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Reengineering production of Mannosylerythritol lipids (MELs): a holistic approach

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Supervisor: Doctor Frederico Castelo Alves Ferreira

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2022

Tæv uforknyt løs

på problemerne,

men vær forberedt på,

at de tæver igen

Problems worthy

of attack

prove their worth

by hitting back.

Piet Hein

Resumo

Lípidos de manosileritritol (MELs) são um grupo de tensioativos glicolipídicos produzidos por *Moesziomyces* spp., que se apresentam como alternativa a tensoativos produzidos quimicamente. MELs podem ser produzidos a partir de vários substratos, incluindo açúcares, lipídios e alcanos. Apesar de suas excelentes propriedades tensioativas, biocompatibilidade e biodegradabilidade, a sua comercialização depende da redução do seu custo de produção. Esta tese tem como objetivo desenvolver soluções que, de forma sustentável, melhorem a eficiência do processo de produção de MELs. O efeito da concentração de cloreto de sódio no metabolismo de *Moesziomyces* spp., nomeadamente na sua capacidade de produzir MELs e lípases, foi investigado, permitindo usar água do mar. Num segundo estudo, recorrendo a catalise pelas lípases produzidas pela levedura, hidrolisou-se os óleos vegetais antes de os adicionar à fermentação como substrato, conseguindo assim reduzir o tempo de fermentação na produção de MEL. Ambos os estudos identificaram condições que promovem a formação de aglomerados ricos em MEL. Assim, foi desenvolvido um novo modulo especificamente projetado para recolher *in situ* os aglomerados formados, permitindo várias recolhas de um produto ao longo de uma fermentação. Descobriu-se que o uso de glicerol na preparação de inóculo promove a formação de fisiologias celulares favoráveis à produção de MEL. A produção contínua de um produto aquoso, sem células, rico em MELs e lípases foi avaliada. Por fim, é proposto um novo processo de purificação para obtenção de MELs com pureza elevada. Este processo apenas usa um solvente, facilitando a sua reciclagem e contribuindo para a sustentabilidade do processo.

Palavras-chave: lípidos de manosileritritol, bioprocesso, biossurfactante, sustentabilidade, downstream

Abstract

Mannosylerythritol lipids (MELs) are a group of glycolipid biosurfactants, produced primarily by yeasts of the *Moesziomyces* genus, which are a promising alternative to chemically synthetised surfactants. MELs can be produced from a range of substrates, including sugars, lipids, and alkanes. MELs have exquisite tensioactive properties, biocompatibility, and biodegradability. However, they competitiveness in the market depends to cost reduction.

This thesis aims to develop solutions and explore different opportunities to, in a sustainable manner, increase MELs production process efficiency. Firstly, the effects of sodium chloride concentrations on *Moesziomyces* spp. metabolism, specifically on their ability to produce MELs and lipases, was study, allowing to use widely available seawater. Secondly, native lipases produced by the yeast were used to pre-hydrolyse the vegetable oils substrates, and effectively shortened the time of the MEL production fermentation. Both studies identified conditions that promote formation of MEL rich beads. Consequentially, a novel device was design for in-situ harvesting of such beads from the bioreactor, enabling multiple solvent-free product recoveries over a fermentation. Investigating inoculum preparation conditions, glycerol was found to promote cell differentiation into physiological structures favourable for MEL production. The continuous production of cell free aqueous product rich in MELs and lipases was assessed. Finally, a novel downstream process was proposed, to obtain MELs of high purity. This process use of only one solvent facilitates solvent recycle, unlike multi-step extractions using mixtures of non-sustainable solvents.

Keywords: mannosylerythritol lipids, process, biosurfactant, sustainability, downstream

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Abbreviations

A – Absorbance (spectrophotometry)

ABS – Acrylonitrile Butadiene Styrene

AC – Activated carbon

DCM – Dichloromethane

DI - Diacylglycerols

EtOH – Ethanol

FAAE – Fatty acid alcohol esters

FABE – Fatty acid butyl esters

FAEE – Fatty acid ethyl esters

FFA – Free fatty acid (alternatively FA – fatty acid)

FAME – Fatty acid methyl esters

GDP - Gross Domestic Product

Glu – Glucose

Gly - Glycerol

LAS - Linear alkylbenzene sulfonates

Mbbl/d – Megabarrels (million barrels) per day

MELs – Mannosylerythritol lipids

MeOH – Methanol

MONO - Monoacylglycerols

NLGs - Natural gas liquids

NPEs - Nonylphenol ethoxylates

PBI – Polybenzimidazole

RSO – Rapeseed oil

SBO – Soybean oil

TRI - Triacylglycerols

WFO – Waste frying oil

YE – Yeast extract

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Chapter 1

1 Introduction

1.1 Motivation and challenges

In the previous decades, society was faced historically unprecedented global population growth, significant technological progress, industrial intensification, and extensive globalization. Such dramatic changes in human society resulted in greatly increased demand for food, water, energy, and consumer products. During this time of rapid growth, the awareness of our impact on the planet increased, calling for a change in our behaviour and practices.

Since the Industrial revolution, virtually all aspects of our lives depend on fossil fuels and petrochemicals. The International Energy Agency (IEA) (IEA 2019) states that in 2018, 64% of the electricity generated globally was obtained from fossil fuels. Furthermore, the same organization states that emissions from transport (road, rail, air and marine) accounted for 24% of global greenhouse gas emissions. To address such stark numbers, newly emerging, but swiftly growing, technologies are being implemented to offer cleaner alternatives to suit the needs of today's human society. As an example, the global market for electric cars has almost doubled every single year in the past decade (IEA 2020). In parallel, price per kWh for solar energy dropped 4 times since 2010. (IRENA 2019) All this sparks some hope that we are on our way to ameliorate our negative impact on the planet.

While electricity/heating, transportation, and land use have the major contribution to anthropogenic greenhouse gas emissions, the often-overlooked industrial manufacturing is currently responsible for 15% of global oil demand. This sector is forecast to become the leading driver for growth of oil and gas consumption in the following decades, with an expected increase in oil consumption to be four million barrels of oil per day. Such an increase is equivalent to the additional use of 5.5 metric tons of oil per second. To make it sound even more sinister, in the time that it took the average reader to read just this paragraph, that equates

to additional three full railroad tank cars carrying oil, on top of what we already use. A significant portion of this oil will be used – for production of chemicals. (Cetinkaya et al. 2018).

The petrochemical industry uses oil and its derivatives, generated in oil refineries, to produce a range of chemical compounds. These are then used by consumers directly, or by other industrial sectors: plastic, rubber, cosmetics and pharmaceutical, among other products needed for maintaining human well-being and life quality. Petrochemical products are used in virtually every aspect of industry, and human society in general – we depend on them for food and medicines production, for making our clothes and for building our houses. While previously mentioned issues regarding energy production and the transportation sector remain relevant, there are promising emerging solutions towards the use of clean alternatives. The change to non-petroleum driven solutions in the manufacturing sector will not be so easy to make. Society is "held hostage" due to its profound dependence on petrochemicals. As access to oil is strategic and petrochemicals are critical goods with uneven geographical distribution, they were the cause of many international conflicts.

Importantly, the very same characteristics of petrochemicals that drew humankind to use them are the reason they are causing such an eco-devastation.

Petrochemicals are immensely resilient. When they enter the ecosystem, they stay there for a tremendously long time, moving through air, water, and soil. In that unnatural circulation through nature, they cause immense damage to the ecosystem. Additionally, most petrochemically derived products are used disposably, as plastic packaging and containers, pharmaceuticals, cosmetics, food additives, detergents, and cleaning agents, making the pollution associated with these products more serious in terms of negative environmental impact. Thus, in terms of ecological damage, use of petrochemically derived products cannot

be compared with other major polluting sectors just by using green-house gas emissions as a parameter.

