WFO
C14:0	-	-
C16:0	12.66	0.13
C16:1	8.88	0
C18:0	59.84	95.43
C18:1	18.62	4.45

In **section B1** it was developed a basic condition (co-substrate strategy, **Figure B7**), with the objective to reduce the use of hydrophobic substrates but at the same time keeping high titres and purities of final crude MELs mixture. Therefore, in the current study, a similar co-substrate cultivation strategy was followed. Following such co-substrate strategy, three different conditions were performed for *M. antarcticus* (**Figure D9 A, C, E)** and *M. Bullatus* (**Figure D9 B, D, F).** Two of these cultivations have started with 40 g/L of glucose and 20 g/L of a hydrophobic carbon

source, where the hydrophobic was algae-derived bio-oils (**Figure D9 A, B)** or WFO (**Figure D9 C, D)**. The third strategy included the use of D-glucose as sole carbon source (**Figure D9 E, F**), but using 60 g/L of D-glucose in order that this condition was fed with a similar carbon molar equivalent than the assays that follow a co-substrate strategy. The results are resumed in **Table D3** and **Figure D9.**

When analysing the patterns of substrate consumption, it is possible to observe that the consumption rate of D-glucose is slightly higher when algae-derived bio-oil is used instead of WFO (19 and 7% for *M. antarcticus* and *M. Bullatus*, respectively). Importantly, the consumption of D-glucose in the presence of either of the oils is relatively high, being virtually depleted at day 4 of the fermentation, which indicates that there is no catabolic repression of oils on D-glucose consumption. For the experiment using D-glucose alone, a higher concentration of this substrate was used and a slightly lower rate of D-glucose was observed. The data also suggests that the consumption of oil is faster for algae-derived bio-oil than for WFO. This pattern can be explained by the composition of the bio-oil, which have an acidic value of 15.3 mgKOH (L and J, 2016), 3.3 fold-higher than the value for WFO (4.67 mgKOH) (Robles Arévalo, 2015). This means that the algae-derived bio-oil has a higher content in free fatty acids and monoacylglycerides or diacylglycerides, while WFO is richer in triacylglycerides. In presence of hydrophobic substrate, *Moesziomyces* spp. have the ability to produce lipases for breakdown of triacylglycerides into free fatty acids to be assimilated by the cell (Ueda et al., 2015). However, to fed the fermentation with a substrate already partially broken down can speed up its assimilation and incorporation of the lipidic molecules into MELs, which will be metabolized through the chain shortening pathway or partial β-oxidation (Kitamoto et al., 1998). Petar Keković, in his PhD thesis (Keković, 2022), as previously described, have shown the effect of using methyl-esters or free fatty acids, instead of triacylglycerides, as substrates, on increasing MELs prosecution and fostering the formation of beads enriched in MELs. Therefore, the use of lipids from *N. oleoabundans* with higher acidic value (richer in free fatty acids), can lead to a faster substrate consumption and higher MELs productivities.

The faster consumption of algae-derived bio-oil, led to a maximum MELs titre of 12.47 ± 0.28 (Day 4) and 5.72 ± 2.32 g/L (Day 7) for *M. antarcticus* and *M. Bullatus*, respectively. The maximum MELs titre was obtained earlier for *Moesziomyces* spp. cultivations using algae-derived bio-oils

than for the ones using WFO as carbon source (day 11 for both strains). Remarkably, there was no significant difference in maximum productivity observed for cultures based on algae-derived bio-oils and WFO, respectively, with values of 1.78 ± 0.04 g/L/day (algae-derived bio-oils) and 1.99 ± 0.12 g/L/day (WFO) for *M. antarcticus* or 1.43 ± 0.58 g/L/day (algae-derived bio-oils) and 1.54 ± 0.01 g/L/day (WFO) for *M. Bullatus*. However, the final titres were 2.86 and 5.57-fold higher for WFO based fermentation than for the ones using bio-oils from microalgae. Namely, when WFO was used, the MELs maximum titres were 21.94 ± 1.31 g/L and 16.98 ± 0.39 g/L, respectively, for *M. antarcticus* and *M. Bullatus* cultures. The high discrepancy on maximum MELs titres could be related with the potential consumption of MELs after day 7, due to the low contents in algae-derived bio-oil, which calls for further fermentation optimization using bio-oils feed batch strategies.

Overall, MELs titres and productivities under all conditions were higher when *M. antarcticus* was used rather than *M. Bullatus*, and this may be due to the capacity of *M. Bullatus* to create reserves of free fatty acids. In fact, this phenomenon is observed when D-glucose is used as sole carbon source, where the low MELs titres and higher yeast lipids accumulation lead to a final purity of 58%.

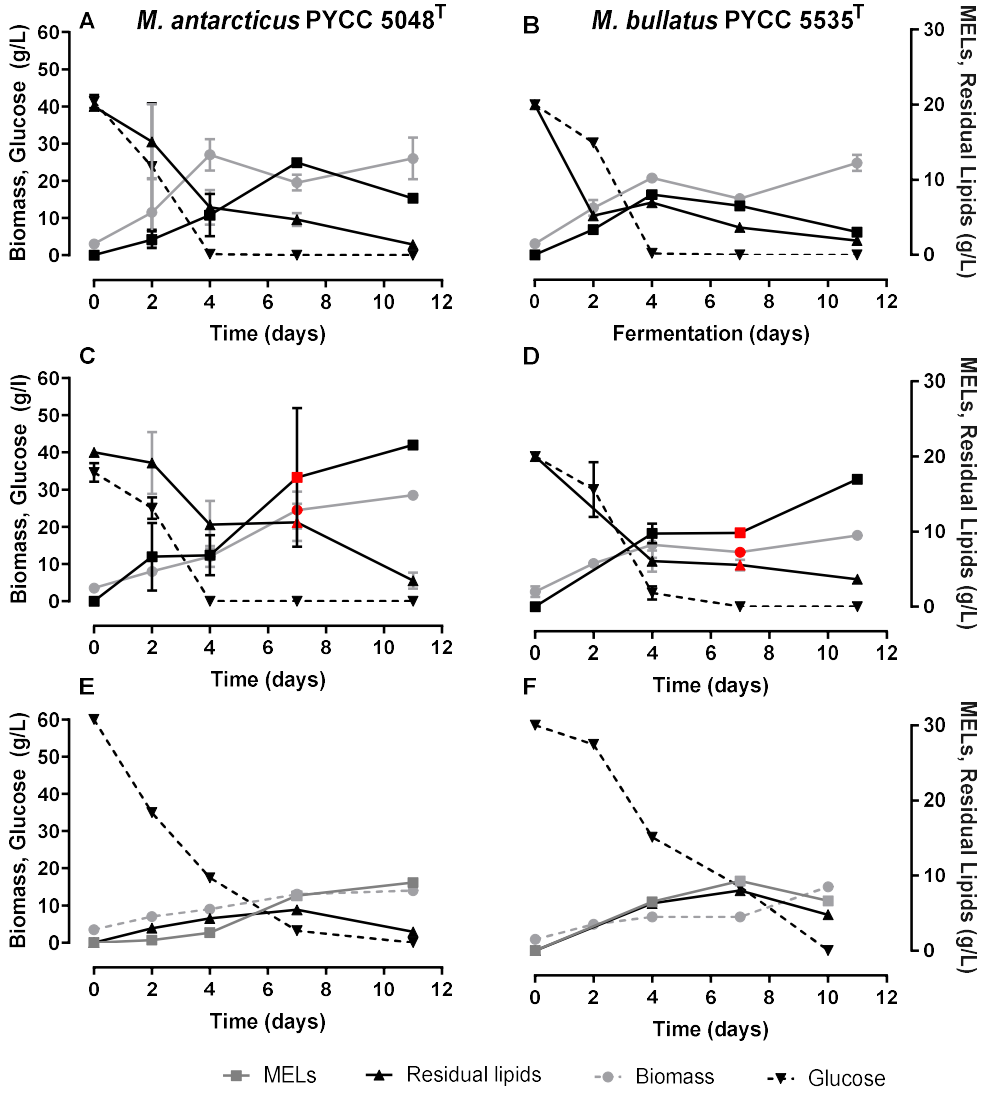


Figure D9: Cultivation of *M. antarcticus* PYCC 5048^T (A, C, E) and *M. bullatus* PYCC 5535^T (B, D, F) using D-glucose (40 g/L) and a hydrophobic source (20 g/L): Algae-derived bio-oil (A, B) and waste frying oil (C, D). As a control for both strains, it was used 60 g/L of D- glucose as carbon source (E, F). Yeast biomass (circles), MELs production (squares), D-glucose (inverted triangles) and residual lipids (crosses). The red point indicates the presence of beads enriched in MELs are formed. Only conditions using algae-derived bio-oil and WFO were performed in biological duplicates. Standard deviations values lower than 1 g/L are not represented.

Table D3: Rate of D-glucose consumption (Rs); maximum biomass produced; Maximum MELs yield obtained (g/L); yield of MELs produced ($g_{MELs}/g_{Substrate}$), maximum productivity (g/L/day) and purity (g/g) for *M. antarcticus* PYCC 5048^T and *M. Bullatus* PYCC 5535^T using 40 g/L of D-glucose with 20 g/L of hydrophobic carbon source (algae-derived bio-oils or waste frying oils).

Parameters	***M. antarcticus* PYCC 5048^T**			***M. bullatus* PYCC 5535^T**		
	Algae-derived bio-oils	**WFO**	**D-glucose**	Algae-derived bio-oils	**WFO**	**D-glucose**
Rs (g/L/h)	0.43 ± 0.02	0.36 ± 0.02	0.31	0.41 ± 0	0.38 ± 0.01	0.36
$Biomass_{max}$ (g/L)	27.0 ± 3.0 (Day 4)	28.5 ± 0.5 (Day 11)	17	24.5 ± 1.5 (Day 11)	28.5 ± 0.5 (Day 11)	14
$MELs_{max}$ (g/L)	12.47 ± 0.28 (Day 7)	21.94 ± 1.31 (Day 11)	8.09 (Day 11)	5.72 ± 2.32 (Day 4)	16.98 ± 0.39 (Day 11)	6.64 (Day 11)
$Y_{MEL/Substrate}$ (g/g)	0.21 ± 0.01	0.37 ± 0.02	0.13	0.1 ± 0.04	0.28 ± 0.39	0.11
$Productivity_{max}$ (g/L/day)	1.78 ± 0.04	1.99 ± 0.12	0.73	1.43 ± 0.58	1.54 ± 0.01	0.60
MELs purity (g/g)	0.84 ± 0.01	0.88 ± 0.03	0.84	0.61 ± 0.06	0.88 ± 0.04	0.58

$Biomass_{max}$ – maximum biomass cell dry weight (g/L); rs – sugar consumption rate (g/L/h); $MELs_{max}$ – maximum MELs produced (g/L); Y MELs/Substrate consumed – maximum MEL yield (g/g); $Productivity_{max}$ – Maximum productivity (g/L/day); MELs purity (g/g) at the end of the fermentation- Ratio of g of MELs to the sum of g of MELs and residual lipids

The WFO and the algae-derived bio-oils have very different compositions, while the former is richer in triacylglycerides and C18 carbon chains, the later have higher contents in free fatty acids and C16 unsaturated chains. Therefore, it was investigated in the type of oil used would affect the chains present on the MELs produced using the different carbon sources (**Figure D10)**. Interestingly, the results show that when microalga's bio-oil is used, for both strains, the content of C8 chains in MELs is 2-fold higher when MELs is produced from WFO or D-glucose. This result is consistent with the algae-derived bio-oils composition, richer on shorter carbon chains, and the hypothesis of partial β-oxidation of free fatty acids fed to the fermentation followed by their integration on MELs molecules, upon their biosynthesis (Kitamoto et al., 1998). The profiles of lipidic MELs chains in the cultures using D-glucose alone it is consistent with the hypothesis that lipids to be integrated into the MELs follow the canonical "de-novo" synthesis of fatty acids chains up to C18, which are before to be incorporated into mannose and erythritol.

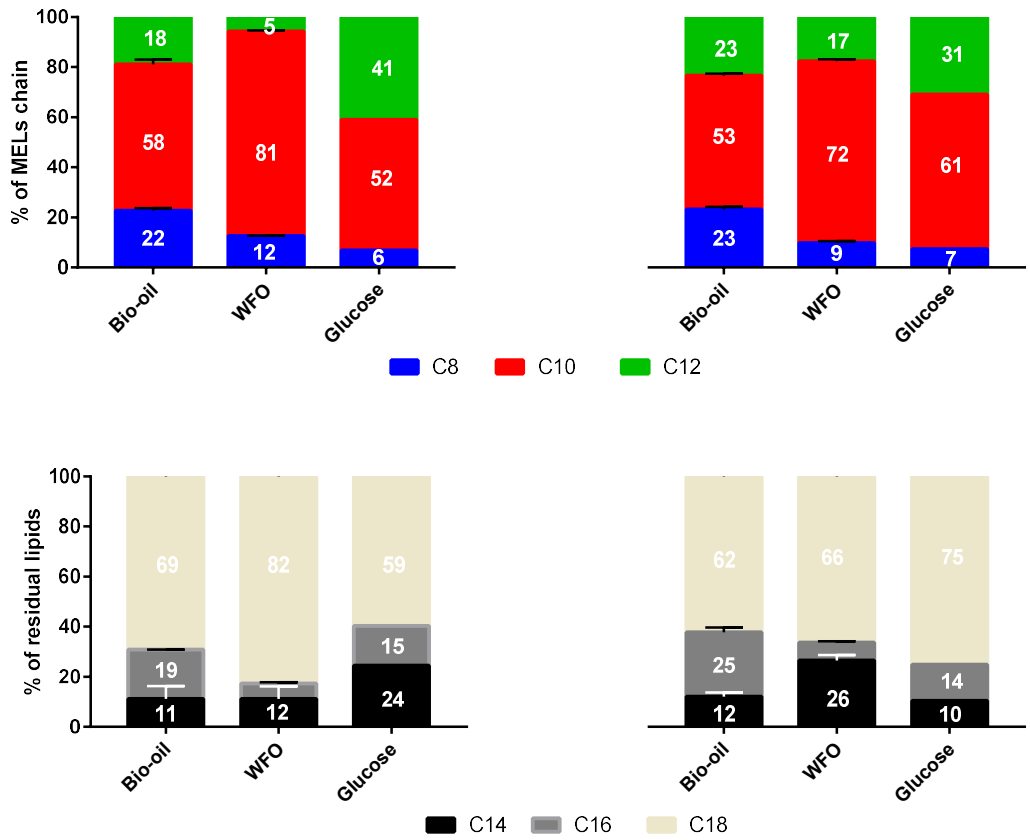


Figure D10: Type of fatty acids chains in maximum MELs titres (C8, C10, C12) and residual lipids (C14, C16, C18) produced by *M. antarcticus* PYCC 5048^{T} (A, C) and *M. bullatus* PYCC 5535^{T} (B, D) with different substrates used: 1) Bio-oils and D-glucose; 2) Waste frying oils and D-glucose and 3) D-glucose alone. Only conditions using algae-derived bio-oil and WFO were performed in biological duplicates. Standard deviations values lower than 1% are not represented.

The production of MELs using lipids produced by others microorganism has been previously reported on the literature, but using oleaginous yeasts, instead of microalgae, for bio-oils production. Namely, Akkermans et al (Akkermans et al., 2020) have used lipids produced from *Cutaneotrichosporon oleaginous* as carbon source for *M. bullatus* and a comparative overview of results from that study and the results obtained here in the presented work can be found on

Table D4. The final MELs titre obtained was 2.4-fold lower than the one obtained in the current study. However, in this study the authors, did not feed *Moesziomyces* spp with an extract of lipids, but instead used *Cutaneotrichosporon oleaginous* cells lysate, obtained by mechanic pre-treatment, as carbon source. Such strategy has the potential to offer a more sustainable approach than the one reported here, since does not rely on the intensive use of organic solvents for lipids extraction. Therefore, further studies using *Neochloris oleoabundans* mechanically obtained lysates are relevant.

Table D4: Summary of the results obtained by Akkermans et al. and the results here presented, that reports the use of two sequential microorganisms for the production of MELs, including type of strain, type of product, titer, yield and productivity. *Values do not present in the article and calculated.

Bio-oil producing strain	Product recovery	MELs producing strain	MELs max (g/L)	Yield (gMELs/gsubstrate)	Productivity (g/L/day)	Ref.
Cutaneotrichosporon oleoginosus	Use of cell lysates	*M. bullatus*	2.3	0.19*	0.5*	Akkermans et al (Akkermans et al., 2020)
Neochloris oleoabundans	Use or organic solvents	*M. bullatus*	5.72 ± 2.32	0.1 ± 0.04	1.43 ± 0.58	This study
		M. antarcticus	12.47 ± 0.28	0.21 ± 0.01	1.78 ± 0.04	

D.2.5 Final remarks

It is reported here, the production of MELs from microalgae-derived oil. When using the same strain, no significant differences were noticed on MELs productivities for conditions using bio-oil or waste frying oils. This study points out an alternative route for research and design of bioprocesses using a more sustainable class of bio-oils for MELs production. Still there is a call for new and more sustainable approaches to extract lipids from microalgae avoiding the use of organic solvents, such as high-pressure homogenizer. Furthermore, further studies should be performed, using a fed-batch fermentation and optimizing MELs production and overcome MELs titres limitations driven from the fast consumption of algae's bio-oils. Finally, the current study illustrates that the properties of different substrates can influence MELs congeners produced, where the algae-derived bio-oils, which comprise a lipid mixture with higher C16 and lower C18 fatty acids content than WFO, promotes the production of MELs mixture with smaller C8 lipidic chains.

D.3 Yeast and microalgae cultivation using the same bioreactor set-up: The way to co-cultivation

D.3.1 Overview

In the previous chapters, it has been studied MELs bioprocess, and strategies to overcome diverse bottlenecks in the process. Specifically, in **section C.2,** the economic analysis to MELs bioprocess, revealed that CO2 emissions are one the major bottlenecks in MELs production, representing a major impact in the final cost of MELs production.

In **Section D.1** (work developed in UFZ, Leipzig), the main parameters to have a successful co-cultivation were defined and studied: 1) selection of the appropriate culture medium (preferably with no competition of microalgae/yeast by the substrate); 2) definition of the inoculum ratio of yeast/microalgae to overcome the differences in growth rates; 3) Time of inoculation, where microalgae should grow alone and stablish before yeast inoculation, in order to give a boost to microalgae growth; and 4) Light regime should be 16:8h (light/dark). Therefore, the main objective was to apply these findings to MELs bioprocess. In **section D.2** it was produced lipids from oleaginous microalgae using bubble column reactors developed in IST laboratories. Later these lipids were used as carbon source for MELs production.

The results achieved in **section D.1**, were performed in a photobioreactor, fully equipped with electrodes (pH, DO; see **sub-section D.1.3.2**). However, the bubble column reactor used in IST laboratories (**section D.2)** are simpler and firstly projected to grow microalgae. In this regard this section aims to improve and redesign the bubble column reactor, able to grow both microalgae and yeasts aiming at a co-cultivation with these microorganisms.

Even though the results here obtained for MELs production in bubble column bioreactor were still far from optimized, this work is important as a foundation for future research on co-cultivation for MELs production at low CO_2 emissions.

D.3.2 Material and methods

D.3.2.1 Yeast and microalgae maintenance

For this section, it was decided to only use *C. vulgaris* CCAP 211/2b, as it will be explained in **section D.3.3**. This microalga was obtained from the Culture Collection of algae & protozoa. The stock culture was maintained with indirect sunlight in an Erlenmeyer shake flask, placed on the laboratory bench, and filled with 1/5 of working volume, corresponding to 50 mL, of Bristol medium. This medium comprises 0.25 g/L of $NaNO_3$, 0.175 g/L of KH_2PO_4, 0.075 g/L of K_2HPO_4; 0.075 g/L of $MgSO_4 . 7H_2O$; 0.075 g/L of $MgSO_4$, 0.060 g/L of Fe-EDTA, 0.075 g/L of $CaCl_2$, 0.025 g/L of NaCl, and 1mL/L of trace elements, i.e. 2.860 g/L of H_3BO_3, 2.030 g/L of $MnSO_4. 4H_2O$, 0.220 g/L of $ZnSO_4$, 0.090 g/L of $CoSO_4. 7H_2O$, 0.060 g/L of $Na_2MO_4. 2H_2O$, and 0.050 g/L of $CuSO_4$. The stock cultures were renewed every two weeks.