What encourages us to use petrochemicals in such a nonchalant fashion is their low price. Globalization and skyrocketing demand drove petrochemical prices to low levels which seem to be the main obstacle for any competing cleaner substitute.

To find an alternative to the use of petrochemicals (i.e., to find a new source for molecules required by the industrial sectors) which is more "natural", we ought to look into nature itself. Our rapidly growing knowledge and understanding of biological systems gave us the ability to harness them in what we would call biotechnologies. Many of such technologies are not novel, with some being used by humans for millennia. Food and beverages were produced through biotechnological processes, with little understanding of their underlying mechanisms. Later, with the advancement of microbiology, the world was reshaped with discoveries such as antibiotics and vaccines.

In the XXI century, use of bioproducts[a] (products deriving from biotechnologies) can be envisioned in almost every aspect of industry. Although we are aware of extraordinary ways in which we could harness microorganisms and their products, these technologies have rarely entered the mainstream. In general, biotechnologies remained mostly "niche" technologies. These include those used to manufacture specialty products and "fine chemicals", such as food additives (vitamins, pigments, emulsifiers), and biopharmaceuticals (insulin, antibodies…), as well as biopesticides. Outside the food industry, bioproducts are rarely used as bulk chemicals,

[a] Even though in a wider sense any products derived from natural sources (including agricultural ones) can be called bioproducts, the term in this thesis signifies exclusively microbially derived products. As the focus of this work is placed on sustainability, bioproducts of microbial origin stand out among others for the efficiency of their production, comparatively lower requirements for energy, water, and cultivation area, as well as other unique properties.

i.e., used in copious amounts and on a global scale. One of these rare examples are biofuels, a line of promising alternative fuels promoted by international regulation and policies, which seem to be nowadays increasingly overshadowed by more convenient renewable sources of energy.

Among bioproducts, microbially-produced alternatives for most chemicals of petrochemical have been developed, including solvents, plastics, pharmaceuticals and food and feed additives. (Brar, Dhillon, and Soccol 2013) The use of microorganisms as producers is beneficial compared to others (animals, plants, as well as mammalian and plant cells) in terms of productivity and material and energy consumption. However, these technologies usually struggle to enter the market and integrate into existing processes, hindered mainly by their higher production costs compared to chemical production processes for petroleum driven products.

Surfactants are the group of chemicals with one of the highest consumption rates globally, due to their use in household cleaning agents, personal care products, cosmetics, pharmaceuticals, plastics, and the textile industry. However, their persistence in nature, combined with their disposable manner of use and potency, even in low concentrations, makes them a great danger to the environment.

Although the surfactant production industry is heavily dependent on petrochemical feedstock, it is possible to substitute these with natural feedstock, mainly using oleochemical materials derived from plants. Soaps and fatty acid esters can be produced in this manner, posing as a renewable alternative to chemical surfactants. (Svensson 2010) However, raw materials used for this production are needed in the food industry and usually need to be in their pure form to be chemically manipulated. The resulting competition between these branches of industry could

endanger food security and potentially raise food prices. Ultimately, products derived in such a manner require additional cumbersome purification steps, increasing solvent and energy requirements.

Indeed, the societal aspect of sustainability is often overlooked. When raising questions on sustainable development, economy and environmental impact are mostly brought up. To come up with a long-term viable alternative to chemical surfactants, and even other chemicals of fossil-fuel origin, any competing technology should be economically lucrative, environmentally friendly, and safe for humans and society.

Biosurfactants, microbially produced surface-active compounds, seem to be a potential substitute to chemical surfactants, often showing superiority where their competitor falls short. They are biodegradable, biocompatible, and renewable, as they can be produced from a wide range of biomass-based feedstock. This means they can be produced anywhere around the globe. Still, biosurfactants struggle to enter the market, even after half of century of research (Arima, Kakinuma, and Tamura 1968).

Truthfully, some of their exquisite properties can be problematic in certain cases. Their biodegradability could affect their shelf life, and their biocompatibility could cause some unwanted interactions with living organisms. With any efforts to place these novel biosurfactants on the market, research should also focus on developing applications suited to their properties. To capture the full potential of microbial surfactants, their strengths enable their use in manners which were not possible with chemical surfactants, which makes this endeavour even more challenging.

However, what primarily prevents biosurfactants from entering the mainstream surfactant market is associated high production costs. This obstacle is a debilitating one in the global profit-driven free market.

The push to upscale biosurfactant production and reduce manufacturing costs should be done, keeping sustainably in focus. Efforts should be made to develop the process to be more economically efficient without affecting its environmental neutrality and societal impact. Still, there is no unique protocol for achieving this, as each biosurfactant has particular properties and production characteristics.

The challenge is to find a successful approach to bioprocess improvement by working on all segments of production as a whole – the upstream, downstream processing, and fermentation itself. Finally, the intended application for the product takes part in the decision-making during process development, as it establishes the final target product standards that the process should deliver. This holistic approach to bioprocess development can be further extended to fit within the concept of circular economy, connecting separate bioprocesses by using waste streams and by-products from one as feedstock for the other. This is the only way to secure that the emerging solutions will be viable on the long term.

1.2 Concept of the thesis

Mannosylerythritol lipids (MELs)[b] are a group of glycolipid biosurfactants with excellent interfacial properties and other appealing qualities. (Morita et al. 2015) The molecule consists of a hydrophilic moiety (4-O-β-D-mannopyranosylmeso-erythritol), and fatty acids and acetyl groups as the hydrophobic moiety (Figure 1.1). 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.

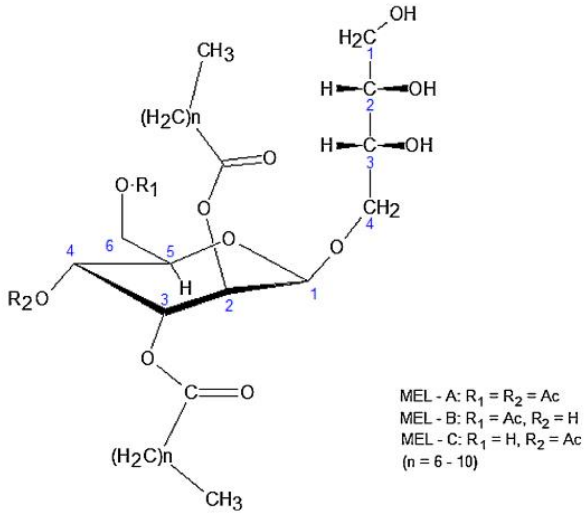

Figure 1.1 Chemical structure of Mannosylerythritol lipids (Valappil et al. 2013)

They can be produced from a wide range of raw materials and are posed to be an attractive substitute to the existing chemical surfactants. While biosurfactants are in general 10-40 times more effective in reducing solution surface tension than chemical surfactants (Roy 2014), MELs outperform other biosurfactants, showing higher effectiveness even in extremely low concentrations. (Kulakovskaya and Kulakovskaya 2014)

[b] In literature sources, MELs are often referred to in a singular form, MEL, as a *singulare tantum* noun. Admittedly, this is also common practice within our group, especially in informal communication, probably due to the fact that the singular noun "mel" stands for "honey" in Portuguese, which greatly resembles the biosurfactant in appearance. However, in this thesis the plural form will be used when addressing the general material itself, especially due to the fact that it comprises of several homologues in varying ratios; except in rare cases when the plural form sounds too coarse, such as when talking about MEL-rich beads.

The focus of this thesis is to improve the production process for MELs contributing to making their entry into the surfactant market more viable. This involves increasing production efficiency, while keeping sustainability in focus, and following an approach that considers the joint improvement of all elements of the process as a whole, as presented in Figure 1.2

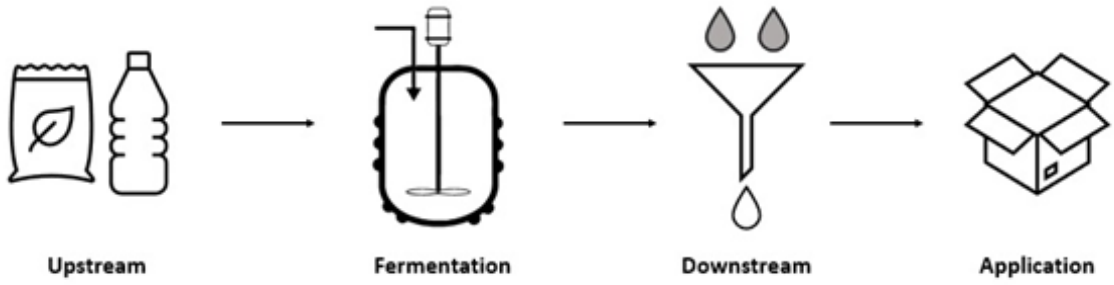

Figure 1.2 Overview of the different sections of the biotechnological production process

In the pictured diagram of the process, key elements include:

- The **Upstream**, part of the process focusing on substrate selection and pretreatment
- The **Fermentation**, where the bioconversion itself takes place, defined by the production setup, fermentation parameters, working microorganism, etc. The efficiency of this segment is mainly characterized by yields, titres, productivities, conversion rates and residual substrates
- The **Downstream processing**, encompassing all unit operations between the fermentation and the final product, including product recovery, purification, and formulation creation
- The **Application** is included in this context as one of the key elements of the process, as it is the driving force in establishing the product value. The target application of the final product defines the criteria needed to be satisfied in the remaining steps, in terms of quality, purity and molecular makeup.

While the thesis focuses on mainly on MELs, it is the intention of the author to develop a rationale which could be followed and applied for the improvement of other bioprocesses aiming to produce similar biotechnological products. The main obstacles that MELs face on their route to enter the market are common for most novel emerging bioproducts and include high production costs, low productivity, downstream processing inefficiency, and a need to find the adequate application that fits specific biosurfactant features.

1.3 Thesis objectives and research questions

The thesis focuses on the development of an integrated production process for MELs from sustainable carbon source, increasing production efficiency and minimization of process environmental impact. The research takes into consideration the *Moesziomyces* genus yeast capacity to produce MELs, to metabolize a wide range of substrates and to secrete large amounts of extracellular enzymes, mainly lipases. (Jan et al. 2017)

This thesis intends to answer the following research question:

- Can the production process of MELs be improved to facilitate a transition to sustainable large-scale production?

This question can be broken down into several more specific challenges to be investigated:

- What are the main obstacles to be overcome to upscale MELs production to an industrial level? (Chapter 2.6)
- Can MELs be produced in salt water? Specifically: i) what is the effect of salinity on the microorganism? ii) What is the potential for marine bioremediation with MELs and MELs-producing microorganisms? iii) Do these conditions enable production of MELs in unsterile conditions? (Chapter 3)

- What is the impact of MELs on the environment, namely its eco-toxicity, considering a model living organisms, and how that compares with other microbial surfactants? (Chapter 3)
- What are the effects of different substrates, including both hydrophilic and lipidic ones, on MELs production? Specifically, what is the effect of substrates on MELs production, microorganism's physiology, and broth properties? (Chapters 4 and 6)
- What are the key process factors that promote the formation of beads rich in MELs? (Chapters 3, 4, 5 and 6)
- How to explore the formation of beads rich in MELs to increase process efficiency? Specifically, how to develop a non-invasive process for MELs bead removal harvesting? (Chapters 5, as well as 4 and 6)
- Can the yeasts be used in resting cell mode for continuous production of an MELs and lipase rich aqueous solution? (Chapter 7)
- Can an efficient downstream process for the treatment of crude MELs be developed to improve MELs purity using sustainable solvents and simple unit operations? (Chapter 8)

1.4 Thesis Outline

Chapter 2 provides a state-of-the-art overview of MELs structure, properties, characterization, and progress made concerning their production. The surfactant market, including biosurfactants, is briefly described. The raising on sustainability concerns for industrial production processes, with efforts to a transition towards cleaner technologies, is discussed. Endeavours undertaken so far to up-scale MELs production reported in scientific literature are highlighted.

Chapter 3 challenges the use of fresh water in the MELs production process and examines increased salinity effects on the performance of the working microorganism. MELs environmental impact in terms of toxicity is assessed.

In **Chapter 4**, the native lipases produced by this yeast are used to pre-hydrolyse of lipidic feeds, in an effort to facilitate bioconversion, and increase productivity by shortening fermentation duration.

Based on observations made in the previous two chapters, **Chapter 5** focuses on the phenomenon of MELs-rich bead formation, underlying factors influencing their appearance, and examines their potential on developing a semi-continuous MELs production process.

In **Chapter 6**, the effect of glycerol and glucose on cell physiology is examined, to develop better fermentation conditions.

Chapter 7 focuses on developing a continuous production process for an aqueous solution rich in MELs and lipases, using resting cells.

Finally, in **Chapter 8** a thorough and sustainable multi-step downstream process is proposed, aiming to produce a pure product with a minimal environmental footprint.

This thesis is structured in a way which allows independent reading of the individual chapters. The reader is asked to bear in mind that this is the reason for some redundancies in information between the separate chapters, mainly in terms of materials and methods, as well as repetition of key data related to the motivation for the work in each chapter.

1.5 Research contributions in publication

Parts of **Chapter 2** are prepared for publication as the review article manuscript "*Bioprocessing strategies for sustainable large-scale production of MELs: a review*", developed with PhD student Miguel F. Nascimento.

Research presented in **Chapter 3** of this thesis is found in the manuscript draft "*Towards Mannosylerythritol lipids (MELs) for bioremediation: Effects of NaCl on M. antarcticus physiology and biosurfactant and lipid production; Ecotoxicity of MELs*".

Parts of **Chapter 4** are prepared as a manuscript draft, to be published under the title "*Production of Mannosylerythritol lipids (MEL) from vegetable oils: Exploring lipase application on substrate pretreatment*". A part of the research results from **Chapter 4** were the topic of an oral presentation given at the 12th European Congress of Chemical Engineering and 5th European Congress of Applied Biotechnology (ECE 12 & ECAB 5), in Florence, Italy, September 2019.

Chapter 6 is prepared for publication in the form of manuscript draft "*Effect of glycerol of M. antarcticus cell quality - impact on cell morphology and MELs production*".

Parts of **Chapter 8** are found in the manuscript draft "*Novel downstream processing setup for biosurfactants produced from lipid-based substrates*", developed with PhD student Miguel F. Nascimento.

Research data published in this thesis is found in two international patent applications. The first patent, related to data from **Chapter 3** regarding bioremediation using MELs, is titled "*Oil dispersant formulation, methods and uses thereof*".

The second one is related to the continuous production of MELs rich beads using the designed separation device, presented in **Chapter 5**, and is titled "*Non-invasive Mannosylerythritol lipids(MEL) beads extraction device*".

Chapter 2

2 State of art in Mannosylerythritol lipids and their potential impact on the surfactant market

2.1 Overview

With increasing awareness of the negative environmental footprint human society is making, efforts are made to meliorate our unsustainable practices. Our dependence on non-renewable materials, mainly fossil fuels, is alarming. The implementation of cleaner alternative methods of production is imperative. Ideally, the impending transition should be made without affecting industrial stability and life quality of the general population. Indeed, although the development of emerging alternatives to produce a wide range of chemicals and materials has to be made soon, it has to be done with consideration of the profound impact that any misapprehension can have overall.

This is especially important for the surfactant production sector of the chemical industry. This widely used group of compounds are used as disposable products, are very potent even in small concentrations, and resilient to biodegradation for extended amounts of time.