Considering the use of *Moesziomyces* species, also for this section, it was decided only to use *Moesziomyces antarcticus* PYCC 5048^T, considering that it is the best MELs' producer strain tested, as shown in previous sections. Therefore, the strain was plated in YM Agar (3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, 10 g/L of D-glucose and 20 g/L of agar) and incubated for 3 days at 30 °C. Stock cultures were prepared by propagation of yeast cells in the liquid media, with similar composition to the one described below for use in inoculum preparation, after which were stored in 20% (v/v) glycerol aliquots, at -70 °C.

D.3.2.2 Yeast and microalgae inoculum

The pre-inoculum of microalgae started by transferring 10% ($V_{inoculum}/V_{culture\ medium}$) of stock solution to an Erlenmeyer shake flask with 1/5 working volume (50 mL) containing Bristol medium, as previously described. The cultivation was carried out for 4 days, at 27 °C and under an agitation of 130 rpm. The light irradiance used was 80 µE/m2/s1 PPFD, with a light regime (light/dark) of 16:8h.

The pre-inoculum preparation of yeast for all experiments started by transferring the stocks cultures of *M. antarcticus* into an Erlenmeyer flask with 1/5 working volume, corresponding to a volume of 50 mL of medium. Such medium contains 0.3 g/L of $MgSO_4$, 3 g/L of $NaNO_3$, 0.3 g/L of

KH_2PO_4, 1 g/L of yeast extract (YE), 40 g/L of D-glucose. These cell cultures were incubated at 27 °C and kept at 250 rpm for 48 h.

D.3.2.3 Design of sparger for bubble column reactor

To design the sparger, it was used the SYLGRAD™ 184 silicone elastomer kit and a GenialLab stamping die as a mold, where each metal pipe having a diameter of 1mm. Initially, , the base and curing agent were mixed in a ratio of 10:1. The resulting mixture was then applied to cover the entire bottom of a petri dish with a total diameter of 16 cm. The petri dish was placed in a vacuum oven (Thermo Scientific™ Vacutherm Heating and drying oven) for 1 hour, in order to degassing the PDMS. Later the stamping die was placed in the Petri Dish and the oven was set to 70°C, allowing to pour into the mold overnight. Finally, the PDMS was pealed from the model, and radial sparger with 8 cm diameter was cut from the PDMS. The specifications of the sparger created are detailed in **Table D*5***.

Table D5: Sparger specifications used for bubble column reactor

Sparger specifications	
Diameter (mm)	8
Holes (#)	192
Holes diameter (mm)	1
Thickness (mm)	0.3

D.3.2.4 Cultivation conditions in bubble column reactor

Initially the bubble column reactors were sterilised using a sterilised solution of 300 g/L of NaCl. The reactor was filled with this solution (~ 600 mL) and let it act during 3 days, to ensure a optimal sterilisation (in-house data have shown that less than 3 days was not enough to kill all microorganism).

For all experiments carried out in this type of reactor with sparger, the temperature was kept at 27ºC, using an external water pump with temperature control for continuous water recirculation.

The light regime used was 16:8 h(light/dark) with a light intensity of 150 μE/m^2/s^1 PPFD. The airflow used was 1 vvm, as it will be explained in **section D.3.4.1**.

For both cultivation of *C. vulgaris* and *M. antarcticus,* the cultivation started by adding 10% (v/v) of inoculum (grown as previously described) to a working volume of 500 mL. For *C. vulgaris* it was used normal culture medium of *M. antarticus*, except for carbon source, and the cultivation period took 12 days. For *M. antarcticus* was used the same culture medium was used, but using 40 g/L of glycerol as carbon source, and the cultivation period took 10 days.

D.3.2.5 Growth and biomass determination

Yeast and microalgae growth was estimated by measuring the cell dry weight (CDW), periodically, over the fermentation period. CDW was determined collecting 1 mL of culture broth, which it was centrifugation at 10 000 x rpm for 6 min. The supernatant was discharged and the cell pellet washed with deionized water (twice) and drying at 60 °C for 48 h. Additionally, for microalgae cultivation, the concentration of cells per mL of culture was also quantified by counting the cells every day using a hemacytometer (Thomas, LW scientific) and a microscope (Axiostar plus, Zeiss). Briefly, a sample of cell cultivation broth was collected and diluted with an appropriate dilution factor (Df), then 15 μL of the resulting solution was added to the hemacytometer and cells on the 4 chambers were counted in duplicated and averaged. The cell density was estimated considering that each chamber has a volume of 10^{-4} mL,as represented in **Equation *17***.

$$\frac{Cell\ number}{mL} = \frac{\left(\frac{Cell\ count}{4}\right)}{10^{-4}\ ml} \times Df$$

Equation 18 : Determination of cell number/mL

D.3.2.6 MELs, residual lipids and substrate quantification

The quantification of glycerol and $NaNO_3$ was performed using HPLC. Culture broth samples were centrifuged at 10000 rpm for 6 min, the supernatants filtered through a 0.22 μm-pore size-filter and injected into HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a Rezex ROA Organic Acid H+ column (300 mm× 7.8 mm, Phenomenex, Torrance, CA, USA), an autosampler (Hitachi LaChrome Elite L-2200), an HPLC pump (Hitachi LaChrome Elite L-2130),

plus Hitachi detectors L-2490 refraction index (RI) and a Hitachi L-2420 UV-Vis VIS detector. Column was kept at 65 ºC using a column heater (Croco-CIL 100-040-220P, 40 cm x 8 cm x 8 cm, 30-99°C) externally connected to the HPLC system. A mobile phase of 5 mM H_2SO_4 was used at a flow rate of 0.5 mL/min. Samples, after centrifugation, were diluted with 50 mM of H_2SO_4 (a dilution 1/20 was normally used for all samples). The final concentration of glycerol and nitrates were estimated using calibration curves.

During the fermentations, 1 mL of the culture broth samples was periodically taken and freeze-dried. The fatty acid content of the biological samples was determined by Gas Chromatography (GC) with a Flame Ionization Detector (FID), after to be transformed to methyl esters by methanolysis as described by Welz et al (Welz et al., 1990). MELs were quantified through the amount of methyl esters comprising fatty acid chains with 8, 10 and 12 carbons long, as previously described (N. T. Faria et al., 2014).

D.3.2.7 Oxygen volumetric mass transference coefficient (kLa) determination

To determine kLa of the bioreactors, 500 mL of working volume was used with all the mineral medium and glycerol (as previously described). It was used a DO probe connected to a control unit cabinet (New Brunswick BioFlo /CelliGen 115, Eppendorf), the same bioreactor used in **section B.1** and **B.3,** allowing to acquire the values of DO and determining kLa. The method used to determine the coefficient was the dynamic method, as described by Linek et al (Linek et al., 1993), where nitrogen is sparged into the bioreactor to remove the oxygen, and then, the stream with air was connected and the dissolved oxygen determined, until reaching the steady state. kLa was determined using **Equation *1***.

D.3.3 Results and discussion

In the development of a co-cultivation system with microalgae and yeasts for MELs production, it was decided to use some of the conclusions obtained in **section D.1,** such as time of yeast inoculation (5 days) and the light regime (16:8 light/dark cycle). However, while in **section D.1** used a well-equipped photobioreactor (pH, DO, electrodes, ect), the current bubble column reactor used for this section, was not sufficiently equipped for a successful co-cultivation system, as will be tailed later.

In this regard, the first objective consisted into designing a new bubble column reactor. Additionally, since the MELs production culture differs from the one used for CA production (see **section D.1**), it was necessary to evaluate the growth of *C. vulgaris* using the culture medium optimised for MELs. Finally, it was tested both solo and co-cultivation of *C. vulgaris*/*M. antarcticus* in the bubble column reactor.

D.3.4.1 Improvement of bubble column bioreactor design

In **section D.2** the growth of microalgae was accessed using bubble column reactor, made of acrylic (see **Figure D*11***), a simple reactor made for a simple purpose of growing microalgae, that do not require so many parameters.

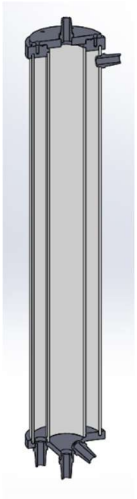


Figure D11: Auto-CAD design of bubble column reactor (1st version)

However, growing yeast in this type of reactors, which required feeds and pH control, poses several challenges. Firstly, sampling is inconvenient as it is done through one of the tubes at the bottom of reactor, where the airflow also originates. Then, the single outlet port in the top of the reactor, makes it impossible to provide a feed, since the airflow is continuously passing through the culture medium. Furthermore, the port to place the temperature sensor, is located at the bottom, and enters in the direct contact with the culture medium, increasing the risk of contamination. While this is not an issue for photoautotrophic microalgae cultures, it poses a significant problem for yeast cultures and can potentially compromise the whole fermentation. Lastly, the water jacket was also modified because it requires more than 0.7 L of water to fill, adding extra weight and pressure, preventing the use of more reactor (not enough power of the external water pump used).

In this regard, a new bioreactor setup was designed (see **Figure D*12***) with the objective to solve the aforementioned issues. In this new setup, the volume of water was reduced to cover only one-third of the total vessel (around 0.25 L), decreasing the overall pressure on the acrylic structure and allowing multiple bubble column reactors to be connected a single external water pump. A sampling valve was introduced at the middle of the vessel (where it is guaranteed a homogenisation of culture medium). Additionally, an extra port was added at the top of the reactor, enabling external feeding, while the air is continuously passes through.

A plastic tube was also included for the temperature sensor, removing the small port in the bottom and eliminating a point of pressure and contamination. Furthermore, this plastic tube can be removed, allowing to attach other types of sensors (e.g DO, pH), which facilitated the characterization of kLa (see **Figure D*14***).

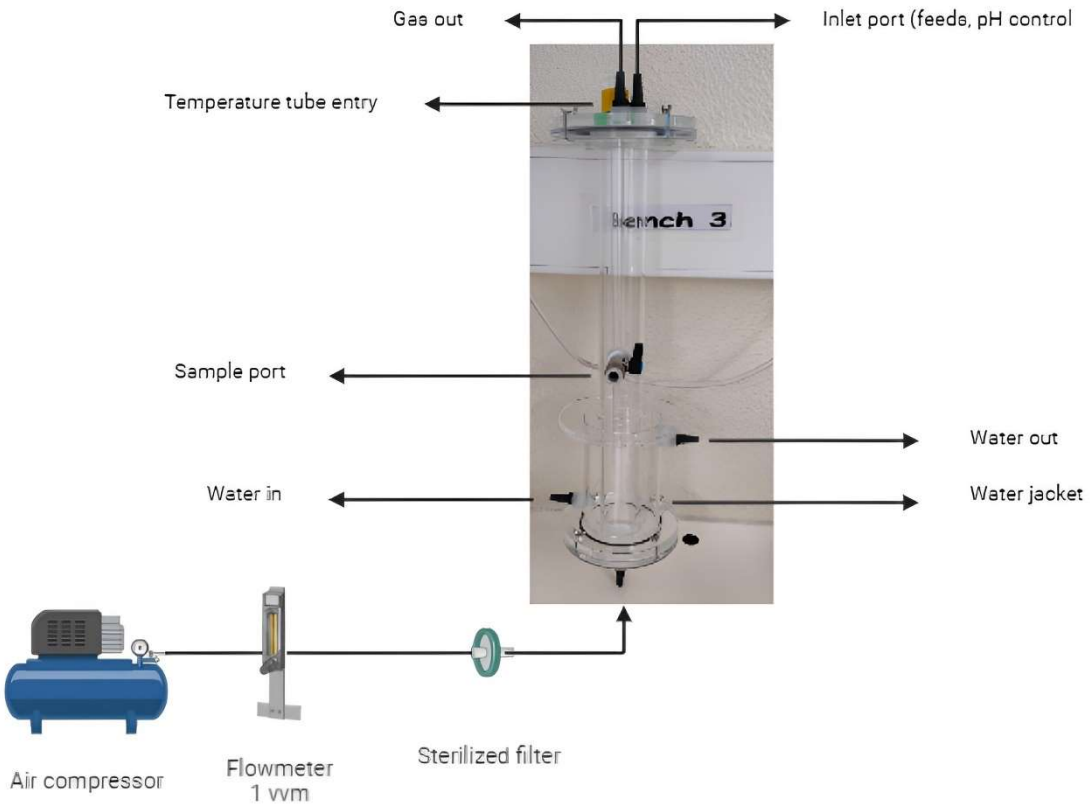


Figure D12: Setup of the modified bubble column reactor (2nd version).

Commonly utilized gas sparger types in the literature include plate type (sieve plate) and pipe type (radial, spider, etc.) (Kulkarni and Joshi, 2011). The spargers are crucial because they allow to reduce the diameter of bubbles, creating a greater gas-liquid interface and longer residence time in the culture medium. This leads to an increase in oxygen transfer (high kLa)) (Kugou et al., 2003)

In the photobioreactor used in **section D.1** the air passes through a tube, leaving by small orifices, creating tiny bubbles (as it occurs in a STR reactor). But for the bubble column reactor, there is no sparger, which is a problem, knowing the importance of oxygen for MELs production (see **section B.1**). In this regard, it was decided to design a sparger, and for bubble column reactor, plate type spargers are favoured when the column diameter is less than 1 m. This is due to the fact that for diameters higher than 1 m, the thickness of the sieve plate would need to

increase in response to high pressure at he bottom (Kulkarni and Joshi, 2011). In this regard, a sparger was designed (see **Figure D*13*** and **Table D*5***, for sparger specifications).

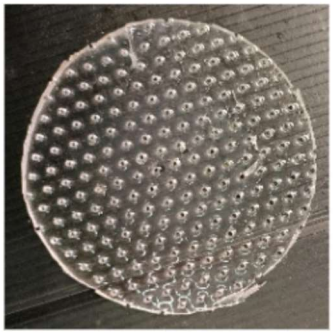


Figure D13: Sparger used in bubble column reactor

For determining kLa (see **Figure D*14***) different vvm values were used. Without a sparger it was it was only possible to use up to 2 vvm, as 3 vvm caused excessive turbulence in the system. However, with a sparger, 0.5 vvm was found to be too low, and the range was adjusted from 1 to 3 vvm. The addition of a sparger resulted in higher kLa, and at 3 vvm the value achieved is 27.7 h^{-1}. However, this is still quite low compared to the value obtained for a STR bioreactor, which is 85 ± 1 h^{-1}, under the optimal conditions for MELs production (2 vvm and 800 rpm). Additionally, when compared to photobioreactors used in **section D.1**, the kLa value is almost double (54.1 ± 0.47 h^{-1}) than the best value obtained for bubble collum reactor. Given the importance of oxygen in MELs production, this will be a challenge for the success of co-cultivation.

Therefore, for the experiments here performed in reactors, it was decided to use the sparger and 1 vvm of airflow. Higher airflows would cause excessive turbulence, increasing shear stress in the cultures. It is important to note that the value of kLa for this reactor (with sparger and using 1 vvm) is 7.3 h^{-1}.

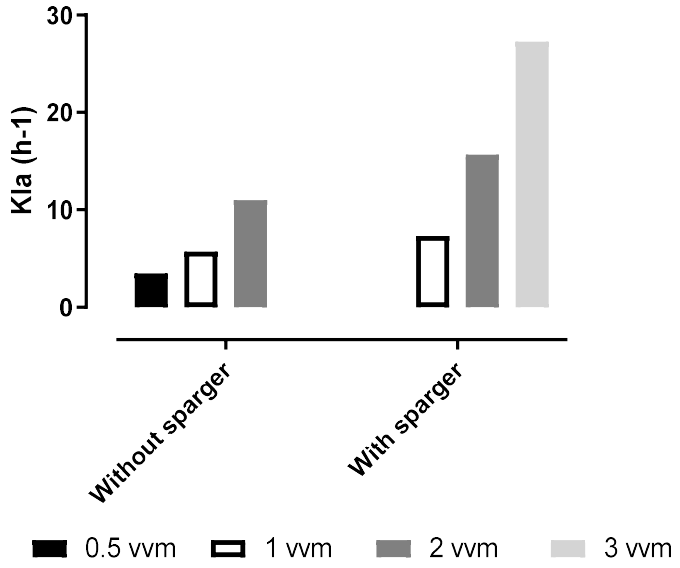


Figure D14: Determination of kLa using different vvm (0.5, 1,2 and 3 vvm) in the absence and presence of a sparger in the bubble column reactor. All tests were performed, using the components of the culture medium (Glucose, mineral salts and YE), as described in material and methods.

D.3.4.2 Mono-cultivation of *C. vulgaris* and *M. antarcticus* in bubble column reactor

Initially it was decided to study the growth of *C. vulgaris* in bubble column reactor. Note that, different from **section D.2**, where it was accessed the production of lipids from *N. oleoabundans,* here it was decided to use *C. vulgaris*, since it's the microalgae with higher growth rate (see **section D.1)**.

This experiment was performed with the objective to evaluate the growth of *C. vulgaris* in these types of reactors (**Figure D*15*** A, C) and also the lipids production. This experiment was also performed in Erlenmeyer flasks as controls for the bioreactor (**Figure D*15*** B, D). The results are represented in **Figure D*15***.

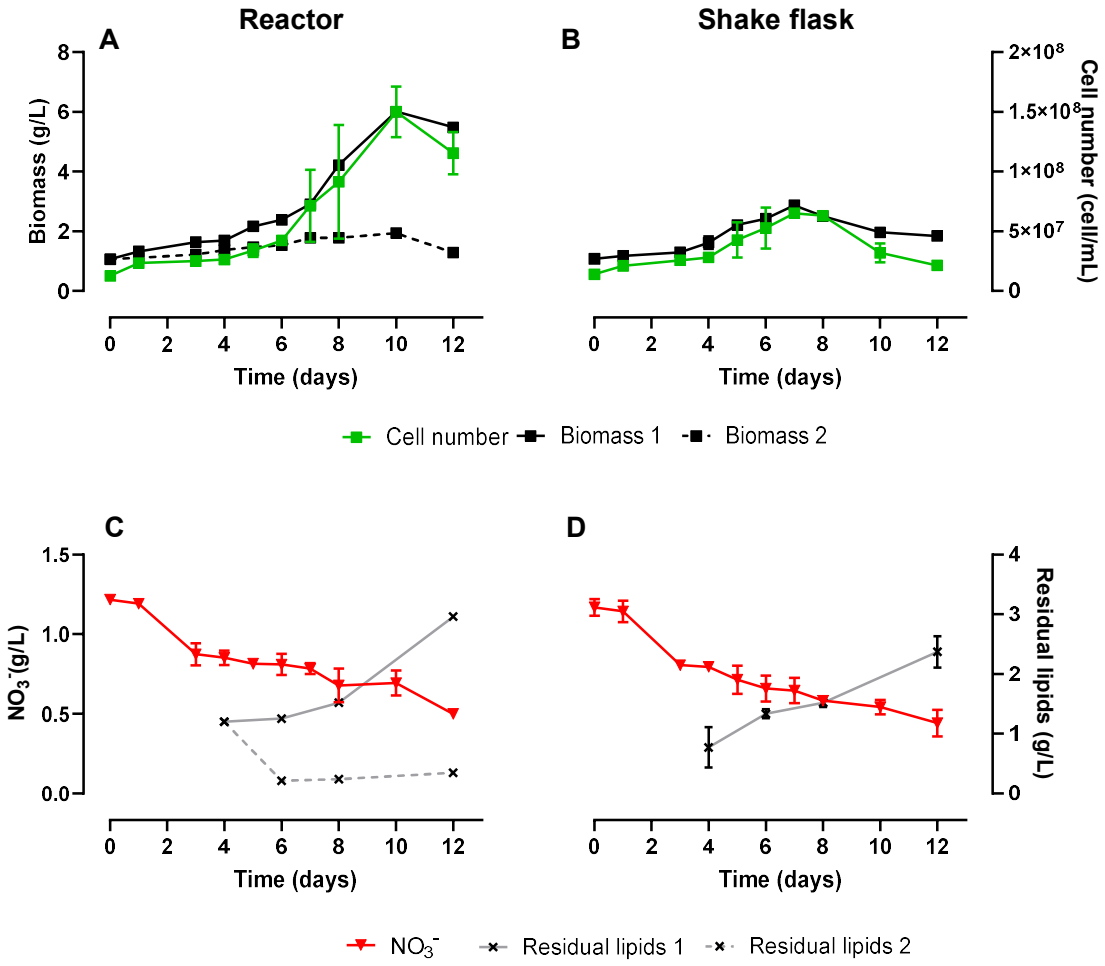


Figure D15: Mono-cultivation of *C. vulgaris* CCAP 211/2b in bubble column bioreactor (A, C) and shake flasks (B, D), for 12 days. (A, B); Biomass (black line with squares) and cell number/mL profiles (green line). (C, D); Consumption of nitrate (inverted triangles) and lipids production (grey line). The dashed and filed line (from same type of line) correspond to each reactor, as two independent replicas. Temperature was kept at 27ºC and 1 vvm was used as airflow.