In the past decades, the scientific community was faced with the emergence of novel microbially produced surfactants, renewable alternatives with a much lower negative environmental impact. Among them, Mannosylerythritol lipids (MELs), a group of potent glycolipid biosurfactants, emerged as one of the most promising ones in this group. Their exceptional properties give them potential to be used in a wide range of applications. Research was focused on improving the understanding of the underlying mechanisms of their production, as well as their properties. Still, MELs are struggling to enter the market while other competing biosurfactants are gaining traction.

In this chapter, an overview is given of the *status quo* of the chemical industry, contemporary research regarding the production of MELs, and the analysis of the potential impact they could have within the wider surfactant market.

2.2 The rising need for green chemicals

In 2015, the United Nations General Assembly approved a blueprint for action on achieving sustainable development goals by 2030. (UN 2015) The set of goals encompasses a range of objectives, focusing on repairing unsustainable social, environmental, and economic practices. Goal 12, related to sustainable consumption and production patterns, urges participating countries to:

- "By 2020, achieve the environmentally sound management of chemicals and all wastes throughout their life cycle, in accordance with agreed international frameworks, and significantly reduce their release to air, water and soil in order to minimize their adverse impacts on human health and the environment."

Although improvement to existing production practices and consumer behaviour would curb the damage chemicals make on the ecosystem, including us as a one of its species, this would not be enough to make a profound and lasting impact. The reliance on fossil fuel for chemicals essential for maintaining the existing life quality, is itself problematic for several reasons. Namely, the uneven distribution of these strategic resources was, historically the cause of social struggle, wars, and economic inequality - by making those controlling the means of their exploitation exorbitantly wealthy. Furthermore, extraction, transportation, and any manipulation of fossil fuels poses an environmental threat, as their inadvertent release into the environment, including oil spills and gas leaks, are bound to happened. With global reserves of fossils on the decline, increasingly aggressive means of their exploitation are practiced. Hydraulic fracking of deep shales, used for capturing reserves of natural gas and petroleum, result in the release of methane, a dangerous green-house gas, which is 40-60% higher compared to exploitation leaks of conventional wells. (Fischetti 2012)

Green chemicals, especially biologically produced chemicals, offer a solution issues caused by the use of petrochemicals. They are produced from renewable raw materials, securing their long-term availability. These resources used for their production are ubiquitous, providing strategic equality, securing market competitiveness, and offering developing countries a chance for increasing their industrial independence. Finally, they are largely biodegradable and non-toxic, curbing the environmental damage caused by any accidental release into the ecosystem or their accumulation in nature.

In this chapter, an overview of the problematic state of the current chemical industry is given, as well as the efforts made to curb these issues. Special focus in this chapter is put on the surfactant production sector within the chemical industry, environmental and health concerns they cause, and the cumbersome road towards finding more alternative substitutes.
Finally, the state-of-the-art of knowledge regarding MELs is given, being one of the promising alternative surfactants with a wide scope of application. Their structure, properties, production, and downstream processing are analysed in detail, with a look at their potential applications and impact on the surfactant market.

2.2.1 Sustainability within the chemical industry

Concerns regarding general sustainability are centuries old (Malthus 1798), and initially were fuelled by the struggle of matching food production rates with the rapidly increasing population numbers. With time the concept of sustainability changed, following the growing understanding of the negative impact industrial activities has on the environment, as well as social struggles caused by the globalized market. Issues such as poverty, famine, social inequality, as well as

pollution, green-house effects, and deforestation, all changed our interpretation of efforts towards sustainable development. (Johnson 2007; Pezzey and Toman 2002)

The epitome of unsustainability of modern industrial operation is the reliance on fossil fuels. It is very obvious how the intense usage of fossil fuels defies one the main principles of sustainability: *Finite materials including fossil fuels should not be extracted at a faster rate than they can be redeposit in the Earth's crust.* (Winnett and Warhurst 2003)

Based on BP's Statistical Review of World Energy (BP 2020), 40% of global fossil fuel use in 2019 was related to crude oil, followed by coal and natural gas. The same report shows how the global oil production has been on the constant rise during the previous half century (Figure 2.1, Left). This trend was largely left unaffected by volatile oil prices, even during the market collapse in the 1980s, and the dramatic Oil Shock of 2007–08 (Hamilton 2009). This variable rise of oil prices over the last decades (Figure 2.1, Right) proved to be uninfluential on the global demand for crude oil, implying that the moment when high oil prices influence industrial demand for oil – is yet to come.

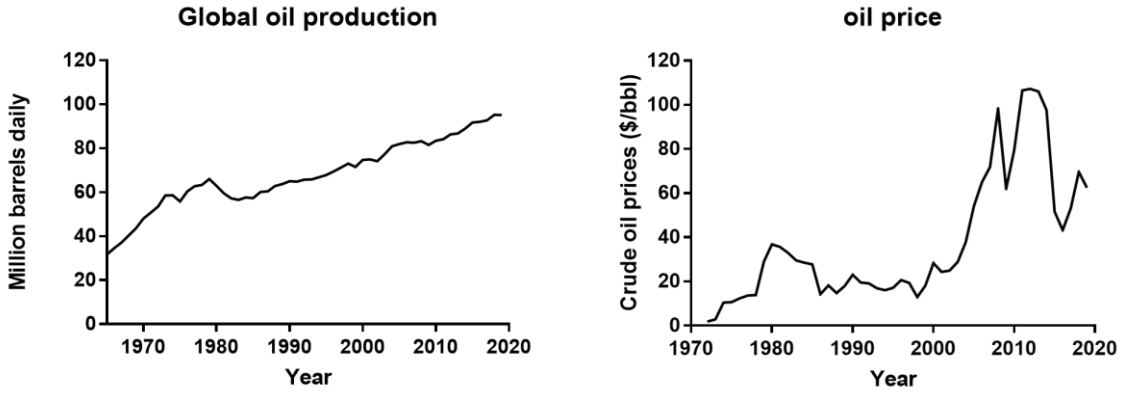

Figure 2.1: Left - Annual Global oil production (Includes crude oil, shale oil, oil sands, condensates, and NLGs); Right - Spot crude prices per barrel (average value based on Dubai, Brent, Nigerian Forcados, and West Texas Intermediate blends) (BP 2020)

The chemical industry contributes to roughly 7% of the global GDP, and supports 120 million jobs. (ICCA 2019; Krueger and Selin 2002) Most of the oil consumption by the chemical industry is associated with the production of petrochemicals. They contribute up to 40% of the total global chemical production. (MoC&F India 2020) This term encompasses derivatives of petroleum, which are used directly or as precursors for more complex chemicals. Petroleum derivatives include olefins and aromatic compounds, which can be converted through chemical modification into a wide range of bulk and specialty chemicals. Plastics, surfactants, solvents, waxes, dyes, and many other compounds, are then widely used to manufacture products in personal care, pharmaceutical, textile, agriculture, and food sectors.

According to the International Energy agency report (IEA 2019) the chemical industry, being one of the most significant industrial sectors, is currently accounting for 15% (13 million barrels per day) of global demand for crude oil. This report recommends implementation of new low CO_2 emissions technologies, approval of legislation that discourages single-use chemicals use, increasing energy production efficiency and to significantly investment in R&D efforts to come up with sustainable alternatives

2.2.2 Pollution and bioremediation of petrochemicals

Environmental pollution is an umbrella term, broadly encompassing antagonistic effects causing damage to the environment on a local and global level. These include deforestation, reduced biodiversity, and depletion of limited natural resources, as well as industrial and societal malpractice, which results in the release and accumulation of harmful chemicals in nature. The seriousness of environmental pollution has increased with the rapid increase of population and industrial development in the past decades. In parallel, our understanding of

these negative effects has increased, resulting in a global call for action. (Appannagari 2017) Water pollution is emerging as one of the most important issues for immediate attention, and rapid urbanization and industrialization without adequate waste management resulted in degradation of water quality, particularly in developing countries. (Goel 2006)

As mentioned before, extensive oil use inevitably results in accidental or intentional oil spills, causing immense harm to marine and terrestrial ecosystems. Besides accidental oil spills caused by oil tanker breakdowns or precarious drilling, oil leaks are caused by intentional damage to the supply infrastructure with the intention of disrupting life quality during wartime and political conflicts. The Trasandino Oil pipeline in Colombia has been the target of Revolutionary Armed Forces of Colombia rebels 18 times between 2010 and 2016, and 25 times in 2019 and 2020. (El Economista 2015; El Espectador 2019; Global Terrorism Database 2017; La Republica 2020; Reuters 2014, 2015; El Tiempo 2020) Most of these attacks resulted in oil spills which destroy water supplies (with one of them affecting drinking water availability for 16000 households) as well as causing harm to endangered wildlife. Many of the attacks have been called “environmental catastrophes”, with oil being carried by rivers into the Pacific Ocean, forcing the Colombian oil companies to scramble in an effort to physically remove the oil from water surfaces and stop the spread of the leak with booms.

Besides physical removal of spilled oil, by skimming or by previously mentioned booms, oil is either burned *in-situ* or dispersed by specialized chemicals. The intention behind the use of such dispersants is just to reduce the amount of free floating oil, and let the native marine microflora degrade it. (Landry et al. 2019) However, a certain trade-off is made here, as the dispersant themselves are toxic to a wide range of sea species, from plankton to vertebrates. (Barron et al. 2020)

The understanding of interactions between common-use chemicals and nature has too often been based on trial and error. An example of this was Dichlorodiphenyltrichloroethane (DDT), an insecticide which earned its inventor a Nobel prize for stopping malaria outbreaks. In the decades that followed, the lasting cancerogenic and teratogenic effects of this chemical came to light. (Guzelian 1982)

Organic chemicals of petrochemical origin rarely biodegrade completely, due to the commonly-present unsaturated bonds and aromatic elements highly resistant to biological degradation. (Vora et al. 2003) Biodegradation is defined as the process of breaking down compounds, catalysed by microorganisms and their metabolism. (Balson and Felix 1995) Sometimes, biodegradation is measured by quantification of decreasing over time of specific chemical properties, such as measuring surface tension activity reduction to assess surfactants degradation. This can be misleading, as some of the intermediary degradation compounds can be more toxic than the original compound. (Ying 2006) Complete biodegradation occurs only when the original compound is degraded into CO_2, ammonia, inorganic salts, biomass and water, which is not very often the case. (Scott and Jones 2000)

2.2.3 Biochemicals – the emerging alternative

Biochemicals include chemical compounds derived from materials of biological origin, usually facilitated by biotechnological conversion. These bio-based chemicals offer an alternative to conventionally used synthetic chemicals of petrochemical origin. (EUBIA 2021) Biochemicals include an array of compounds - small molecules which are used as fuel or raw material for synthesis of more complex chemicals (biohydrogen, alcohols and organic acids, monomers,

fatty acids, carbohydrates, and bioaromatics), as well as consumer chemicals and fine chemicals used for specialty purposes – pharmaceutics, medicine (vitamins, peptides), and so on.

Although the term biochemicals is usually applied to fermentation products, it also includes chemicals generated by chemical synthesis from biological raw materials and/or microbially-derived precursors. An example biochemical would include syngas fuel derived from CO released by pyrolysis of biomass and hydrogen of algal origin. A common for biochemicals is to reach near net zero carbon emissions in their life cycle, which requires exclusive use of renewable materials and production processes designed to be sustainable.
Raw materials for biochemicals include ubiquitous and cheap renewable substrates and waste streams from several industries. Among these materials are biomass waste, vegetable oils, animal fats, agricultural products, and so on.

In 2018, the European Commission updated their Bioeconomy strategy (European Commision 2018), which, among other topics, highlight the importance on the sustainable development of bio-based processes, reduction of reliance on fossil fuel raw materials, and adding value to biowaste though the encouragement of circular economy. The programme included a package of funds aiming to stimulate research and industrial development, with the goal of achieving 20% of biochemicals participation among general chemical products. To regulate the emerging market of bio-based products, the Commission previously released a series of standardization mandates, including M/491, concerning the development for bio-surfactants and bio-solvents. (European Commision 2011)

Current global leaders in the still relatively small bioproduction industrial sector are the US, Brazil, and a few Asian countries, mostly focused on production of biofuels and feedstock chemicals using sugar and starch feedstock, as well as vegetable oils. (EUBIA 2021) The EU

industrial bioproduction lags behind compared to other world regions and bioplastic production is its only significant contribution to the sector, accounting for 25% of global bioplastic production. Despite this, due to thorough government strategy and intense financial support of R&D efforts, the EU is projected to join the global leaders of bio-based industries soon, with 30% of oil-based chemicals and materials to be replaced by bio-based ones by 2030. (Natrass et al. 2016; Scarlat et al. 2015)

2.2.4 Biorefineries

Existing petrochemical refineries work using the platform-chemical approach. This means that, starting with the raw material, first a few chemical intermediates are produced, which are later used elsewhere for the synthesis of a number of more complex chemical products. (Werpy and Petersen 2004) These chemical processing facilities work independently, generating polluting waste and consuming enormous amounts of energy and water.

Most waste streams from the production of biochemicals are more valuable and less toxic than those in petroleum refineries (as they are usually not fully degraded). This enables multiple bioprocessing facilities creating various products to be joined together. In this scenario, biological raw material (i.e., lignocellulosic biomass) would enter the facility, from which energy and a plethora of valuable chemicals and materials would be generated. This concept is dubbed as the biorefinery – a sustainable alternative to conventional oil refineries. The social aspect of biorefineries is therefore amplified, as they offer job security and development for rural areas.

Renewable feedstocks used in a biorefinery can be agricultural and forestry waste, waste streams, by-products and leftovers from industry and households, and aquaculture raw materials. (Cherubini 2010)

One of the main advantages of the biorefinery is its potential efficiency. Some biotechnological production processes are already implemented in an industrial scale (biofuels, organic acids). However, they often require the use of raw materials competing with the food industry (sugar- and starch-based materials), Indeed, biorefineries sustainability success depends on some crucial decisions. Namely, the selection of feedstock has a high impact, competition with food and feed applications should be avoid and the need for pre-treatment methods, which would increase production costs, carefully considered. The biorefinery would need to be strategically located near the sources of these wastes – agricultural and industrial areas, to reduce needs for transporting the bulky raw material. Also, currently waste management of existing facilities is a burden for the producers, as the waste streams are usually wet and rich in organic matter, making their transportation and processing costly. Under the "single roof" of the biorefinery waste from one stream can be used as part of the feed for the other, and with adequate planning organic waste from the facility can be reduced to near zero.

2.3 Surfactants

The term surfactant covers a wide group of chemical compounds that lower surface tension between two phases. The term itself was coined by Antara Products in 1950, as an abbreviation for SURFace ACTive AgeNTs (Rahman and Sekhon 2015). Surfactant molecules are amphiphilic and consist of a polar head and a non-polar tail group. Besides affecting surface tension, they increase solubility of immiscible liquids and mobility of dispersions. Due to this property, they are widely used in an array of applications, including personal care and cleaning

products, many industries (pharmaceutical, food, textile, petrochemical), agriculture, etc, making them an integral part of our everyday lives. Surfactants can be used as detergents (to enable washing/solubilization of non-polar compounds and particles), wetting agents (to enable better contact between water and hydrophobic surfaces), foaming agents, dispersants (to form dispersions of solids in a liquid solution), or emulsifiers.

Most surfactants have at least a partial petrochemical origin. As we rely on surfactants in our everyday life, questions are raised regarding their long-term sustainability. Due to their generally poor biodegradation and ecotoxic effects, they pose an immense danger to the environment.