C. vulgaris was able to growth on an autotrophic regime using *M. antarcticus* culture medium. In fact, observing cell number/mL (**Figure D*15*** A), at day 10 it is achieved a maximum of 1.5E+8 cell number/mL, a value higher than the one obtained for *N. oleoabundans* when cultivated during 15 days (2E+7 cell number/mL, see **Figure D*8***, **section D.2).** This can be due to the use of sparger, but also because *C. vulgaris* has a higher growth rate than *N. oleoabundans.* Nevertheless, it is possible to observe that both replicas of reactor had different behaviours

concerning the biomass and lipids production, and this might be explained maybe because of one the inoculum used in the reactor was already under stress. Interestingly for shake flask and for reactors, the production of lipids started at day 4, while there is still nitrate available. This might indicate that lipids production was stimulated by the consumption of other component (potentially magnesium or potassium), instead of nitrate, as observed for *N. oleoabundans.*

In **Table D*6*** it is represented the lipidic profile for each reactor. As expected, both reactors had different profiles, since even the production of biomass was different (5.4 and 1.3 g/L of biomass). One way that to address this problem in the future, is to control pH or even CO2 emissions (which due to a failure in the equipment was not possible to address in this section).

Table D6: Fatty acid composition of the lipids produced by *C. vulgaris* CCAP 211/2b after 12 days of cultivation for two distinct reactors. * means that there was an overlap in the C18:0 and C18:1 peaks.

Fatty acid chain (%)	Reactor 1	Reactor 2
C16:0	11.37	18.75
C16:1	17.61	17.53
C18*	71.02	63.52
Total lipids content	20.74	10.12

Even though the optimal condition for producing MELs is to start with 40 g/L of glucose and 20 g/L of WFO (see **section B.1**), for this proof-of concept, it was decided to use only glycerol as carbon source. This decision was made for two reasons: 1) *C. vulgaris* has a negligible consumption of glycerol (see **section D.1)**, and it is know that *C. vulgaris* in heterotrophic mode can consume 20 g/L of glucose in less than 48 hours, as shown by (Schüler et al., 2020).: 2) Based on results obtained in **section D.2**, WFO was not used to observe the lipid profiles produced at the end of co-cultivation.

As performed by *C. vulgaris,* production of MELs was followed in reactor with sparger (**Figure D*16*** A, C) and shake-flask (**Figure D*16*** A, C). Unfortunately, but expected, MELst titre in reactor using sparger (0.74 ± 0.04 g/L) is even lower than the one obtained in shake-flask (1.26 ± 0.04 g/), indicating that bubble column reactor is really not suitable for MELs process. However, there

are no differences of final biomass between shake-flask (11.00 ± 0.10 g/L) and reactor (10.81 ± 0.13 g/L).

Nevertheless, based on these results, it was decided not to proceed with co-cultivation, as the aim was to produce MELs, which is not feasible with this type of reactor. In the future, production should be shifted to photobioreactors (such as the ones used in **section D.1**, having a kLa of 54.1 ± 0.47 h^{-1}) or even using STR with integrated LED lights.

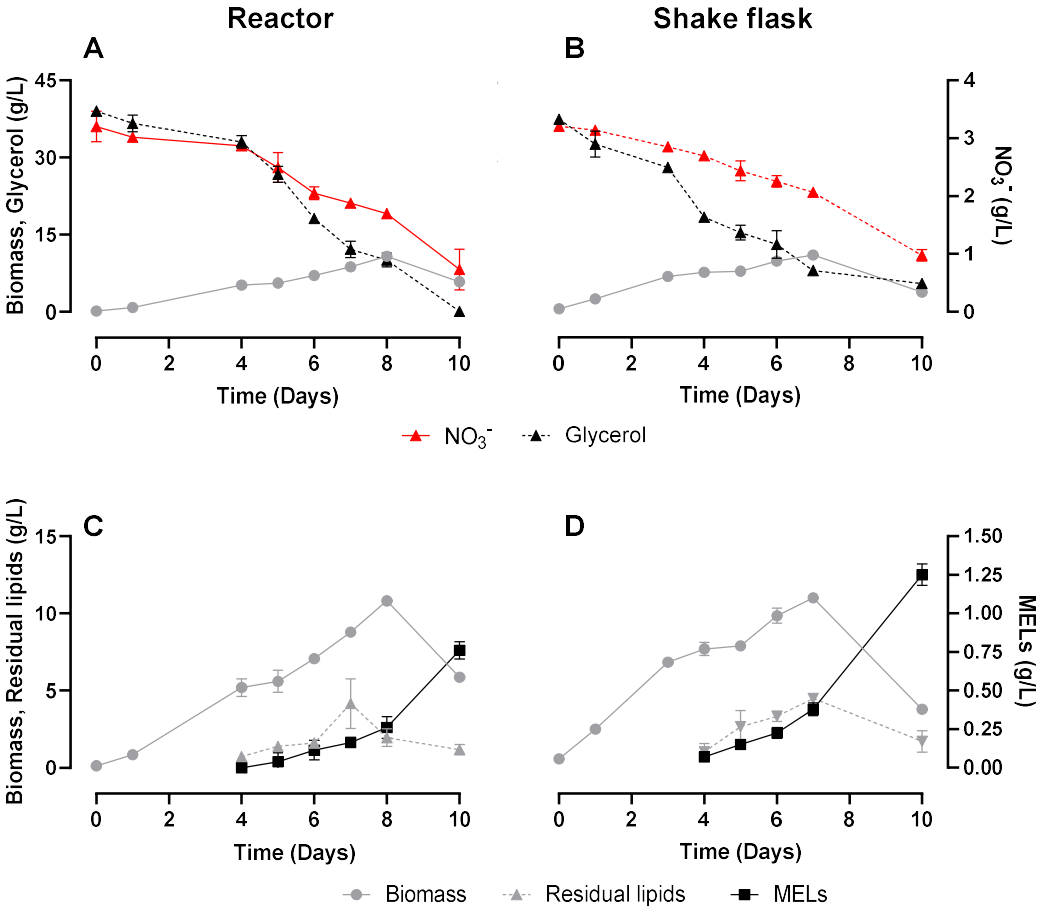


Figure D16: Mono-cultivation of C. vulgaris CCAP 211/2b in bubble column bioreactor (A, C) and shake flasks (B, D), for 10 days. (A, B); Production of Biomass (grey circles), consumption of glycerol (dashed black line with triangles) and nitrate (filled red line with triangles). (C, D); MELs (black line with squares) and lipids production (dashed line with triangles). The experiment was performed in duplicates.

D.3.4 Final remarks

The aim of this section was to develop a co-cultivation process for MELs production. For that it was used the conclusions drawn in **section D.1**. Initially the bubble column reactor was re-design for a better growth of strains and control of the process (less probability of contamination). Then it was constructed a sparge for better gas transference in reactor (5.4 and 7.8 h^{-1} for reactor without and with sparger, respectively).

It was found that the growth of *C. vulgaris* is not inhibited by using yeast culture medium, and it was achieved a biomass of 1 g/L in just 12 days. However, the mono-cultivation of *M. antarcticus,* only rendered 0.74 ± 0.04 g/L of MELs. This value is very low, and due to the low oxygenation conditions observed for this type of reactor.

Based on these results, it was decided to not proceed with co-cultivation in these types of reactors. In the future it is mandatory to shift the production to other bioreactors.

Section E: Conclusions and future perspectives

E.1.1 Conclusions

The aim of this thesis was to develop new strategies to produce MELs and other high value products (organic acids, such as citric acid) from waste streams. This involved exploring the features of non-conventional yeasts, alone or synergistically in combination with microalgae, to enhance the environmental performance of production processes and contributing to the creation of a biorefinery that supports circular economy approaches.

To achieve this main objective, the thesis was divided into three main sections. Each one reached different conclusions, but all shared the common goal of creating a robust and environmentally friendly MELs-production bioprocesses.

E.1.1.1 Section B

Section B focused on intensifying the production process by improving MELs titres and productivity through different cultivation conditions and carbon source feeding regimes. Simultaneously, agro-industrial side streams were attempted to replace culture medium components (e.g carbon source). The main conclusions drawn from this section were:

- The use of co-substrates with opposite polarities increased MELs titres and productivities, without comprising the final purity of the product. Moreover, when using this approach in combination with a fed-batch fermentation, one of the highest yields reported in the literature was obtained (is 0.6 ± 0.1 mol of C_{MELs}/mol of $C_{substrate}$).
- *Moesziomyces* spp. can produce β-galactosidases, and consequently consume lactose as carbon source. Lactose, but in particular galactose, are strong inducers of β-galactosidase production by these yeasts.
- Cheese-whey, side stream of cheese industry and reach in lactose, can be used simultaneously as carbon source and replacement of mineral and yeast extract. This conclusion is important to reduce overall costs of the process and increase the sustainability of MELs bioprocess.
- The use of pomace oil (side-stream of olive oil industry) as hydrophobic carbon source for MELs production by *Moesziomyces* spp. led to the highest titre obtained in this thesis

(> 70 g/L of MELs). This study also provided the hypothesis that the use of substrates with high content of free fatty acids (and consequent high acidic value), leads to higher titres/productivities of MELs than using common vegetable oils as hydrophobic carbon source).

E.1.1.2 Section C

The focus of this section was to integrate MELs bioprocess by developing: i) a green and efficient technology capable of purify MELs; ii) a mathematical model from MELs bioprocess, performing an economic assessment and identifying the main bottlenecks of the processes. The main conclusions drawn from this section were:

- If MELs crude mixture are still contaminated with TAG, methanol should be used, since it is the solvent that allows the better separation between these two components.
- The use organic solvent nanofiltration technology allowed to reach 98% of purity with only 11.6% of MELs losses and the possibility of solvent recycling, achieving one of the best values reported in the literature for the extraction/purification of MELs. Moreover, the technology here proposed in this section, can be applied to others mBS, such as SLs and RMs.
- For the first time it was designed a mathematical model for the entire MELs bioprocess, performing economic evaluation and identifying important parameters, such as:
    - The scale-up of the process leads to a reduction of unit cost of production, but only until a scale of 20 m3 (a final cost of 25.5 €/Kg_{MELs}).
    - The way to overcome equipment downtime, is to identify the bottleneck equipment (in this case, the main bioreactor) and then different equipment can be coupled in a stagger mode. The optimal scenario uses four main bioreactors of 20 m^3.
    - It is favourable to use more bioreactors with a lower capacity rather than using a low bioreactor number, but with higher capacity. In the performed model , the use of 4 reactors of 20 m^3 leads to a final payback time of 5.4 years, while the use of 1 bioreactor of 100 m3 leads to 7.8 years.

- Maintaining the same conditions, and just by replacing carbon source and culture medium by cheese-whey leads to a reduction of payback time when compared with normal scenario (5.08 and 5.4 years).
- Overall CO_2 emissions, culture medium and productivity are the main bottlenecks of this process.

E.1.1.3 Section D

The main objective was to design a system to couple phototrophic microalgae and non-conventional yeasts culture, especially in co-cultivation, for the production of organic acids (CA) and MELs. Overall, the most important parameters of co-cultivation found here and that are transversal to every bioprocess were:

- Selection of culture medium is important, and it must favour both microorganisms. Specifically, there must not exist co-competition for the same components, such as carbon source or nitrogen source.
- It is necessary to find the optimal ratio of yeast to microalgae. This can be done by decreasing the ratio of yeast to microalgae inoculum.
- Since microalgae have a lower growth when compared to yeasts, co-cultivation process should be de-coupled in the beginning, allowing microalgae to lead a head start for growth. It was found here in this thesis that delaying the co-cultivation process for 5-7 days (until achieve the stationary phase), leads to an increase in CA productivity of 87%, when compared with co-cultivation where both strains were added at the same time.
- A light regime (light/dark) of 16:8h is the appropriate to have

It was also discovered that MELs can be formed by using lipids previously produced by oleaginous microalgae. This supports the possible creation of a biorefinery. Unfortunately, MELs production was not achieved when using co-cultivation, since bubble column reactor are not suitable for MELs production (kLa of 7.3 h^{-1}), and future studies are required to use a different photobioreactor (same as used in **section D.1,** with a kLa of 54.1 ± 0.47 h^{-1}), or even using

photobioreactor with Rushton impellers. Nevertheless, it was found that *C. vulgaris* growth is not inhibited by yeast culture medium.

E.1.2 Future perspectives

E.1.2.1 Use of industrial side-streams

The integration of side-streams in MELs bioprocess is crucial to have a sustainable process that promotes a circular economy approach, but also to tackle one of the major bottlenecks of the process (price of glucose and culture medium components). Remarkably, in this thesis was found that the use of cheese whey as carbon source and culture medium replacer has no impact in the final productivity, when compared with the optimal condition (glucose + culture medium). However, these results were achieved only in shake-flask, and future works should use CW in bioreactor, to understand if it continues to do not affect the final productivity of the process.

On this thesis was used pomace oil, previously extracted with hexane, which is not sustainable. Moreover, these residues are obtained from a factory (3-phase decanter), also generates other type of side-stream (liquid oil mill wastewater streams, OMW). Considering that PO residues requires a prior extraction with organic solvents, focus should be shifted focus should be redirected to the OMW side-stream. This fraction is fully enriched in residual lipids and only requires a centrifugation process to separate water from these residues, avoiding the use of organic solvent (work on-going).

Nevertheless, other side streams should be tried, especially lignocellulosic residues (the most abundant side-stream in planet earth, or potentially wastewater, that also have the potential to replace culture medium.

E.1.2.2 Novel bioreactor processes and scale-up

In order to have a competitive process with SLs, it is necessary to improve the final titre (> 100 g/L), and this might be achieved by using different bioreactors setup. For example, the use of marine impellers, instead of Rushton can have a potential impact in MELs bioprocess and, so far was never attempted it. Also, it is important to use in the future computational fluid dynamics (CFD) methods, that will allow to have a better characterisation of the reactors regarding fluid and oxygen dispersion, allowing to achieve the optimal condition for MELs bioprocess.

Furthermore, it is crucial to integrate artificial intelligence (AI) with MELs bioprocess in the future. With the appropriate model and data, AI will be able to predict the best feeding regimes and anticipate several scenarios (especially if the reactor should be stopped). Using these tools will allow to have a bioprocess more efficient and robust.

Additionally, it is important to validate the processes at different scales (at least to scales of 50-150 L), which will allow to discover potential problems that might appear (e.g foam appearance) and validate the process regarding final titre and purity of MELs.

E.1.2.3 Co-cultivation process

The parameters here defined to have a successful co-cultivation process are promising. However, as described in this thesis, MELs production is entirely dependent on oxygen, and the use of air-lifts bioreactors are not ideal for MELs bioprocess. Therefore, in the future it is necessary to use other types of bioreactors, such as stirred-tank or tubular photobioreactors, that will allow a better oxygen transference. This will allow to have a better co-cultivation process, and transference of O_2-CO_2 between microalgae/yeast.

E.1.2.4 Exploring innovative applications

Even though it was not the case of study of this study, aiming new MELs applications it is crucial for the success of this bioprocess, since it what will validate the potential of the molecule in a wide range of areas. For these studies, more than just testing one formulation, for each application, it is important to validate the multifunctionality properties of MELs, aiming the development of new formulations that can act in different areas.

Furthermore, while the process is not yet fully established and the production costs are still high, initially it is also crucial to focus in high-value applications (such as medicine, pharmaceutical, and cosmetics).

E.1.2.5 LCA analysis and economic studies

To fully understand the environmental impact of MELs bioprocess it is essential to perform a life-cycle analysis (LCA). This analysis includes all sections of bioprocess, from upstream and substrate pre-treatment to product extraction, use and disposal. For instance, merely using an industrial side stream does not guarantee a more sustainable process when compared with

process using normal culture medium. Factors such as pre-treatment, and the distance between the factory and facilities where the bioprocess will happen, can potentially impact the sustainability. Therefore, it is important in the future to develop a LCA for MELs bioprocess, and use the values obtained for CW and pomace oil, to understand the final impact in the environment.

Considering the economic analysis, even though if it was created a model, it is important to update with new data (e.g actual price of bioreactor), and test different scenarios, such as: 1) Different industrial side streams and the impact of their pre-treatment; 2) Different feed regimes; and 3) Different downstream techniques, such as nanofiltration here developed.

E.1.3 Final remarks

This thesis established important milestones in the MELs bioprocess, helping to bridge the gap with other established microbial biosurfactants (mBS) in the market, such as rhamnolipids (RMs) and sophorolipids (SLs). Additionally, some of the results here obtained (use of CW; co-cutlivation parameters; nanofiltration; economic analysis) can be used in other types of bioprocesses.

To conclude I would like to share a quote from professor Leon C. Megginson, which stated ‘’*According to Darwin’s Origin of Species, it is not the most intellectual of the species that survives; it is not the strongest that survives; but the species that survives is the one that is able best to adapt and adjust to the changing environment in which it finds itself.*’’ (Megginson, 1963). This raises a question: Are we capable of adapting to the changing environment, by adopting new green solutions and processes, or we will keep paving the easy way, by relying on fossil-fuel products?

Section F: Appendix

F.1 Supporting information for section B.1:

Production of MELs in shake-flask: The importance of oxygen In MELs production

Table F1: Rate of glucose (Rs) and nitrate consumption (RNaNO3); maximum biomass produced and the respectively yield (Yx/s); maximum MELs obtained (g/L), the respective yield (gMELs/gSubstrate) and productivity (g/L/day), MEL purity and the final concentration of residual lipids in the fermentation broth for *M. bullatus* PYCC 5535T, cultures incubated during 7 days at 27ºC using 40 g/L of glucose and 20 g/L of WFO as carbon source, varying the ratio of volume headspace and medium volume (0.25, 1.5, 4 and 9 for a total volume of 200, 100, 50 and 25 mL, respectively).

Parameters	**Ratio volume headspace/medium volume**			
	0.25	**1.5**	**4**	**9**
rNano3 (g/L/h)	**0.004 ± 0**	**0.009 ± 0**	**0.015 ± 0**	**0.016 ± 0**
rs (g/L/h)	0.06 ± 0.01	0.13 ± 0.02	0.21 ± 0	0.18 ± 0
$Biomass_{max}$ (g/L)	11.5 ± 1.5 (day 4)	13 ± 0 (day 4)	18 ± 0 (day 7)	18 ± 0 (day 7)
$Y_{Xmax/S}$ (g/g)	0.19 ± 0.03	0.22 ± 0	0.3 ± 0	0.3 ± 0
$MELs_{max}$ (g/L)	6.5 ± 0.5 (day 7)	10.52 ± 0.2 (day 7)	20.4 ± 3.19 (day 7)	25.63 ± 0.07 (day 7)
$Y_{MELs/Substrate}$ (g/g)	0.11 ± 0.01	0.18 ± 0	0.34 ± 0.05	0.43 ± 0
Y mol C MELs/ mol C Substrate (mol/mol)	0.06 ± 0	0.1 ± 0	0.2 ± 0.03	0.25 ± 0
$Productivity_{max}$ (g/L/day)	0.93 ± 0.07	1.5 ± 0.03	2.91 ± 0.46	3.66 ± 0.01
MELs purity (g/g)	0.26 ± 0.01	0.81 ± 0.02	0.77 ± 0.02	0.89 ± 0.03

Parameters	Ratio volume headspace/medium volume			
	0.25	**1.5**	**4**	**9**
Residual lipids (g/L)	18.4 ± 2.7	2.42 ± 0.41	6.12 ± 0.16	3.2 ± 0.82

Biomass $_{max}$ – maximum biomass cell dry weight (g/L); r_s – sugar consumption rate (g/L/h); $Y_{Xmax/S}$ – maximum biomass yield (g/g); MELs $_{max}$ – maximum MELs produced (g/L); Y $_{MELs/Substrate}$ – maximum MELs yield (g/g)

Figure F1: Typical beads enriched in MELs that appear during the cultivation in shake-flask

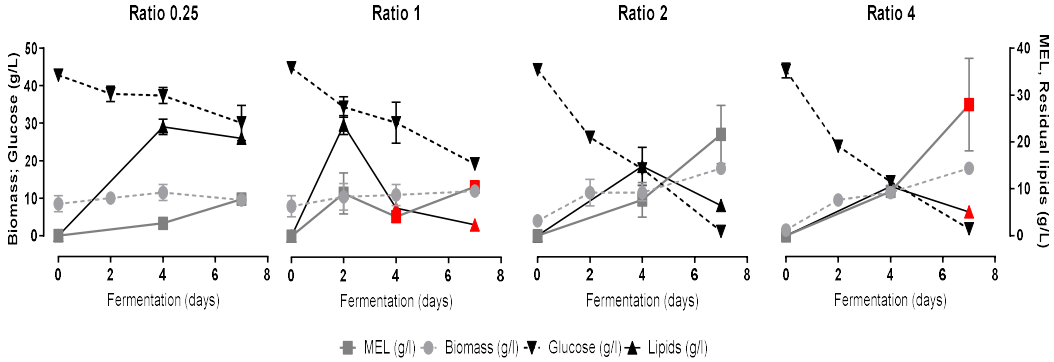


Figure F2: Profile growth (grey filled line with circles), glucose consumption (black dashed line with inverted triangles), MELs production (gold filled line with squares) and residual lipids (black filled line with triangles) for *M. bullatus* PYCC 5535^{T} cultures incubated during 7 days at 27ºC using 40 g/L of glucose and 20 g/L of WFO as carbon source, varying the ratio of volume headspace and medium volume (0.25, 1.5, 4 and 9 for a total volume of 200, 100, 50 and 25 mL, respectively). The red point at specific days indicates the presence of beads.

Production of MELs in bioreactors

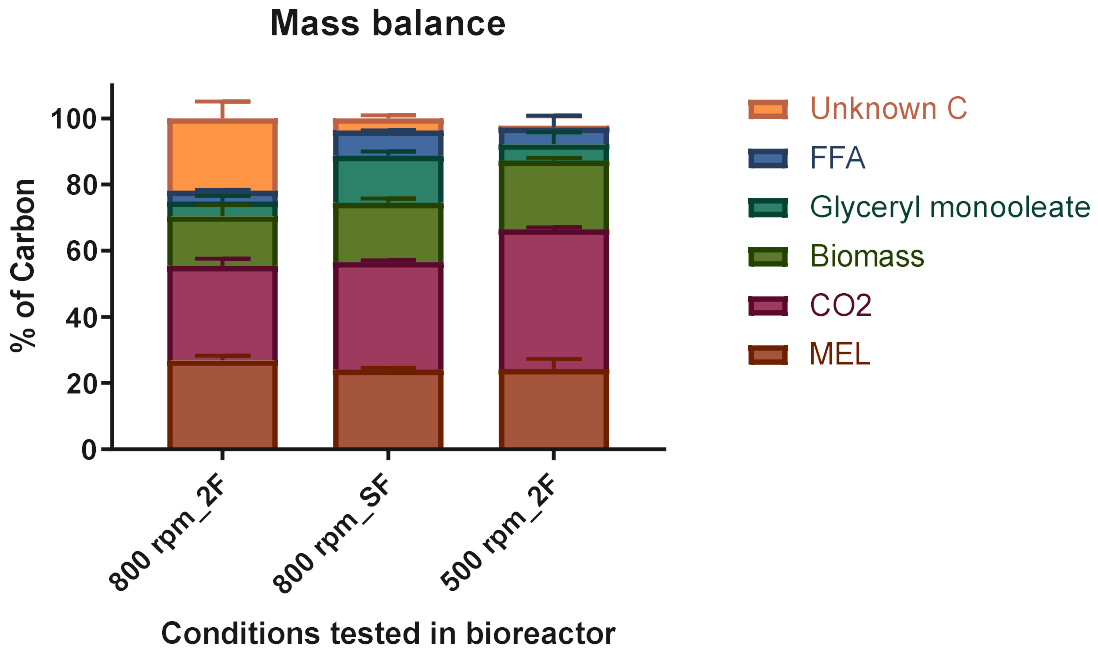


Figure F3*:* Mass balance in carbon for the bioreactors performed: 1) The case where the cascade system varied from 150-800 rpm and airflow from 0.5-2vvm, using two large feeds of 20 g/L of WFO (800 rpm_2F) or small feeds of 6.66 g/L of WFO every day until day 6 (800 rpm_SF); 2) Cascade system varied from 150-500 rpm and airflow from 0.5-1vvm. This mass balance in carbon included: MELs, CO2 emissions, biomass, glyceryl monooleate (MAG), free fatty acids (FFA) and unknown C

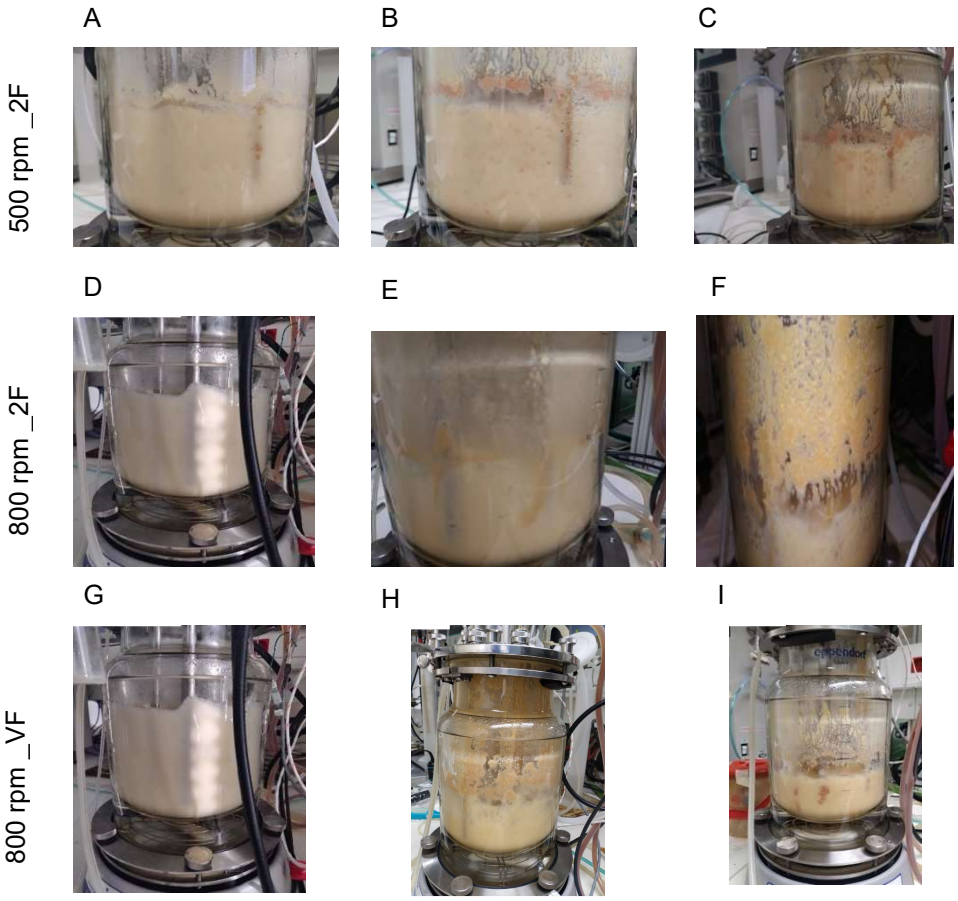


Figure F4: Pictures taken at specifi days for the bioreactors performed: 1) Cascade system varied from 150-500 rpm and airflow from 0.5-1vvm (A, B, C); 2) cascade system varied from 150-800 rpm and airflow from 0.5-2vvm, using two large feeds of 20 g/L of WFO (D, E, F) or small feeds of 6.66 g/L of WFO every day until day 6 (G, H, I). A, D, G – day 3 of fermentation; B, E, F – Day 6 of fermentation; C – Day 12 of fermentation and F, I – day 8 of fermentation.

Lipase activity for both strains when using 40 g/L of glucose as sole carbon source.

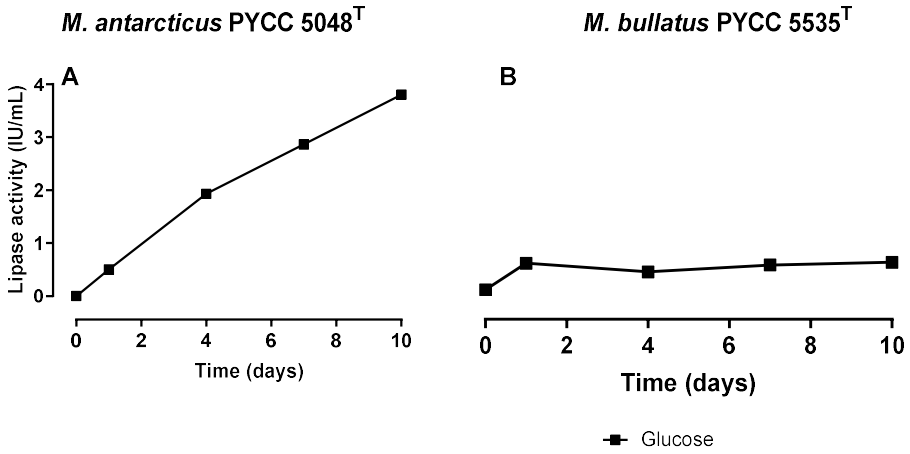


Figure F5: Individual profiles of lipase activity by *M. antarcticus* PYCC 5048^T and *M. bullatus* PYCC 5535^T, using 40 g/L of glucose as carbon source.

F.2 Supporting information for Section B.2:

Characterization of Cheese-whey before and after pasteurization.

Table F2: Analysis of the components present in cheese whey, before and after pasteurization. The D-lactose is hydrolysed into D-glucose and D- galactose**.**

CW analysis	Before pasteurization	After pasteurization
Ammoniacal nitrogen (g/L)	0.12	0.12
Kjeldahl nitrogen (mg/L)	2.81	1.08
Content of D-glucose in hydrolysate* (g/L)	56	62
Content of D-galactose in hydrolysate (g/L)	64	71
Content of total sugars in hydrolysate (g/L)	113	128
Chloride* (g/L)**	0.56	0.56
Phosphorous (g/L)**	0.89	0.63
Content of oil (% m/m)	0.070	0.014
Sodium (g/L)**	0.56	0.54
Potassium (g/L)**	2.10	2.00
Calcium (g/L)**	1.10	0.40
Magnesium (g/L)**	0.23	0.18

*The hydrolysate was obtained after placing a sample of CW for 30 min at 120 ºC in the autoclave, in the presence of sulphuric acid (2 M). The resulting sugars were analysed using HPLC-IR (as described in Materials and Methods section).

**Obtained by FAAS - Flame Atomic Absorption Spectrometry

***Potentiometric titration

Enzymatic profiles β-galactosidase and lipase for different conditions

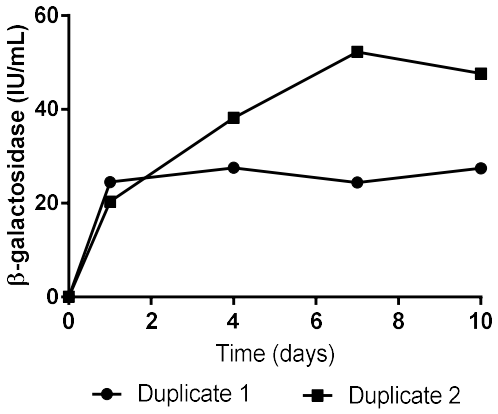


Figure F6: Individual profiles of β-galactosidase extracellular activity produced by M. bullatus PYCC 5535^{T}, using 40 g/L of lactose as carbon source and without $NaNO_3$.

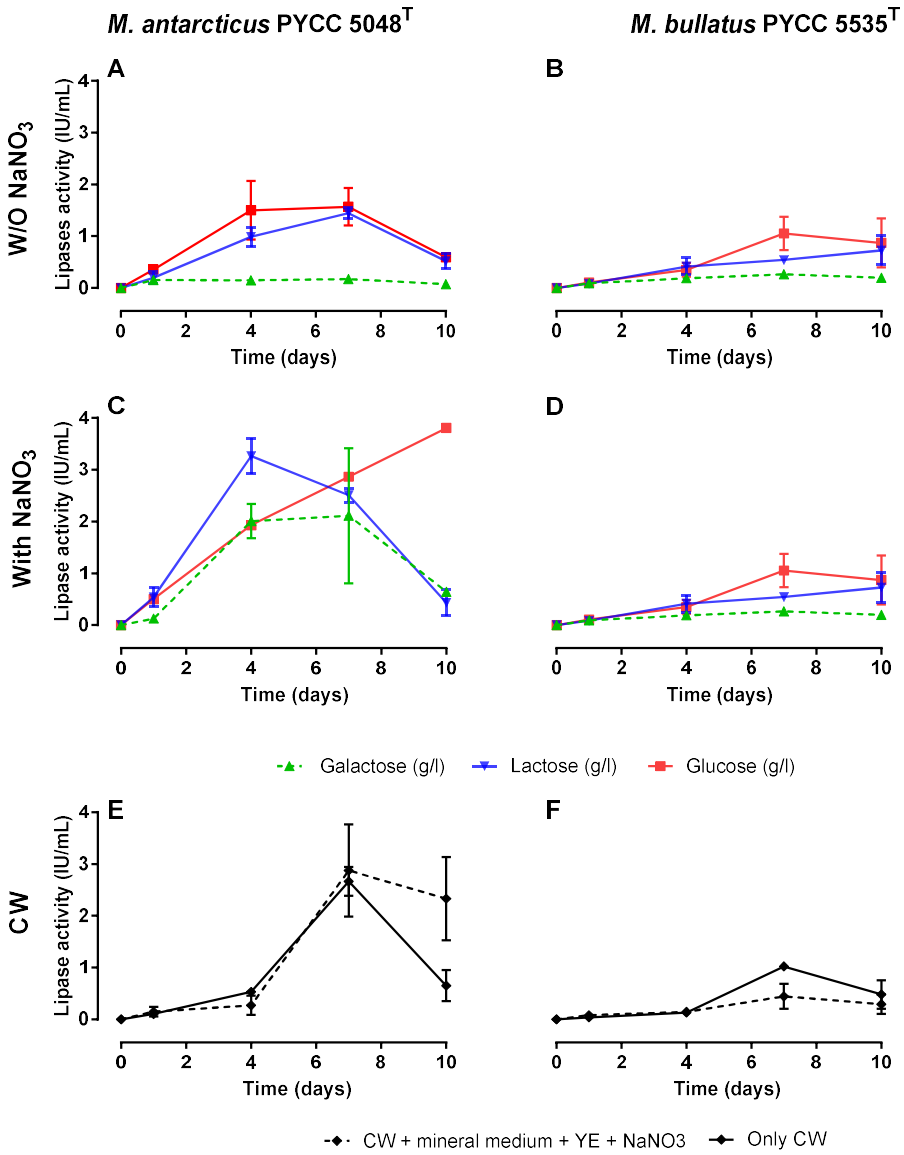


Figure F7: Lipase production (IU/mL) in *M. antarcticus* PYCC 5048^T (A, C, E) and *M. bullatus* PYCC 5535^T (B, D, F); cultures incubated during 10 days at 27 ºC, in mineral medium with YE, using different carbon sources: D-galactose (40 g/L), D-glucose (40 g/L) and D-lactose (40 g/L) in the absence (A, B) or presence (C, D) of $NaNO_3$. Also, lipase production when using CW as solo carbon source, in the presence (dashed line) and absence (filled line) with mineral medium, YE and $NaNO_3$. Standard deviation values lower than 1 IU/mL are not represented.

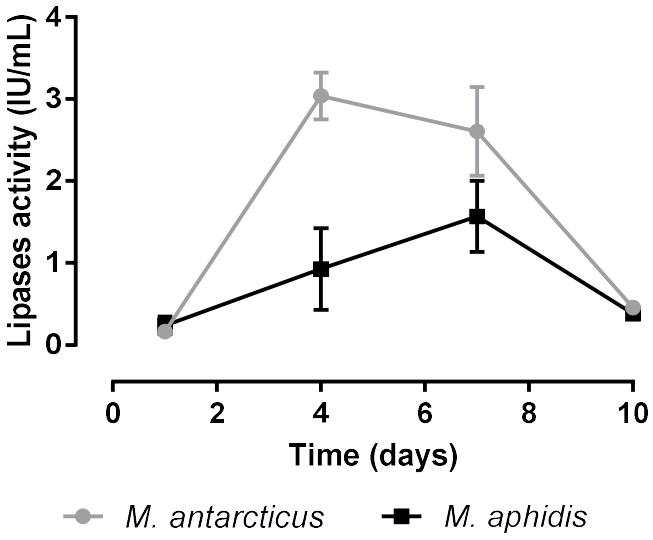


Figure F8: Lipase production in *M. antarcticus* PYCC 5048^{T} (grey filled line with circles) and *M. bullatus* PYCC 5535^{T} (black filled line with squares), using a mixture of D-galactose (20 g/L) and D-glucose (20 g/L), as carbon source in the presence of mineral medium with YE and $NaNO_3$. Cultures incubated during 10 days at 27ºC. Standard deviations values lower than 1 IU/mL are not represented.

Determination of different parameters for different conditions

Table F3: Rate of consumption (Rs), maximum biomass produced and the respectively yield (Yx/s), maximum MEL obtained (g/L); yield of MEL produced ($g_{MEL}.g_{Substrate}^{-1}$); volumetric (IU/mL) and specific activity ($IU/g_{biomass}$) for *M. bullatus* PYCC 5535^T and *M. antarcticus* PYCC 5048^T using of D-galactose (40 g/L) and a mixture of of D-galactose (20 g/L) and D-glucose (20 g/L), as carbon sources, in the presence of mineral medium with YE and $NaNO_3$.