2.3.1 Properties

By the nature of their hydrophilic head group, surfactants are classified as anionic, cationic, non-ionic, and amphoteric (zwitterionic). (Singh and Hui Mei 2013)

Anionic surfactants are compounds consisting of hydrophobic tail, usually of alkyl nature, and a hydrophilic negatively charged head, consisting of a sulphonate, phosphate, carboxylate, or similar group. Due to this property, they interact with ions present in the water, such as those present in hard water, which affects their performance. Soaps, the oldest human-made form of surfactants, fall into this category.

Cationic surfactants, on the contrary, contain a positively charged hydrophilic head (usually quaternary amine group). They are rarely used for washing, due to their strong binding to

hydrophobic surfaces (causing damage to hair and skin irritation) (Rhein 2007), and are mostly commercially used in fabric softeners or antistatic agents.

Non-ionic surfactants' polar head has no charge, making them suitable for use in hard water. Besides this, they are soluble in a range of organic solvents. Most of the microbial biosurfactants fall into this group, due to lack of ionic groups in their structure.

Amphoteric, or zwitterionic, surfactants have a polar head whose charge can be positive or negative depending on the pH value of the solution. They are less irritating to the skin and hair, making them suitable for body care products for which they are used in the past decades, and have a higher biodegradability rate. (FernLey 1978)

The most important parameter for surfactants is the concentration at which they start forming micelles in the solution, called the critical micelle concentration (CMC). Typically, the surface tension of an aqueous solution of surfactant decreases with increasing surfactant concentration from zero to it reaches CMC and above that threshold the increase on surfactant concentrations results on higher amounts of micelles, but without consequence on the solution surface tension. As the properties of surfactant solutions vary greatly below and above the CMC, it is the reference value for establishing concentrations in formulations for various applications. In other words, surfactants with lower CMC values are usually used in lowered concentrations and can thus be considered more potent.

2.3.2 Production

Most commercially used synthetic surfactants have a petrochemical origin. Surfactant producers mainly depend on ethylene and its derivatives as the main feedstock for synthesis. In 2019, 33% of fossil fuels processed by the petrochemical industry was converted to ethylene. (Grand View Research 2020) Ethylene can be directly oxidized into ethylene oxide, which in turn is converted into ethylene glycol in the presence of water (catalysed by acids, bases, or elevated temperature). Alternatively, it can react with ammonia to form ethanolamines. These two groups of compounds are the main feedstock materials used for production of chemical surfactants. (Frauenkron et al. 2001)

Ethanolamines are classified based on the how many carbon items the amino group is attached into monoethanolamine (MEA), diethanolamine (DEA) and triethanolamine (TEA). MEAs and TEAs are often used as precursors to produce cleaning agents, surfactants used in the textile industry, plasticisers, and wetting agents. DEAs are more commonly used in agriculture and laundry detergents. (IARC 2013) Due to the possibility of cancerogenic nitrosamine presence in final products, DEA-derived surfactants are prohibited for use in the European Union in personal care products and cosmetics. (Dodson et al. 2012)

Ionic surfactant production, besides the need for the petrochemically-derived organic backbone of the molecule, requires the use of inorganic raw materials for generation of the ionic head. This negatively affects the production process even further in terms of sustainability. For instance, production of alkyl sulphates, a major group of anionic surfactants, consumes massive quantities of chlorosulfuric acid. Cationic surfactants require substantial amounts of ammonia for their synthesis, which is in turn generated by the energy consuming Haber-Bosch process. Amphoteric surfactants have the highest cost of production and raw material requirements, and

are usually sold as highly active specialty chemicals at premium prices. (Levinson, Zoller, and Sosis 2008)

Some surfactants are partially produced by raw materials of biological origin. These include those with hydrophobic tails originating from vegetable oil fatty acids. Their toxicity is generally lower, due to the absence of branched or aromatic structures, and their biodegradation is easier. Still, they cannot be considered sustainable as the production of these surfactants requires the use of non-renewable chemicals and inorganic compounds for the polar head synthesis. They also rely on the use of food-grade purity substrates, and still may contain traces of toxic compounds in the final product.

2.3.3 Environmental impact and toxicity

In the past century, society went through several cycles of use and prohibition of some of the most popular surfactants, switching to new ones when issues with the previous ones became known. After the transition from soaps to detergents, propylene tetramer benzene sulphonate (PT benzene) became one of the most used surfactants until the 1960s in household and personal care products. Subsequently, the appearance of foaming in rivers near urban areas, and issues emerging in sewage treatment facilities showed that PT benzene has no apparent ability to biodegrade, due to its branched structure. (Dee and Snell 1958; Holmberg 2019; Scott and Jones 2000) Then, the use of linear[c] alkylbenzene sulfonates (LASs) was encouraged. They were found to be easily removed from wastewater by activated sludge and other biological oxidation treatments (95-99% removal) (McAvoy, Eckhoff, and Rapaport 1993), as well as to biodegrade more easily that non-linear alkylbenzene sulfonates when reaching open water bodies.

[c] Indicating linearity of the hydrocarbons used in their synthesis, not in the surfactant itself. LASs are non-linear molecules, and include an aromatic ring and sulfonate group. (Vora et al. 2003)

(Rapaport and Eckhoff 1990) However, their degradation products were not as harmless. The use of sludge from wastewater treatment plants as agricultural fertiliser is usual practice in the UK and other countries. Therefore, any remaining LASs or their degradation products in such sludge can cause dangerous contamination of soil. (Nielsen, Borch, and Westh 2000)

Anionic surfactants in general tend to be bio-toxic, due to interactions with proteins and macromolecules, often disrupting normal metabolic activity of cells by affecting enzymes and membranes. Most anionic surfactants show some level of skin irritation in humans, while others might have more sinister effects on the human body by interacting with DNA and metabolic enzymes. (Cserháti, Forgács, and Oros 2002; Nielsen et al. 2000)

Compared to anionic surfactants, cationic surfactants are proven to be drastically more toxic to humans and dangerous to the environment, and little is known about any degradative process they go through in the ecosystem. (Scott and Jones 2000) Luckily, they perform poorly as cleaning agents when used alone, and are most often used in small amounts in formulations for fabric softener products. (Steber 2007) Still, even in these small quantities they can wreak havoc in contact with living cells. As an example, cetyltrimethylammonium bromide (CTAB), sometimes used for hair conditioning products and some specialty antiseptic products, is proven to disrupt the normal function of ATPsynthase in cells, leading to disruptive effects in sensitive ecosystems. (Schachter 2013) As some chemical surfactants have intense anti-bacterial properties, they are used as additives in certain personal care products. However, their accumulation in nature could result in raising bacterial resistance to bactericides. United States Food and Drug Administration (FDA) issued in 2016 an announcement (FDA 2016), calling for strict control and monitoring of the use of non-biodegradable chemical surfactants with strong antibacterial properties.

Surfactant pollution has adverse effects on human health as well. Some reports (Soares et al. 2008; Sonnenschein and Soto 1998) estimate that nonylphenol ethoxylates (NPEs), a group of alkylphenol ethoxylates (APEs), commonly used in various detergent formulations, might have an impact on testicular and breast cancer, as well as reduced sperm count, by mimicking the hormone oestrogen, thus disrupting the normal hormonal activity in exposed humans. Appearance of feminized male fish near a sewage outlet in the UK troubled researchers, who later found that most probably intermediary products originating from the biodegradation of APEs disrupted the normal endocrine functioning of the animals. (Purdom et al. 1994) These compounds persist in sewage sludge and river sediments for extended periods of time due to their physico-chemical properties. Still, there was a lack of sufficient international compliance with imposed bans on APEs. A more recent study reported that in developing countries dangerous levels of accumulated alkylphenol ethoxylates are still present and can be inhaled as dust, with exposure rates as low as some $ng.day^{-1}$ affecting children and young adults. (Abafe et al. 2017)

Due to their physico-chemical interactions with hydrocarbons, some surfactants are used for bioremediation of water and soil after oil contamination. Research shows that non-ionic surfactants can aid bioremediation of polycyclic aromatic hydrocarbons, by making them more bioavailable for endogenous microbial consortium able to catalyse such molecules mineralization. (Bueno-Montes, Springael, and Ortega-Calvo 2011) However, they are not an ideal solution, as they themselves pose a danger to the ecosystem, as discussed before.