Parameters	*M. antarcticus* PYCC 5048^T			*M. bullatus* PYCC 5535^T		
	D-galactose	Co-substrate		D-galactose	Co-substrate	
		D-glucose	D-galactose		D-glucose	D-galactose
rs (g/L/h)	0.32 ± 0.02	0.15 ± 0.00	0.21 ± 0.02	0.29 ± 0.01	0.17 ± 0.01	0.19 ± 0.01
$Biomass_{max}$ (g/L)	10.0 ± 3.0 (Day 4)	20.0 ± 1.0 (Day 4)		10.5 ± 1.5 (Day 7)	14.5 ± 0.5 (Day 4)	
$Y_{Xmax/S}$ (g/g)	0.25 ± 0.08	0.50 ± 0.02		0.26 ± 0.04	0.36 ± 0.01	
MELs max (g/L)	2.20 ± 0.50 (Day 7)	3.20 ± 0.17		0.38 ± 0.07 (Day 4)	1.70 ± 0.52 (Day 4)	
$Y_{MELs/Substrate}$(g/g)	0.05 ± 0.01	0.08 ± 0.00		0.01 ± 0.00	0.04 ± 0.01	
β-galactosidase (IU/mL)	505.2 ± 3.1 (Day 7)	36.0 ± 1.2 (Day 7)		127.0 ± 31.2 (Day 4)	31.4 ± 0.4 (Day 7)	
β-galactosidase ($IU/mg_{biomass}$)	63.1 ± 6.1	3.3 ± 0.1		11.2 ± 1.0	3.8 ± 0.7	

Biomass $_{max}$ – maximum biomass cell dry weight (g/L); r_s – sugar consumption rate (g/L/h); $Y_{Xmax/S}$ – maximum biomass yield (g/g); MELs $_{max}$ – maximum MELs produced (g/L); $Y_{MELs/Substrate}$ – maximum MELs yield (g/g); β-galactosidase (IU/mL) max – Maximum volumetric activity of β-galactosidase; β-galactosidase ($IU/g_{Biomass}$) max – Maximum specific activity β-galactosidase;

Table F4: Rate of consumption (Rs), maximum biomass produced and the respectively yield (Yx/s), maximum MELs obtained (g/L); yield of MELs produced (gMELs/gSubstrate); volumetric (IU/mL) and specific activity (IU/gbiomass) for *M. bullatus* PYCC 5535^T and *M. antarcticus* PYCC 5048^T using D-galactose (40 g/L) or D-glucose (40 g/L), as carbon sources, in the presence of mineral medium with YE and in the absence of NaNO3.

Parameters	***M. antarcticus* PYCC 5048^T**		***M. bullatus* PYCC 5535^T**	
	D-galactose	**D-glucose**	**D-galactose**	**D-glucose**
rs (g/L/h)	0.20 ± 0.00	0.21 ± 0.00	0.21 ± 0.00	0.27 ± 0.00
$Biomass_{max}$ (g/L)	8.5 ± 1.5 (Day 7)	14.0 ± 1.5 (Day 7)	10.0 ± 0.1 (Day 7)	8.0 ± 0.5 (Day 4)
$Y_{Xmax/S}$ (g/g)	0.25 ± 0.04	0.35 ± 0.00	0.25 ± 0.00	0.20 ± 0.50
MELs max (g/L)	1.50 ± 0.20 (Day 10)	4.52 ± 0.34 (Day 7)	2.2 ± 0.08 (Day 10)	3.4 ± 0.19 (Day 7)
$Y_{MELs/Substrate}$ (g/g)	0.04 ± 0.0	0.11 ± 0.00	0.05 ± 0.00	0.08 ± 0.00
β-galactosidase (IU/mL)	154.3 ± 14.6 (Day 10)	2.7 ± 0.6 (Day 7)	8.6 ± 3.3 (Day 10)	1.6 ± 0.0 (day 10)
β-galactosidase ($IU/mg_{biomass}$)	24.1 ± 4.0	0.19 ± 0.02	1.3 ± 0.4	0.2 ± 0.0

Table F5: Rate of consumption (rs), maximum biomass produced and the respectively yield (Yx/s), maximum MEL obtained (g/L); yield of MEL produced (gMEL/gSubstrate); volumetric (IU/mL) and specific activity ($IU/g_{biomass}$) for *M. bullatus* PYCC 5535^T and *M. antarcticus* PYCC 5048^T using D-lactose (40 g/L) as carbon source, in mineral medium with YE and either in the presence or in the absence of NaNO3.

Nitrogen source	**Parameters**	***M. antarcticus* PYCC 5048^T**	***M. bullatus* PYCC 5535^T**
		D-lactose	
With NaNO3	rs (g/L/h)	0.32 ± 0.01	0.39 ± 0.04
	$Biomass_{max}$ (g/L)	18.0 ± 4.0 (Day 4)	13.5 ± 1.5 (Day 4)
	$Y_{Xmax/S}$ (g/g)	0.45 ± 0.10	0.33 ± 0.04
	MEL max (g/L)	2.93 ± 0.53 (Day 10)	1.91 ± 0.01 (Day 4)
	$Y_{MEL/Substrate}$(g/g)	0.07 ± 0.02	0.09 ± 0.00
	β-galactosidase (IU/mL)	44.50 ± 3.30 (Day 7)	37.52 ± 10.00 (Day 10)
	β-galactosidase (IU/mgbiomass)	5.10 ± 0.77	4.66 ± 0.1
Without NaNO3	rs (g/L/h)	0.24 ± 0.00	0.38 ± 0.02
	$Biomass_{max}$ (g/L)	17.0 ± 1.0 (Day 4)	10.5 ± 1.5 (Day 7)
	$Y_{Xmax/S}$ (g/g)	0.42 ± 0.03	0.26 ± 0.03
	MEL max (g/L)	4.93 ± 0.53 (Day 10)	2.20 ± 0.10 (Day 10)
	$Y_{MEL/Substrate}$(g/g)	0.12 ± 0.04	0.05 ± 0.00
	β-galactosidase (IU/mL)	16.74 ± 0.21 (Day 10)	7.67 ± 0.00 (Day 10)
	β-galactosidase (IU/mgbiomass)	1.90 ± 0.52	1.10 ± 0.01

Table F6: Intracellular and extracellular β-galactosidase activity (Volumetric (IU/mL) and specific activity ($IU/g_{biomass}$) at day 2 and 4 of fermentation, for for *M. bullatus* PYCC 5535^T and *M. antarcticus* PYCC 5048^T using: D-galactose (40 g/L), D-lactose (40 g/L); or a mixture of D-glucose (20 g/L) with D-galactose (20 g/L), in the presence of mineral medium YE and $NaNO_3$.

Carbon source	Strain	Fermentation (days)	Extracellular		Intracellular	
			Volumetric act (IU/mL)	Specific activity ($IU/mg_{biomass}$)	Volumetric act (IU/mL)	Specific activity ($IU/mg_{biomass}$)
D-galactose	*M. antarcticus* PYCC 5048^T	2	2.2 ± 0.8	0.22 ± 0.08	1.5 ± 0.3	0.15 ± 0.03
		4	90.6 ± 1.5	9.9 ± 2.8	525.9 ± 16.3	57.2 ± 15.5
	M. bullatus PYCC 5535^T	2	56.7 ± 22.5	3.9 ± 1.8	17.0 ± 209.6	1.2 ± 0.5
		4	118.8 ± 15.6	11.3 ± 0.9	86.3 ± 19.5	8.3 ± 2.3
D-lactose	*M. antarcticus* PYCC 5048^T	2	2.3 ± 0.9	0.24 ± 0.08	11.2 ± 3.3	1.2 ± 0.23
		4	29.4 ± 2.9	1.5 ± 0.04	11.7 ± 1.5	0.6 ± 0.0
	M. bullatus PYCC 5535^T	2	21.4 ± 3.9	1.94 ± 0.00	9.2 ± 0.6	0.8 ± 0.1
		4	37.2 ± 0.93	2.8 ± 0.2	5.4 ± 0.6	0.4 ± 0.1
D-galactose and D-glucose	*M. antarcticus* PYCC 5048^T	2	1.6 ± 0.4	0.13 ± 0.00	0.4 ± 0.2	0.03 ± 0.02
		4	27.0 ± 1.3	1.4 ± 0.005	14.5 ± 1.8	0.7 ± 0.06
	M. bullatus PYCC 5535^T	2	1.2 ± 0.4	0.07 ± 0.02	6.3 ± 0.4	0.5 ± 0.06
		4	30.2 ± 0.4	2.8 ± 0.1	7.5 ± 0.2	0.7 ± 0.05

Table F7: Rate of consumption (Rs); maximum biomass produced; yield (Yx/s); Maximum MELs obtained (g/L); yield of MELs produced ($g_{MEL/}g_{Substrate}$); volumetric (IU/mL) and specific activity (IU/gbiomass) for *M. bullatus* PYCC 5535^T and *M. antarcticus* PYCC 5048^T using pasteurized CW (40 g/L in D-lactate), in the presence or absence of mineral medium, YE and $NaNO_3$.

Condition	Parameters	***M. antarcticus*** PYCC 5048^T	***M. bullatus*** PYCC 5535^T
With mineral medium and YE	rs (g/L/h)	0.41 ± 0.07	0.32 ± 0.00
	$Biomass_{max}$ (g/L)	11.0 ± 1.0 (Day 4)	14.0 ± 1.0 (Day 10)
	$Y_{Xmax/S}$ (g/g)	0.28 ± 0.03	0.35 ± 0.03
	MEL max (g/L)	1.75 ± 0.20 (Day 7)	1.02 ± 0.02 (Day 10)
	$Y_{MEL/Substrate}$(g/g)	0.04 ± 0.01	0.03 ± 0.00
	β-galactosidase (IU/mL)	305.16 ± 62.01 (Day 4)	296.36 ± 26.36 (Day 7)
	β-galactosidase (IU/mgbiomass)	27.46 ± 3.14	24.70 ± 2.20
	Lipases (IU/mL)	2.88 ± 0.63 (Day 7)	0.45 ± 0.17 (Day 10)
	Lipases (IU/mgbiomass)	0.29 ± 0.06	0.03 ± 0.02
Without mineral medium and YE	rs (g/L/h)	0.25 ± 0.01	0.29 ± 0.01
	$Biomass_{max}$ (g/L)	11.5 ± 0.5 (Day 10)	13.0 ± 0.0 (Day 10)
	$Y_{Xmax/S}$ (g/g)	0.29 ± 0.01	0.33 ± 0.00
	MEL max (g/L)	1.94 ± 0.32 (Day 10)	0.77 ± 0.11 (Day 4)
	$Y_{MEL/Substrate}$(g/g)	0.05 ± 0.01	0.02 ± 0.03
	β-galactosidase (IU/mL)	137.54 ± 10.43 (Day 10)	186.04 ± 45.86 (Day 4)
	β-galactosidase (IU/mgbiomass)	11.89 ± 0.34	15.50 ± 3.82
	Lipases (IU/mL)	2.66 ± 0.20 (Day 7)	1.03 ± 0.03 (Day 7)
	Lipases (IU/mgbiomass)	0.23 ± 0.01	0.09 ± 0.03

$Biomass_{max}$ – maximum biomass cell dry weight (g/L); r_s – sugar consumption rate (g/L/h); $Y_{Xmax/S}$ – maximum biomass yield (g/g); $MELs_{max}$ – maximum MELs produced (g/L); $Y_{MEL/Substrate}$ – maximum MELs yield ($g.g^{-1}$); (IU/mL) max – Maximum volumetric activity of β-galactosidase/Lipases; ($IU/g_{Biomass}$) max – Maximum specific activity β-galactosidase/Lipases

Profiles of biomass, substrate consumption, and MELs production for different conditions

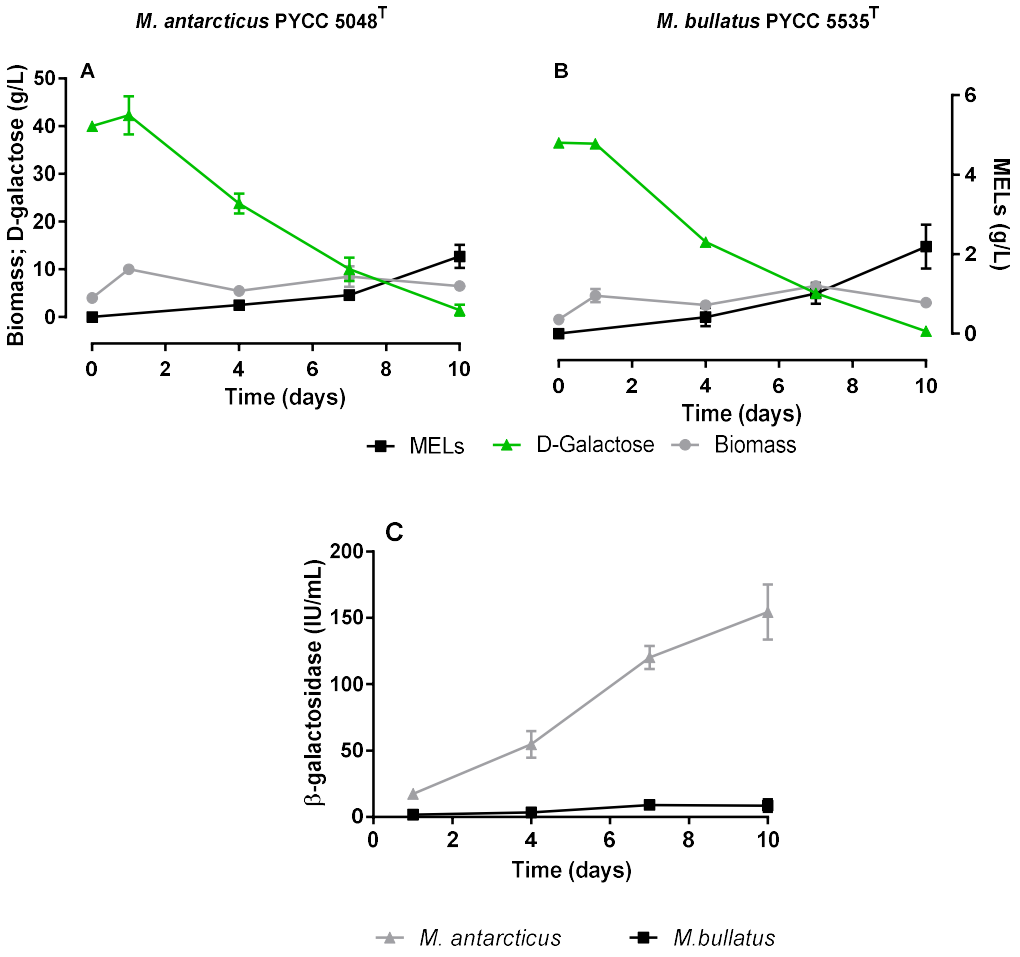


Figure F9: Cultivation of *M. antarcticus* PYCC 5048^T (a, c) and *M. bullatus* PYCC 5535^T (b, c) in D-galactose (40 g/L) in the presence of mineral medium with YE, but in the absence of $NaNO_3$, during 10 days at 27 ºC. Yeast biomass, D-galactose consumption and MELS production (A, B); Extracellular β-galactosidase profile activities (C). Standard deviation values lower than 1 g/L and 1 IU/mL are not represented.

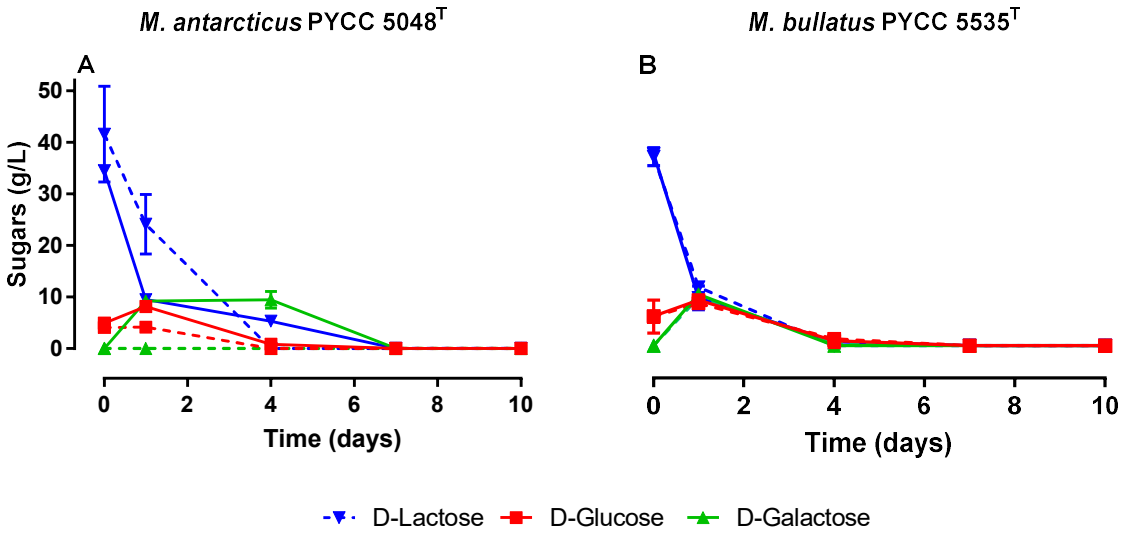


Figure F10: Monomers resulted from the hydrolysis of CW. Glucose (red dashed/filled line with squares), and D-galactose (green dashed/filled line with triangles) for *M. antarcticus* PYCC 5048^T (A) and *M. Bullatus* PYCC 5535^T (B) cultures incubated during 10 days at 27ºC using CW (40 g/L in D-lactate) as carbon source (blue dashed/filled line with inverted triangles), in the presence (dashed lines) and absence (filled lines) of mineral medium with YE and $NaNO_3$. Standard deviations values lower than 1 g/L are not represented.

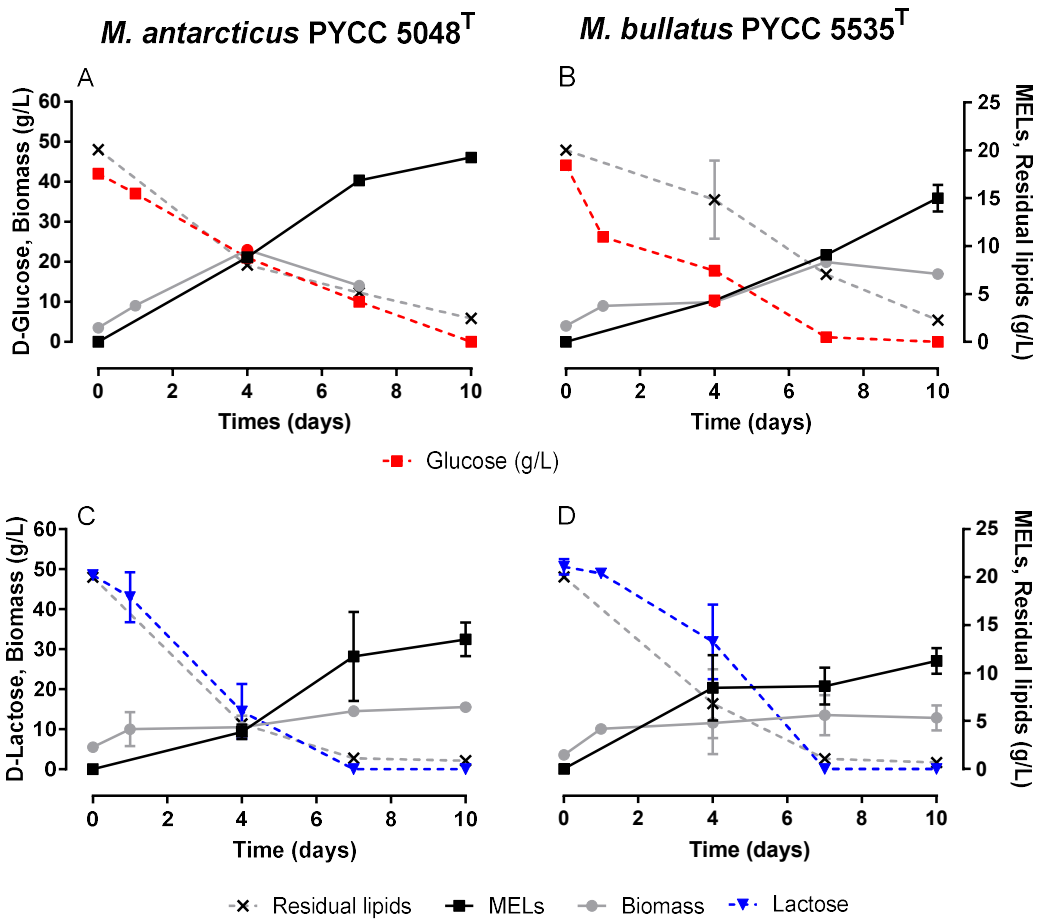


Figure F11: Cultivation of *M. antarcticus* PYCC 5048^T (A, C) and *M. bullatus* PYCC 5535^T (B, D) using as carbon source a combination of D-glucose (40 g/L) (a, b) or D-lactose (40 g/L) (c, d) with WFO (20 g/L) in the presence of mineral medium with YE and $NaNO_3$, during 10 days at 27 ºC. For all graphics patterns of yeast biomass and MELs production; D-lactose and residual lipids (driven from WFO) consumption are shown. The red point indicates the appearance of beads enriched in MELs and residual lipids. Standard deviation values lower than 1 g/L are not represented.

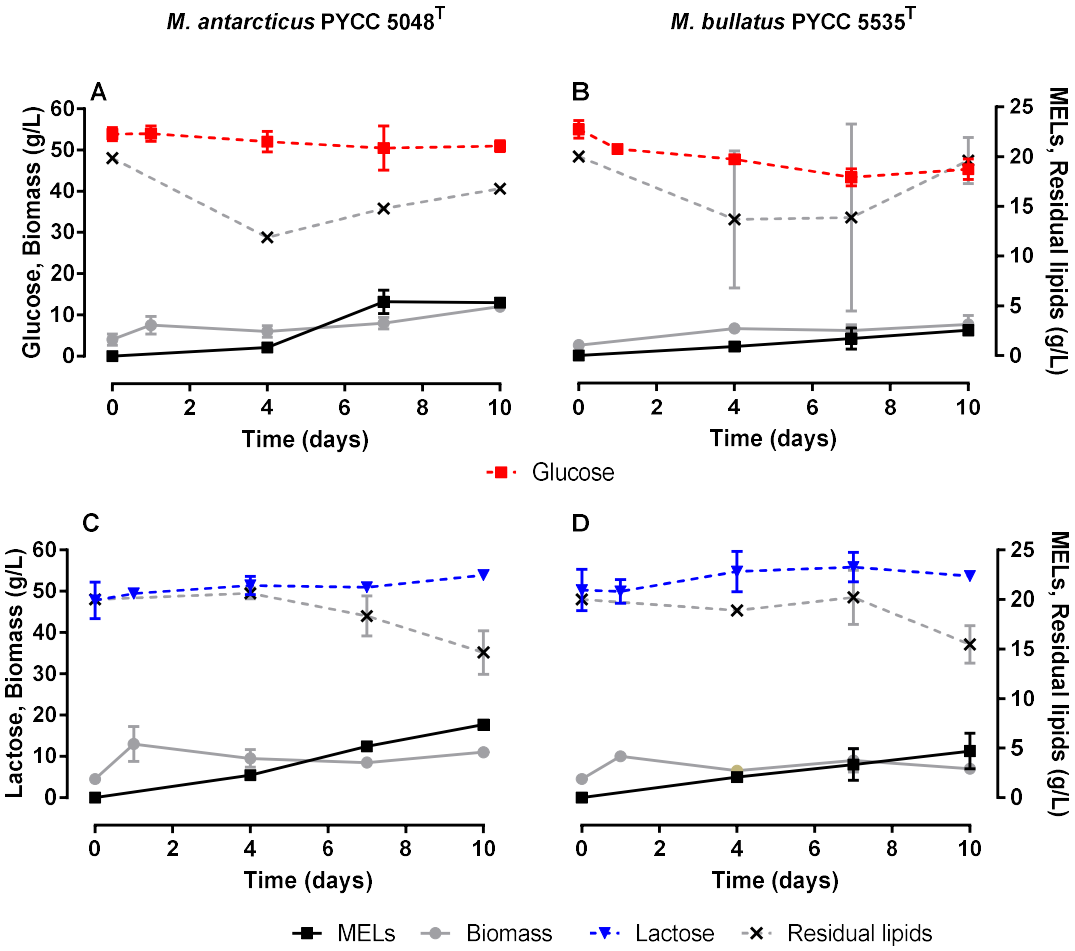


Figure F12: Cultivation of *M. antarcticus* PYCC 5048^T (A, C) and *M. bullatus* PYCC 5535^T (B, D) using as carbon source a combination of D-glucose (40 g/L) (a, b) or D-lactose (40 g/L) (c, d) with WFO (20 g/L), in the absence of mineral medium, YE and $NaNO_3$, during 10 days at 27 ºC. For all graphics patterns of yeast biomass and MEL production; D-lactose and residual lipids (driven from WFO) consumption are shown. Standard deviation values lower than 1 g/L are not represented.

F.3 Supporting information for Section B.3:

Production of MELs, in shake-flask, using different hydrophobic carbon sources.

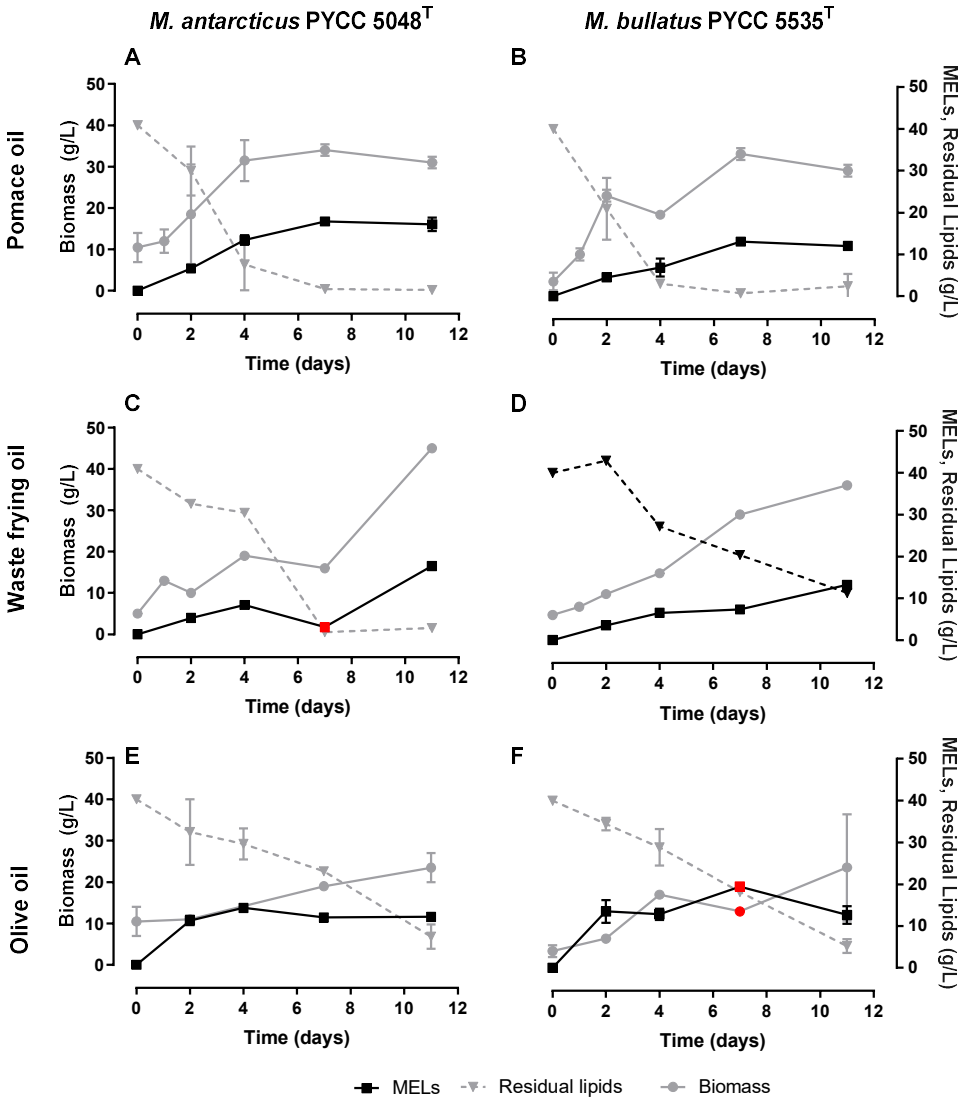


Figure F13: Cultivation of *M. antarcticus* PYCC 5048^T (A, C, E) and M. bullatus PYCC 5535^T (B, D, F) in 40 g/L of pomace oil (PO) (A, B), WFO (C, D) and processed olive oil (E, F), during 11 days at 27 ºC and 250 rpm in the presence mineral medium. For all graphic it is represented yeast biomass, MELs production and residual lipids consumption. The red point, at specific days, represent the appearance of beads enriched in MELs and residual lipids.

Characterization of hydrophobic carbon sources; Pictures and characterization of MELs produced.

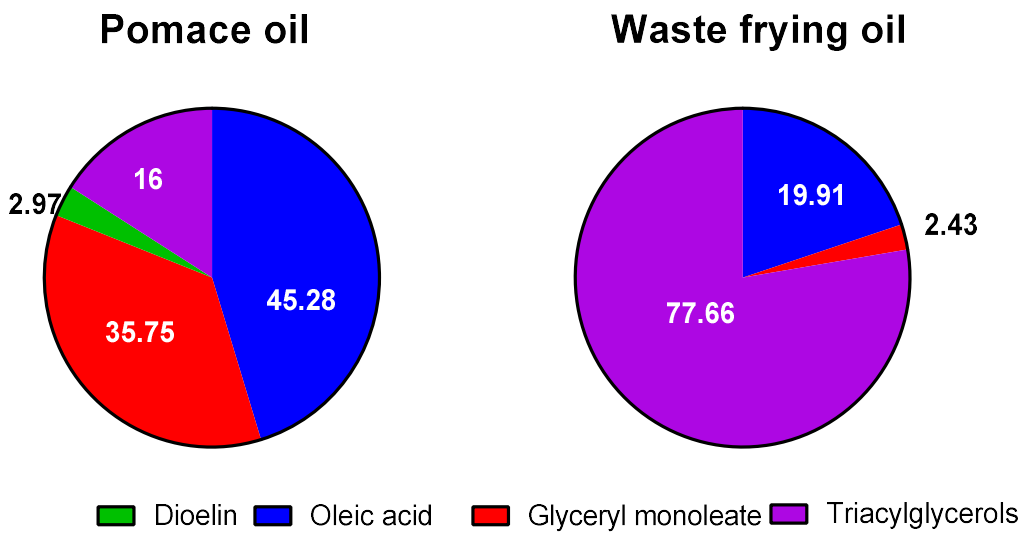


Figure F14: Composition (%) of Pomace oil and waste frying oil, for different compounds: Dioelinx, Oleic acid, Glyceryl monoleate and triacylglycerols. This quantification was performed in HPLC, as described in **section B.3.3.6**.

Figure F15: MELs production in bioreactors by *M. antarcticus* PYCC 5048^{T}*, revealing the presence on beads enriched in MELs at day 5 of fermentation.*

Figure F16: Crude MELs produced by *M. antarticus* PYCC 5048^{T}, using 40 g/L of PO (A) and WFO (B) as carbon source.

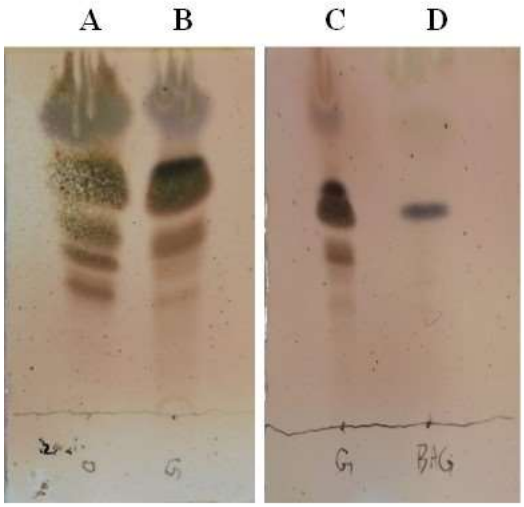


Figure F17: TLCs performed for different crudes MELs produced from different conditions: 40 g/L of WFO (A); 10% of OP residues (B, C) and OP residues before fermentation (D).

Figure F18: TLCs performed for detection of Chlorophyl and β-carotene: A) MELs produced from pomace oil and B) Pomace oil.

F.4 Supporting information for section C.1:

Nanofiltration

Table F8: Experimental values of MELs, residual lipids rejection (%) and flux (L/m^2/h) for each membrane tested using EtOAc or MeOH as organic solvents. For each membrane it was also calculated the theoretical minimum DV and the correspond MEL losses (%) to achieve 97% of purity. The membranes marked at [1] means that to the slow flux, only 10 mL were permeated, instead of 25, and 30 bar of pressure was used.

Solvent	Membrane	MELs Rejection (%)	Residual lipids rejection (%)	Flux (L/m^2/h)	Theoretical Minimum DV (-)	Theoretical MELs losses (%)
EtoAC	GMT-oNF-2	87.1 ± 0.6	32.0 ± 0.4	69.0	3	32.0
	PuraMem-600	84.3 ± 4.5	38.4 ± 21.5	25.5	3	38.1
	PBI 22%	73.1 ± 0.5	32.6 ± 8.4	36 ± 1.5	4	66.00
	PBI 22%-X	78.4 ± 0.5	60.8 ± 3.6	33 ± 1.5	7	78.5
	PBI 24%	92.3 ± 4.0	68.0 ± 7.3	16.5 ± 0.0	7	38.1
	PBI 24%-X	84.0	56.0	21 ± 0.0	6	61.7
	PBI 26%	97.0 ± 1.0	71.0 ± 3.0	9.0	6	16.5
	PBI 26%-X[1]	99.2 [1]	96.3 [1]	2.7	-	-
MeOH	DuraMem-500	88.4 ± 0.7	74.9 ± 6.2	27 ± 0.0	9	66.0
	PBI 22%	67.7 ± 9.5	26.6 ± 12.3	39.5 ± 1.5	4	73.3
	PBI 22%-X	83.1 ± 0.9	60.1 ± 5.2	37.5 ± 0.0	5	69.5
	PBI 24%	93.0 ± 2.0	52 ± 10.2	43.5 ± 1.5	5	24.2
	PBI 24%-X	93.3 ± 1.3	67.8 ± 1.7	34.5 ± 3.0	7	18.9
	PBI 26%	97.1 ± 0.9	75.1 ± 8.7	30 ± 3.0	7	18.9
	PBI 26%-X	98.1 ± 0.8	77.5 ± 3.6	25.5 ± 3.0	8	14.7

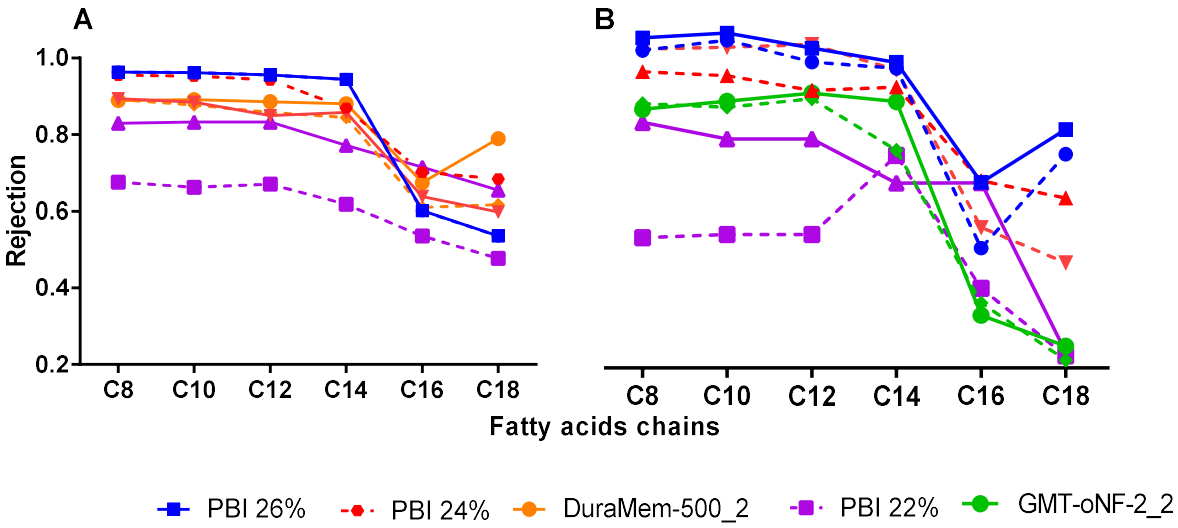


Figure F19: Rejection of different OSN membranes for MELs (C8, C10, C12 and C14) and residual lipids (C16 and C18), using MeOH (A) and EtOAc (B) as organic solvents. The existing species on permeate and feed are submitted to methanolysis and the obtained concentrations of methyl esters obtained in permeate (C_P) and feed (C_F) and rejections are calculated as 1- C_P/C_F.

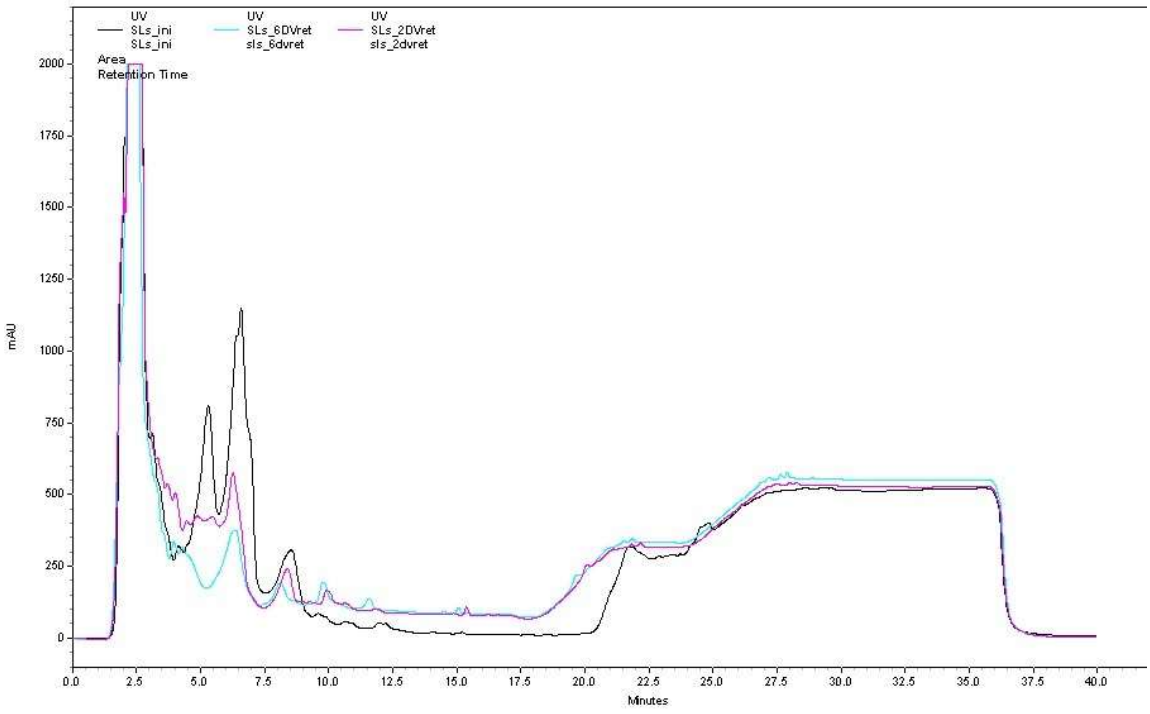


Figure F20: HPLC chromatogram of SL sample diluted in methanol. Black line – initial sample. Purple line – retentate sample after 2DV. Cyan line – retentate sample after 6DV.

The used HPLC method is able to discriminate between individual lipid groups, with free FFA occurring between t = 2.5-7.5 min, MAG between t = 5-20 min, DAG between t = 20-25 min and TAG between t = 25-35 min. One can be observed a reduction of the peaks on the region of the FFA and MAG. From the chromatogram it can be observed that the nanofiltration of SLs successfully removed small lipidic contaminants from the crude SL mixture.

F.5 Supporting information for section C.2:

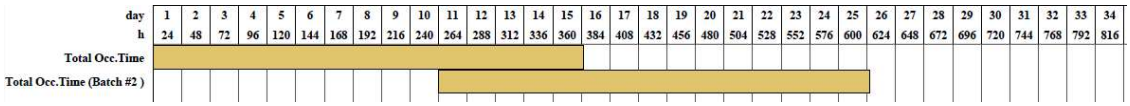
Comparison of different factory capacity

Table F9: Comparison of process and economic parameters for MELs production process, varying the capacity of bioreactor (1, 10, 20, 50 and 100 m^3) while having a fixed selling price of 70 €/kg MELs. Values retrieved from Super Pro Designer®.