2.4 Biosurfactants

Biosurfactants are compounds with biological origin showing tensioactive properties. They reduce the surface tension of solutions and facilitate the formation of emulsions and other structures. (Desai and Banat 1997) They are composed of a hydrophilic moiety – carbohydrate, peptide or acid, and a lipid-based hydrophobic tail. (Kapellos 2017)

Glycolipid surfactants (one of which is the focus of this thesis) consist of a carbohydrate and a lipid section. (Morita et al. 2016) Besides MELs, other most researched glycolipid biosurfactants are sophorolipids and rhamnolipids, with sophorose and rhamnose as hydrophilic moieties, respectively.

Microorganisms produce biosurfactants for several reasons , usually related to increasing their survivability. For flagellates, reducing surface activity of their surrounding enables better, more efficient motility. (Kearns and Losick 2003) Cells can use these poorly soluble compounds for biofilm creation (Satputea et al. 2016). On the other hand, biosurfactants are also known to inhibit biofilm formation. (Banat, De Rienzo, and Quinn 2014) Control of biofilm formation can take a crucial role in competition for space and resources between species, a phenomenon that is known to occur among bacteria (Kim, Oh, and Kim 2009). Finally, the emulsifying properties of biosurfactants can increase uptake of hydrophobic organic substrates. (Ocampo 2016) Understanding the biological role of microbial products is important, as understanding the natural phenomena in which these compounds participate opens the door towards biomimetic applications and triggers concepts to design enhanced production processes.

Compared to chemical surfactants, which rarely have beneficial interactions with biological systems, the profound bioactivity of biosurfactants can be utilized for many novel applications.

In the future, we are bound to see pharmaceuticals based on biosurfactants, and their application in the medical field.

The use of biosurfactants was hypothesised to aid with the ongoing COVID-19 pandemic. Namely, based on the anti-viral and anti-inflammatory properties of many biosurfactants, Subramaniam et al. (Subramaniam et al. 2020) suggest utilizing these potentials for the treatment of patients with heavy lung inflammation.

On a commercial scale, non-microbial biosurfactants (those produced by plants and animals) have limited potential to be sustainable, as their production requires intense needs for space, time, water, and energy. Poractant (Ainsworth et al. 2000), Infasurf, and Beractant (Bloom et al. 1997), are commercial formulations containing biosurfactants extracted from bovine and porcine lungs, used for treatment of neonatal respiratory distress syndrome in prematurely born infants. These surfactants are naturally produced in the lungs of mammals and play a role in the prevention of the collapse of alveoli during breathing. Commercial production includes extraction of these compounds from the lungs of neonatal calves and piglets. Despite the highly unsustainable method of production, biocompatibility of these compounds is the ultimate motivation for their use, indicating the significance of this trait in certain applications. A report by Suresh and Soll (Suresh and Soll 2005) states the superiority of these animal surfactants to synthetic ones in neonatal treatment, while highlighting the need for cheaper “third-generation” (microbial) biosurfactants to be developed, which would lower their price and make this life-saving treatment available to vulnerable groups.

2.4.1 Market potential of biosurfactants

The global market of surfactants reached $39.90 billion in 2019. and it was predicted to reach 52.42 billion by 2025. (Markets and Markets 2020) Non-ionic surfactants were prospected to have the fastest growth, while application-wise surfactants used in agriculture and home and personal care were projected to gain footing in the global market in the following years.

Biosurfactants were estimated to have a market size of $3.99 billion in 2016, and a projected size of $5.52 billion by 2022, with a higher compound annual growth rate (CAGR) of 5.6% than general surfactants (4.5%). (Markets and Markets 2017) A previous report by the same organization (Markets and Markets 2015) estimated a lower CARG of 4.3 for the period up to 2020, indicating the unexpected expanse of the market size. However, these values might be misleading, as they include biosurfactants in a wider meaning, i.e., surfactants for which at least one segment of the molecule had biological origin, including chemicals derived from plants, thus including semi-synthetic surfactants.

A recent report strictly focused on microbial biosurfactants (360 Research Reports 2020) projects their global market size to be $18 million in 2020, and to reach $23 million by 2026, with a CAGR of 4.8%. A rough comparison of the market size estimates from these sources would indicate that microbial biosurfactants partake in the global biosurfactant market by roughly 0.5%, and 0.046% of the global general surfactants market. Key players in the biosurfactant market were indicated to be Evonik (Germany), Jeneil Biotech (US), and Ecover (Belgium).

In this small market segment, MELs partake with 3 million in 2019, and expected to grow to $4.4 million by 2027. (Coherent Market Insights 2020) Largest producers of MELs were

indicated to be Toyobo Co. Ltd., Biotopia Co. Ltd., Damy Chemicals Co. Ltd., and Kanebo Cosmetics.

There are several reasons for the lack of market presence for MELs and other microbial biosurfactants. For one, they are a novel product which is still in development, with knowledge about their properties and production only emerging in the recent decades. Then, more research is to be made regarding their applications, as their properties require a different manner in which they are formulated and used, compared to chemical surfactants. Finally, their high price prevents them from competing with chemical surfactants, which are valued at roughly $2/t. (Santos et al. 2016) Thus, so far microbial biosurfactants found their place only in niche segments of the market and for highly specialised applications.

2.5 Mannosylerythritol lipids

In 1955, Haskins et al. (Haskins, Thorn, and Boothroyd 1955) reported of ether-soluble “oils” produced by fungi of the *Ustilago* genus. Later (Boothroyd, Thorn, and Haskins 1956), characterised it’s structure as a glycolipid, composed of D-mannose, meso-erythritol, and a number of fatty acids. What they discovered were MELs, a group of glycolipid biosurfactants which were later found to show interesting biochemical interactions and great tensioactivity. In recent decades, MELs came into the spotlight of the scientific community as one of the most promising alternatives to chemical surfactants.

The name of *Moesziomyces antarcticus*, currently the most popular producer strain for MELs, stems from the continent where the microorganism was first isolated – from the bottom of Lake Vanda in the Wright Valley, Antarctica. The microorganism was initially classified as *Sporobolomyces antarcticus*, and the name was later adjusted after phylogenetic analyses.

(Goto, Sugiyama, and Iizuka 1969) The lake is meromictic, meaning that the separate layers of the lake are static and do not mix due to the drastic salinity and temperature gradient. The lake acts as a solar energy trap. Despite the freezing Antarctic weather at the surface of the lake, the water at the bottom maintains a steady temperature of 25° C and a salinity of 14% - four times higher than sea water level. (Gibson 1999; Schutte et al. 2020; Wilson and Wellman 1962) The same temperature would later be determined as optimal for the growth of the microorganism. (Ishii 1993) The search for microorganisms in the frozen wastelands of the Antarctic was motivated by the need for extremophilic cultures with enzymes showing activity at low temperatures, to develop more energy-efficient bioconversion processes.

Although MELs are produced from a range of diverse carbon sources, present studies for the production of glycolipid biosurfactants in general focus on combinations of lipid-based substrates and carbohydrates. Similarly, the work in this thesis focuses on production of MELs from substrates containing sugars, vegetable oils and glycerol.

The production process of MELs can be separated in three main segments, as previously mentioned in Chapter 1.2. Thus, this section is structured to follow that rationale. Sections 2.5.2 and 2.5.3 deal with the main concerns regarding the Upstream part of the process. Sections 2.5.4, 2.5.5, and 2.5.6 relate to the fermentation, i.e., the bioconversion of substrate into the product itself. Section 0 provides an overview on downstream processing of MELs, their recovery and purification. Finally, an overview of existing and hypothesised applications for MELs is made in section 0.