Parameters	Bioreactor capacity (m3)				
	1	**10**	**20**	**50**	**100**
Investment (k€)	3 823	17 975	27 395	70 301	150 669
Net Annual Operating Costs (k€)	177 €	1 057	1 564	2 941	4 257
Annual Revenues (k€)	177 €	1 838	3 460	7 568	12 433
Batch Time (h)	324	341	362	423	514
Cycle Time (h)	222	229	240	274	330
Batches per year	35	34	32	28	23
Production rate (kg/batch)	72	772	1 545	3 861	772
Production rate (Ton/year)	3	26	49	108	178
Net Production Cost (€/Kg MELs)	195.7	39.9	31.7	27.1	25.5
Gross Margin (%)	-179.6	42.5	54.8	61.2	63.6
ROI (%)	0.6	12.1	14.0	13.8	12.8
Pay Back Time (years)	151.7	9.9	7.2	7.3	7.8
IRR - after taxes (%)	-	7.1	9.8	9.5	8.4
NPV - 7% interests (k€)	- 3 181	160	4 020	9 442	4 000

Ghant chart for 20 m^3 bioprocess, with different number of bioreactors

A



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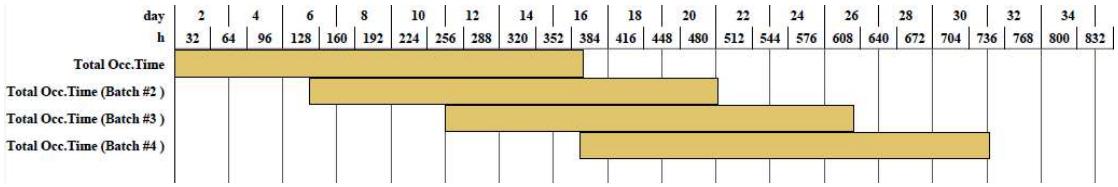

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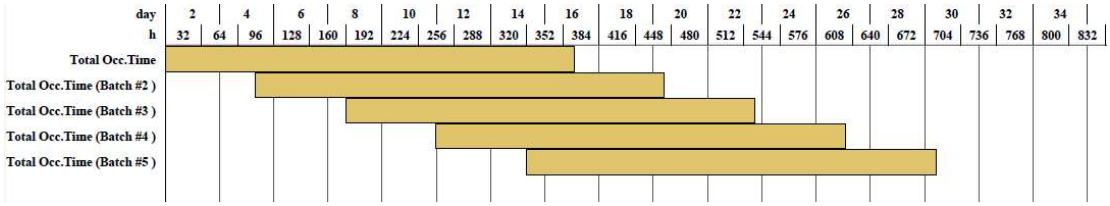

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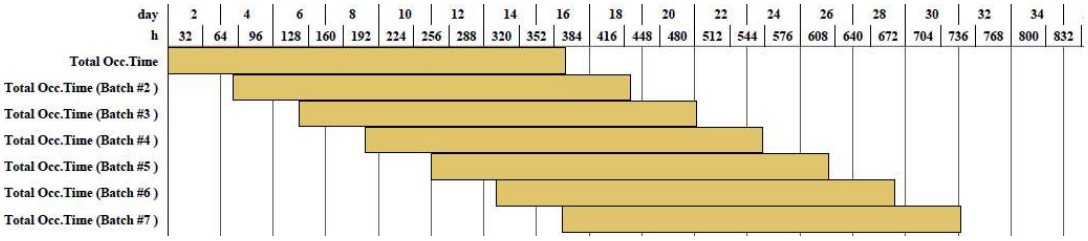

E

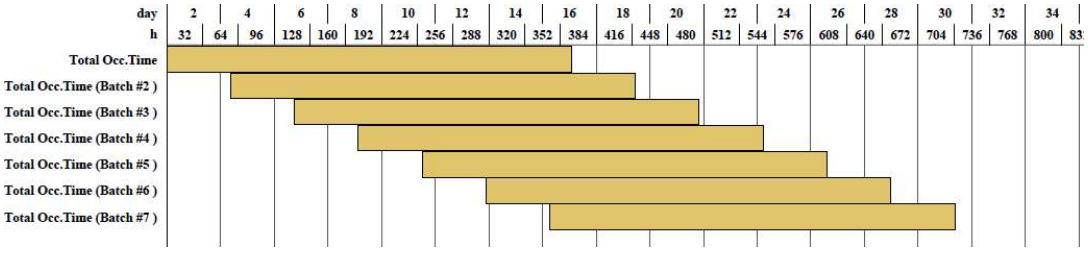

Figure F21: Ghant chart for operations for a process using 20 m3 as main bioreactor, varying the number of bioreactors in stagger mode: A) 0; B) 1; C) 2; D) 3 and E) 4.

Comparison of different processes with different bioreactors size.

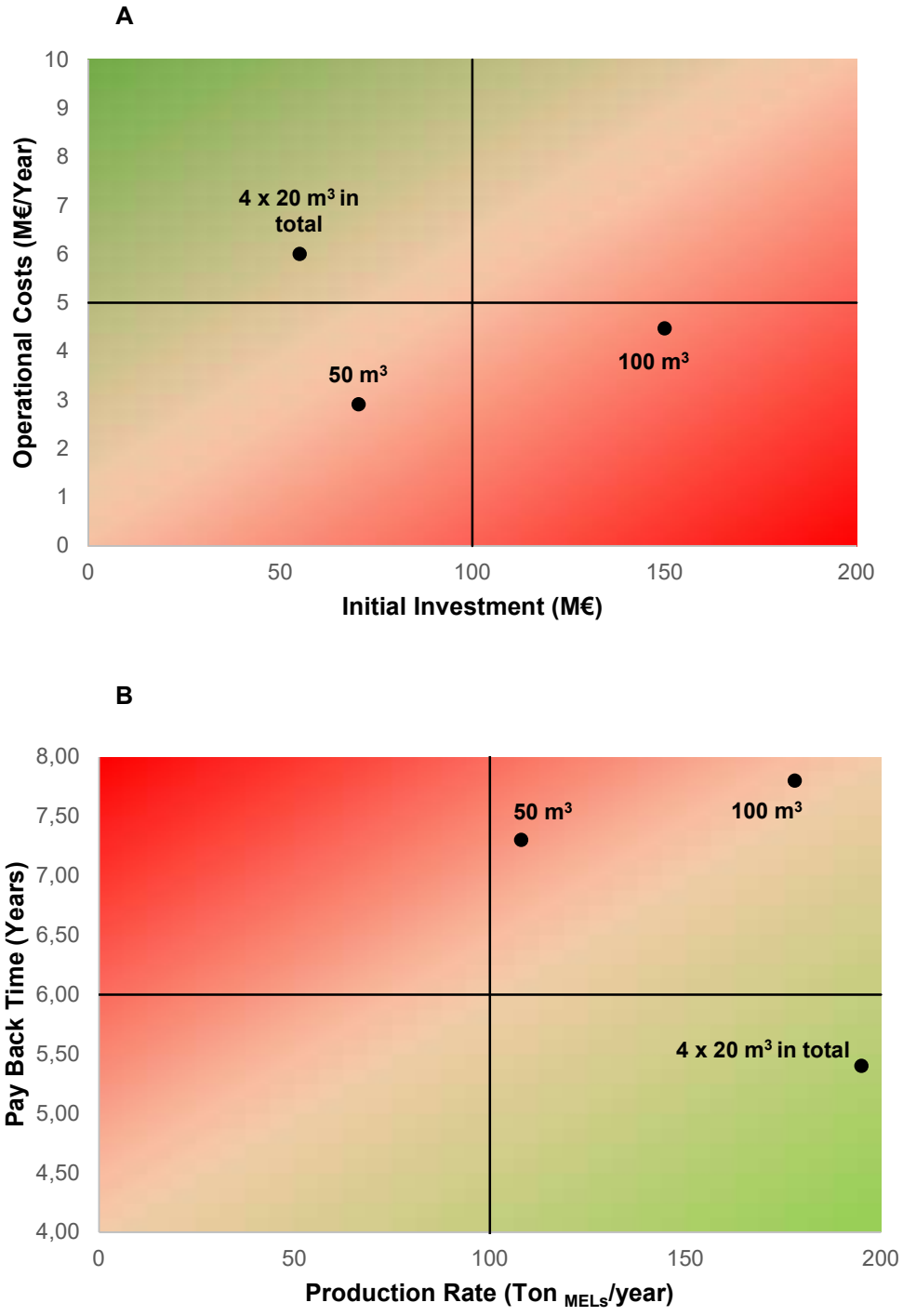

Figure F22: Comparison between different processes (50 and 100 m^3 without stagger mode, and 20 m^3 bioprocess, with 3 reactors in stagger mode. A) Operational costs (M€) vs Initial capital investment; B) Payback time (year) vs Production rate (Ton MELs/year).

Comparison of processes using different side streams

Table F10: Comparison of economic parameters for MELs production, using the optimal process (4 bioreactors of 20 m^3 in stagger mode), while varying the type of substrate used: Glucose + WFO; Cheese whey (replacing glucose and mineral medium) and finally using glucose but replacing WFO with pomace oil.

Parameters	Raw material		
	Glucose + WFO	**CW + WFO**	**Glucose + Pomace oil**
Investment (k€)	55 180	55 104	53 976 €
Net Annual Operating Costs (k€)	6 237 €	5 409 €	5 616 €
Annual Revenues (k€)	13 622 €	13 622 €	14 067 €
Batch Time (h)	362	362	386
Cycle Time (h)	60	60	66
Batches per year	126	126	115
Production rate (kg/batch)	1 545	1 544	1 748
Production rate (Ton/year)	195	197	201
Net Production Cost (€/Kg MELs)	32.1	28.1	27.2
Gross Margin (%)	54.7	60.3	61.2
ROI (%)	16.7	19.7	20.8
Pay Back Time (years)	5.4	5.08	4.81
IRR - after taxes (%)	14.9	16.0	17.1
NPV - 7% interests (k€)	2 617	3 011	3 359

Comparison of different variables using the software Matlab.

Program in MATLAB that resulted in the graph that compares the profit (Z), the production cost (X) and the titre (Y):

```
[X,Y]=meshgrid(2:40,40:80);

Z = (Y.*20)+(0.1197.*(Y.*20)).*126*(70-X);

surf (X,Y,Z)
```

Program in MATLAB that resulted in the graph that compares the profit (Z), the production cost (X) and the number of batches per year (Y)

```
[X,Y]=meshgrid(2:40,99:350);

Z = (61.*20)+(0.1197.*(61.*20)).*Y.*(70-X);

surf (X,Y,Z)
```

F.6 Supporting information for section D.1:

Medium composition

Table F11: Medium composition of trace elements added on Behrens (mg/mL) and modified Šetlik (g/mL) medium.

Components	Behrens medium (mg/mL)	Modified Šetlik medium (mg/mL)
$CaCl_2.2H_2O$	-	0.5
H_3BO_3	5.7	0.3
$MnSO_4.5H_2O$	4.0	0.1
$CoSO_4.7H_2O$	0.5	0.24
$CuSO_4.5H_2O$	4.0	0.12
$ZnSO_4.7H_2O$	-	0.14
$(NH_4)_6Mo_7O_{24}.4H_2O$	-	0.18
$ZnCl_2$	2.1	-

Crude glycerol composition

Table F12: Composition of different fractions of crude glycerol (P105 and RO70104).

Composition (%)	Crude glycerol (P105)	Crude glycerol (RO70104)
Glycerol	81.3	80
Water	10.9	13.8
Methanol	0.14	0.02
Potassium or sodium hydroxide	-	-
Free fatty acids	-	-

Photobioreactor characterisation

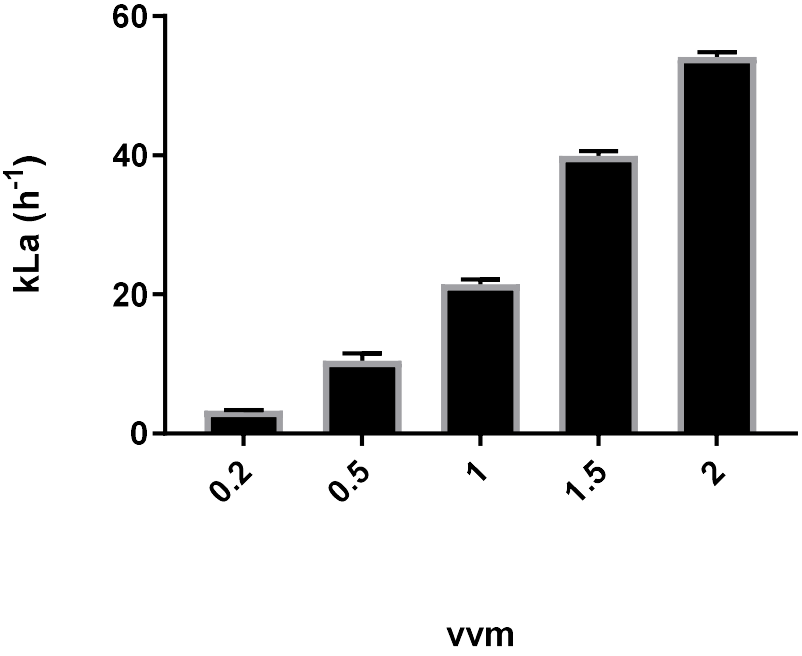

Figure F23: kLa (oxygen gas coefficient) determination at different vvm. kLa was determined using the dynamic method, for different vvm (0.2, 0.5, 1,1.5 and 2 vvm) using modified Šetlik medium.

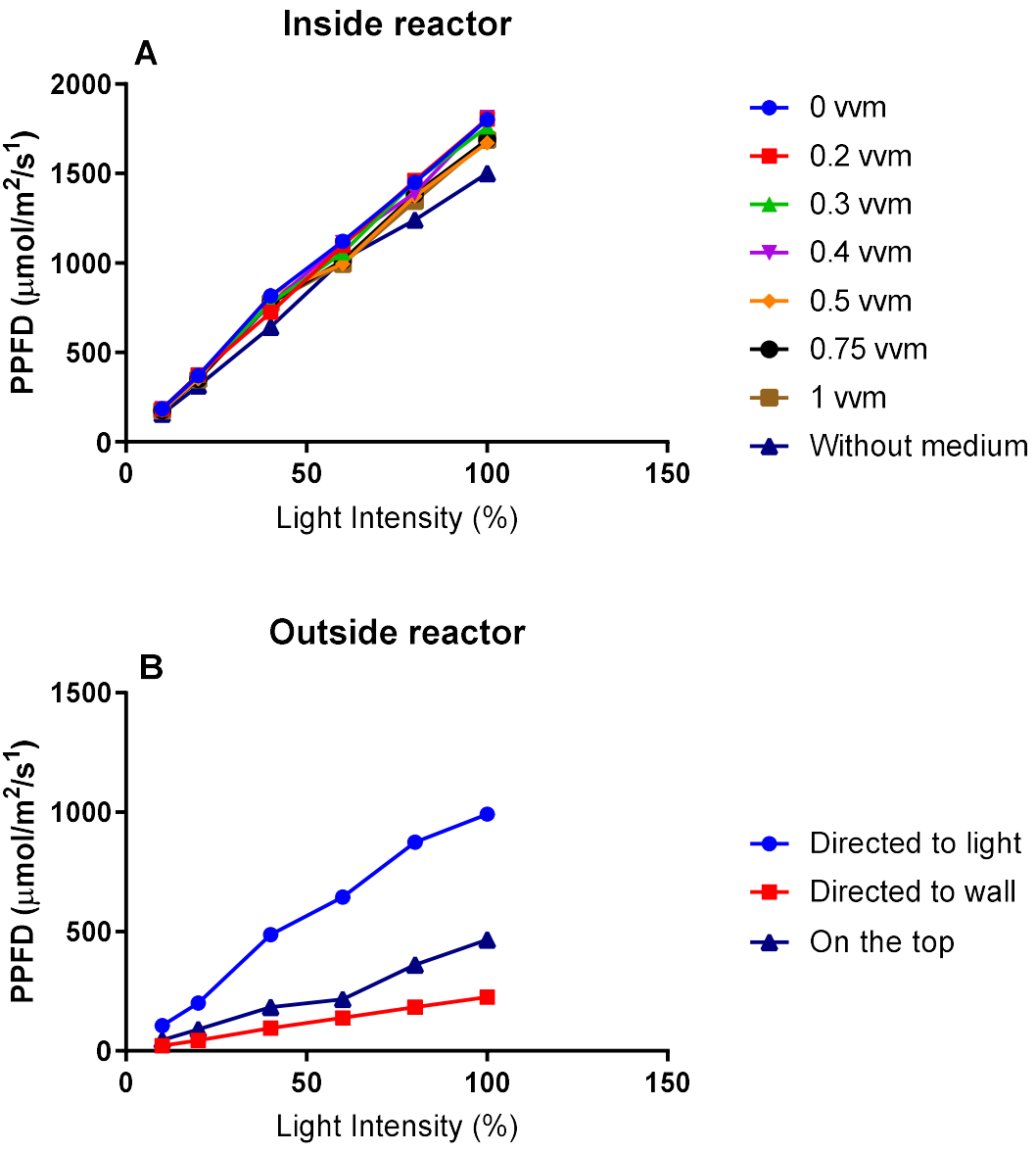

Figure F24: Light intensity characterization (µmol/(s·m²)) in bioreactors used in this experiment. Different measures were performed for each intensity (10, 20, 40, 60, 80 and 100%): A) Inside of reactor, with setilk medium and air-flow rate (0, 0.2, 0,3, 0.4, 0.5, 0.75 and 1 vvm) and without Šetlik medium; B) Outside reactor, re-position the device directed to light, Wall and on the top.

Shake-flask cultivations:

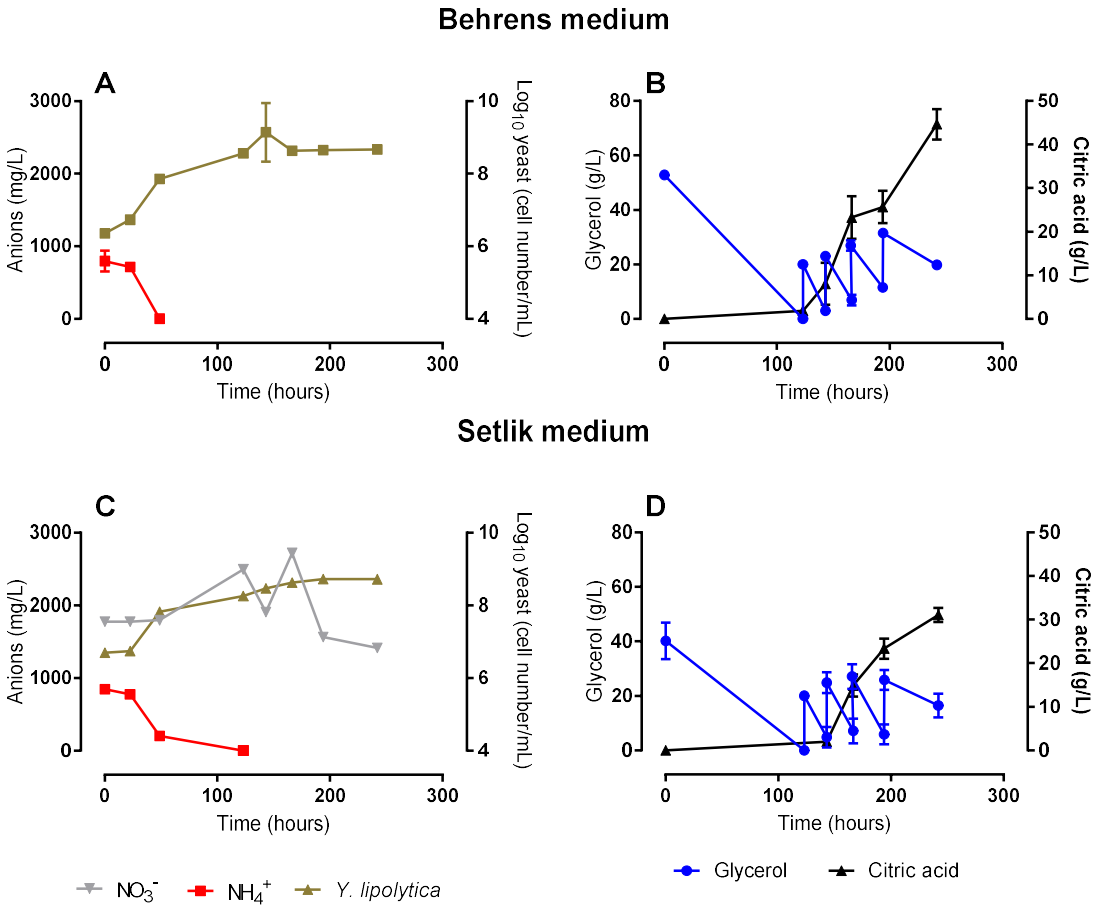

Figure F25: Cultivation of *Y. liplolytica* H181 in Behrens (A, B) and Šetlik (C, D) medium, starting with 50 g/L and followed by feeds of glycerol at of 20 g/L Glycerol at 123, 143, 165 and 194, during 242 h at 26ºC using an agitation of 130 rpm. pH was manually controlled at 6.5 every day, using 25 and 27% of H_2SO_4 and NaOH, respectively. Ammonium consumption (squares) and yeast cell number (hexagons) (A, C); Glycerol consumption (circles) and citric acid production (triangles) (B, D). Standard deviations values lower than 1 g/L are not represented.

Table F13: Final titre, productivity, yield of citric acid and final biomass, for different carbon sources (glucose, glycerol and 2 different fractions of crude glycerol), with a final concentration of 50 g/L, used in mono-cultivation of *Y. lipolytica* H181 in shake flaks with baffles, during 240 h at 130 rpm and 26ºC. Feeds of 20 g/L were added to the system at 91, 164, 188 and 213 h.

Carbon source	Final titre (g/L)	Overall Productivity (g/L/h)	Yield (g/g)	Biomass (g/L)
Glucose	47.05 ± 5.04	0.2 ± 0.02	0.35 ± 0.04	10.86 ± 0.21
Pure glycerol	40.18 ± 0.32	0.17 ± 0	0.3 ± 0.03	10.68 ± 0.23
Crude glycerol (P105)	45.87 ± 1.2	0.19 ± 0.01	0.34 ± 0	10.01 ± 0.37
Crude glycerol (RO174)	46.27 ± 2.64	0.2 ± 0.01	0.34 ± 0.02	10.65 ± 0.27

Table F14: Citric acid maximum titre (g/L); total glycerol, unconsumed glycerol (g/L) and the correspondent yield (gCA/gconsumed glycerol); final biomass (g/L) and final ratio of algae cells/total cell number for different conditions: 1) The use of different inoculum percentages of yeast (Y) and microalgae (A) (10%Y-0%A; 0.01%Y-0%A; 0.01%Y-10%A; 0%Y-10%A); 2) De-coupling co-cultivation process, where the cultivation starts with only C. vulgaris 212-2b, for specific days (0; 2; 5; 7 and 10). After these days, yeast is added (0.01% Vinoculum/Vculture medium) along with NH4Cl (1.5 g/L), thiamine (1 mg/L), $FeSO_4.7H_2O$ (6.3 mg/L), glycerol (50 g/L) and trace elements (1mL). Co-cultivation was carried out for 240 h (10 days), followed by feeds of glycerol (20 g/L), to avoid a concentration below than 10 g/L. The temperature was kept at 26ºC, using an agitation of 130 rpm, and a light regime (light/dark) of 12:12, with an intensity of 60 µmol/(s·m²). pH was manually controlled at 6.5 every day, using 25 and 27% of H_2SO_4 and NaOH, respectively.

Experiment	Condition	Titre max (g/L)	Yield (g/g)	Ratio yeast/algae end	Biomass
Different ratios	10%Y - 0%A	31.13 ± 1.17	0.3 ± 0.02	-	13.26 ± 0.49
	0.01%Y - 0%A	25.35 ± 1.16	0.27 ± 0.01	-	10.54 ± 0.97
	0.01%Y - 10%A	28.63 ± 4.93	0.46 ± 0.05	5.07 ± 0.31	12.74 ± 0.22
	0%Y - 10%A	-	-	-	0.77 ± 0.12
Decoupling the co-cultivation process (0.01%Y - 10%A)	0	28.63 ± 4.93	0.46 ± 0.05	4.55 ± 0.21	12.74 ± 0.22
	2	20.35 ± 4.66	0.24 ± 0.05	4.11 ± 0.65	11.23 ± 0.26
	5	26.05 ± 3.61	0.31 ± 0.02	7.66 ± 0.27	10.85 ± 0.5
	7	51.64 ± 0.47	0.54 ± 0.01	9.76 ± 0.13	11.74 ± 0.15
	10	39.05 ± 2.5	0.43 ± 0.03	8.44 ± 0.26	14.62 ± 0.03

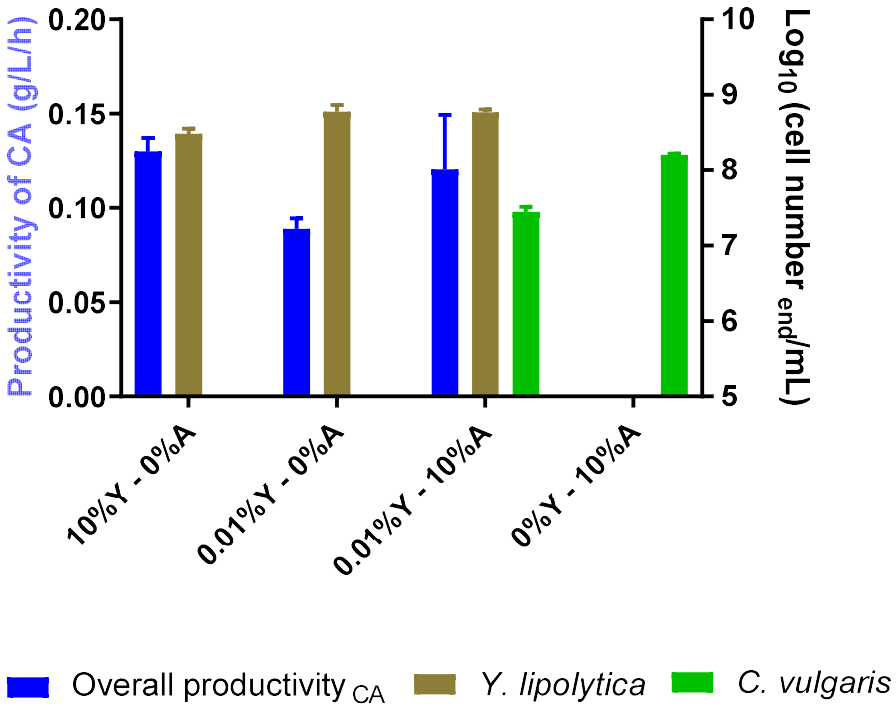

Figure F26: Cultivation of *Y. liplolytica* H181 and *C. vulgaris* 212-2b using Glycerol (50 g/L) as carbon source, varying the volume of yeast/algae inoculum added on the beginning of the cultivation, where it is represented final productivity of citric acid and the log10 of final yeast and microalgae cell number. This experiment was performed in a fed-batch mode, feeding 20 g/L of glycerol into the shake-flask in different days, to avoid a concentration bellow than 10 g/L. The cultivation was performed during 240 h at 26ºC with an agitation of 130 rpm and using a light regime (light/dark) of 12:12, with an intensity of 60 µmol/(s·m²). pH was manually controlled at 6.5 every day, using 25 and 27% of H_2SO_4 and NaOH, respectively.

Mono-cultivation of *Y. lipolytica* in photobioreactors

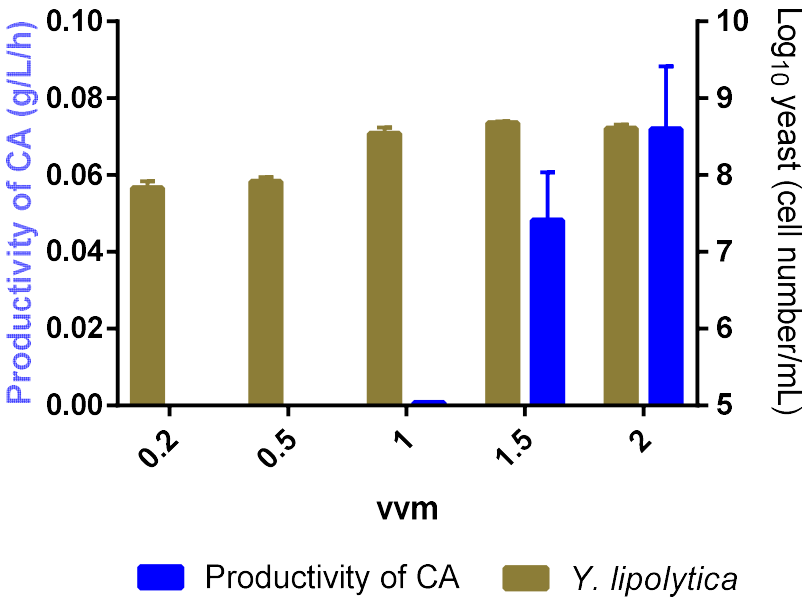

Figure F27: *Y. liplolytica* H181 was cultivated in bioreactors using Šetlik medium, testing different vvm (0.2; 0.5; 1; 1.5 and 2). Cultivation started by adding 0.01% ($V_{inoculum}/V_{culture\ medium}$), using 50 g/L of glycerol as carbon source, followed by a feed of 20 g/L of glycerol at 141 hours (for conditions using 1;1.5 and 2 vvm). The cultivation was carried out for 240 hours at 26ºC, with an agitation provided by a magnet at 120 rpm, and a light regime of 12:12 (light/dark) with an intensity of 500 µmol/(s·m²). It is represented overall productivity (blue bar) and final cell number (brown bar).

Mono-cultivation of *C. vulgaris* in photobioreactors

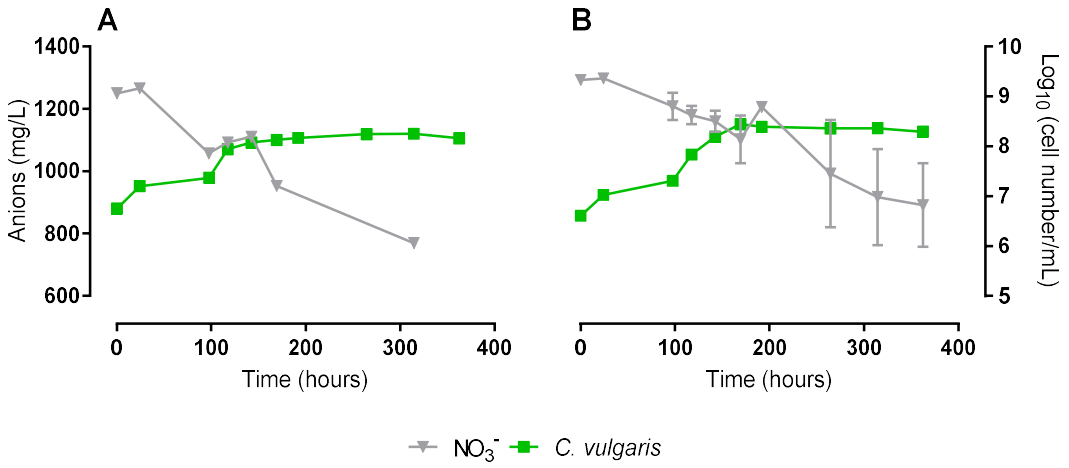

Figure F28: Mono-cultivation of C. vulgaris 212-2b, varying light intensity. A) Using only 50% of light intensity; B) During 5 days light intensity was kept at 50%, and then changed for 75%, during 10 more days. The experiment was performed in bioreactors during 15 days, at 26ºC. It was used a light regime (light/dark) of 12:12, with an intensity of 500 µmol/(s·m²). pH was manually controlled at 6.5 every day, using 27 or 25% of NaOH and H2SO4, respectively.

Co-cultivation in photobioreactors

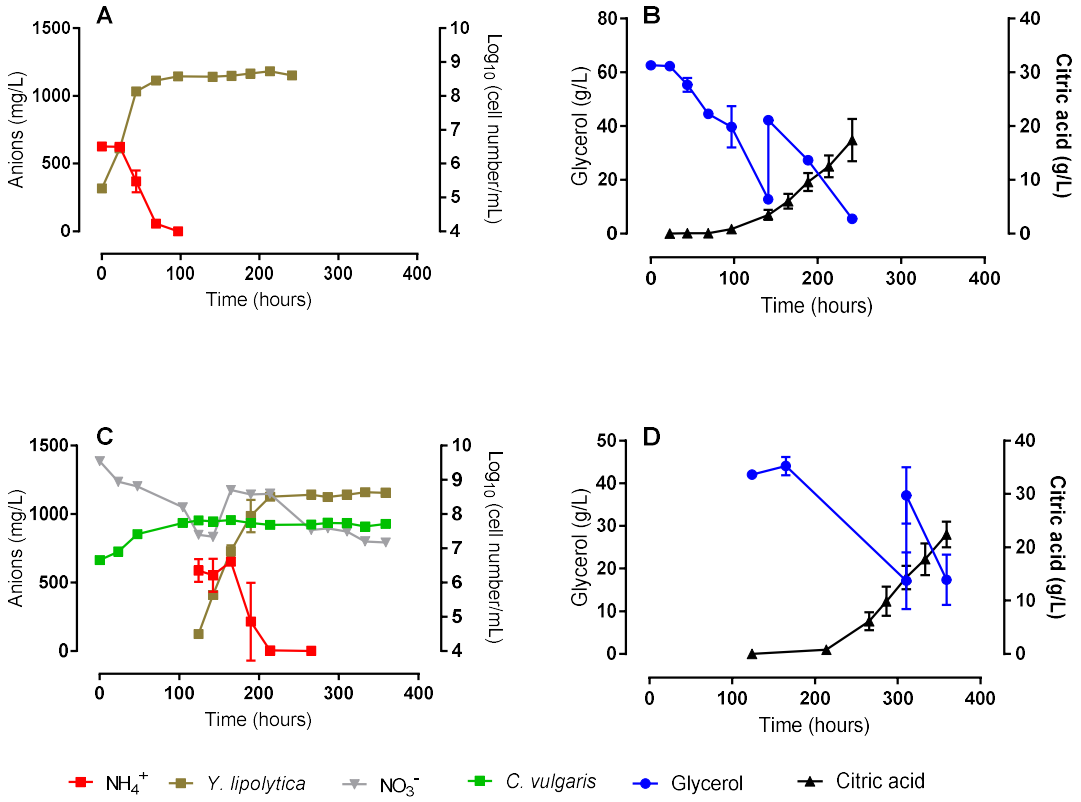

Figure F29: *Y. lipolytica* H181 was cultivated alone (A, B) or co-cultivated with *C. vulgaris* 212-b in bioreactors using a modified Šetlik medium. In the mono-cultivation of the yeast, glycerol at a concentration of 50 g/L was used initially, followed by a glycerol feed of 20 g/L at 141 hours. Co-cultivation started after 5 days of C. vulgaris growth, by adding 50 g/L of glycerol, thiamine, $FeSO_4.7H_20$, and NH_4Cl, followed by one feed of 20 g/L of glycerol at 310.25 h. The temperature was maintained at 26ºC with an air-flow rate of 2 vvm and agitation at 120 rpm using a magnet. The pH was automatically controlled at 6.5 with a 3% NaOH solution. The light regime was set at 12:12 (light/dark) with an intensity of 500 µmol/(s·m²). Ammonium (squares), nitrate consumption (inverted triangles), yeast (hexagons) and algae cell number (hexagons) and (A, C); Glycerol consumption (circles) and citric acid production (triangles) (B, D). Standard deviations values lower than 1 g/L are not represented.

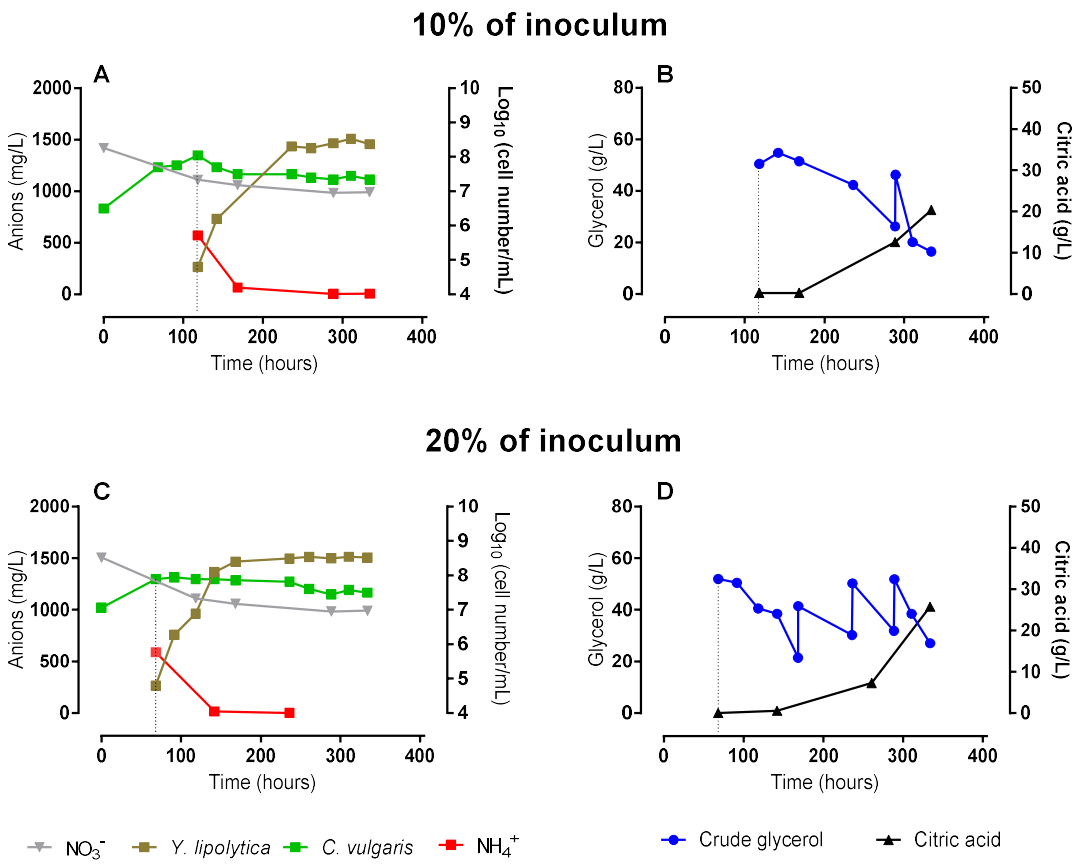

Figure F30: Citric acid production was carried out through co-cultivation of *Y. lipolytica* H181 and C. vulgaris 212-2b, using crude glycerol as carbon source. It was tested the effect of varying the initial % of *C. vulgaris* inoculum, 10% (A, B) and 20% (C, D). The co-cultivation process began by adding 0.01% of yeast (Vinoculum/Vculture medium) along with NH_4Cl (1.5 g/L), thiamine (1 mg/L), $FeSO_4.7H_2O$ (6.3 mg/L) and crude glycerol (50 g/L). Co-cultivation for the 10% inoculum started on the 5th day, while for the 20% inoculum, it started on the 3rd day. The duration of cultivation was 354 hours and 364 hours, respectively. Glycerol feed of 20 g/L was added at 213, 282, and 330 hours for the 10% inoculum, and at 165 and 239 hours for the 20% inoculum. The temperature was maintained at 26ºC with an air-flow rate of 2 vvm and agitation at 120 rpm using a magnet. The pH was automatically controlled at 6.5 with 3% NaOH solution. The light regime was set at 12:12 (light/dark) with an intensity of 500 µmol/(s·m²). Ion NH4+ (squares), ions NO3- (inverted triangles), yeast (hexagons) and algae cell number (hexagons) and (A, C); Glycerol consumption (circles) and citric acid production (triangles) (B, D). Arrow dashed line indicates the beginning of co-cultivation (addition of yeast to the bioreactor). These experiments do not have replicates.

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