2.5.1 Structure

As all surface-active compounds, the structure of MELs consists of a hydrophilic and a hydrophobic moiety. The hydrophilic head of the molecule is a D-mannose linked to an erythritol molecule. The hydrophobic tails, as is the case with most biosurfactants, consists of fatty acids of varying length. Acyl groups can be present, and based on the level of acetylation, MELs have several homologues with distinct properties. If two acyl groups are positioned on the C4 and C6 atoms of mannose, the molecule is classified as MEL-A. MEL-B and MEL-C is monoacylated on positions C6 and C4, respectively, while the non-acylated homologue is denominated as MEL-D. (Coelho et al. 2020a) The structure of the molecule and its main homologues is presented in Figure 2.2.

Additionally, a third acyl group can be present on the end of the erythritol molecule, forming a tri-acylated MEL. This structure might form due to an additional esterification of MEL-A by enzymes in the presence of residual fatty acids. (Fukuoka et al. 2007) Interestingly, the acyl group can be present on an alternative position, substituting the fatty acid connected to the atom C2. Namely, the yeast *Sporisorium sp. aff. sorghi SAM20* was reported to produce a tri-acylated MEL with this structure, labelled MEL-A2. (Alimadadi, Soudi, and Talebpour 2018) This variance of structure among the different MEL homologues shows the structural diversity of these biosurfactants. Possibilities exist of modifying the structure of the molecule further and creating novel compounds with new properties and biological interactions.

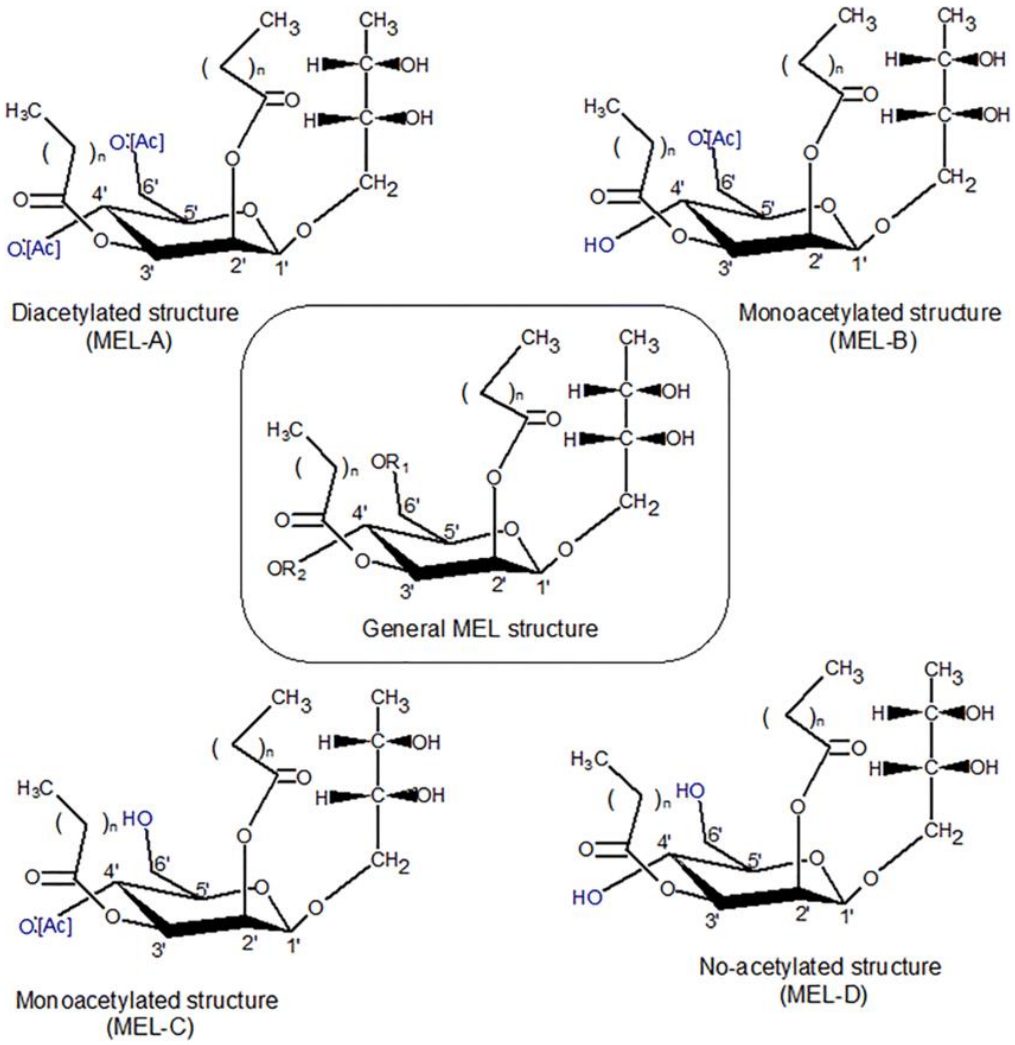

Figure 2.2: Chemical structure of Mannosylerythritol lipids and its four main homologues (Coelho et al. 2020b)

Distinct species produce different MELs derivatives in varying ratios. Yeasts strains *Moesziomyces bullatus* (formerly *aphidis*) and *M. antarcticus* were found to produce predominantly MEL-A, followed by the monoacylated homologues, with small fractions of MEL-D. (Kitamoto et al. 1990; Rau, Nguyen, Schulz, et al. 2005) The fungus *Ustilago scitaminea* NBRC 32730 was reported to predominantly produce MEL-B. (Morita et al. 2009a) Based on the fact that these different MEL homologues have distinct properties, selection of working microorganism in MELs production can result in a product with variable properties, and can be adopted depending to the desired application. (Varvaresou and Iakovou 2015; Yu et al. 2015)

The various MEL homologues participate in the formation of different structures due to their self-assembling properties. Imura et al. (Imura et al. 2006) reported that MEL-A and MEL-B form large unilamellar vesicles at very low concentrations. Furthermore, the report states that, with the further increase of biosurfactant concentration, MEL-A started forming sponge structures with surprisingly wide channels, which were formed by separate laminar bilayers. On the other hand, MEL-B tended to form multi-lamellar vesicles rather than sponge structures, signifying the role of the number of acetyl group on the curvature of formed multimolecular structures.

2.5.2 Metabolic pathways for MELs productions

After the discovery of yeasts able to efficiently produce high concentrations of MELs, effort increased to study the underlying mechanisms of production of this biosurfactant. One of the earliest reports studying MELs metabolic pathway, comes from Kobayashi et al. (Kobayashi, Ito, and Okamoto 1987). In their report, glucose supplemented with casein amino acids was used to produce ME (mannosylerythritol), which they hypothesised plays a role in the emulsification of lipids, enabling easier metabolization.

Phylogenetic analysis of the *M. antarcticus* genome sequence reveals its relation to the well-studied *S. cerevisiae*. (Morita et al. 2014) The two yeasts have a similar number of genes, and a large group of common genes encoding enzymes taking part in the primary carbon metabolism. However, *M. antarcticus* has more genes related to lipid synthesis, secondary metabolism, the pentose phosphate pathway, and others, enabling it to metabolise a wider range of substrates. Understanding of the sugar transport mechanisms for *Moesziomyces* spp. is limited.

For intracellular assembly of the complex glycolipid biosurfactant, the cell's metabolic pathway needs to assemble the building blocks of the molecule – mannose (in the activated form as GDP-Mannose), erythritol (to construct with mannose the previously mentioned mannosylerythritol), fatty acids (in acyl-CoA form, for the lipidic hydrophobic tails of the molecule) and acetate groups (as acetyl-CoA, for acetylation of the MEL-D molecule). (Santos 2017) This pathway for the synthesis of MELs from sugars, glycerol, and vegetable oils is presented in Figure 2.3 (Li et al. 2015; Santos 2017).

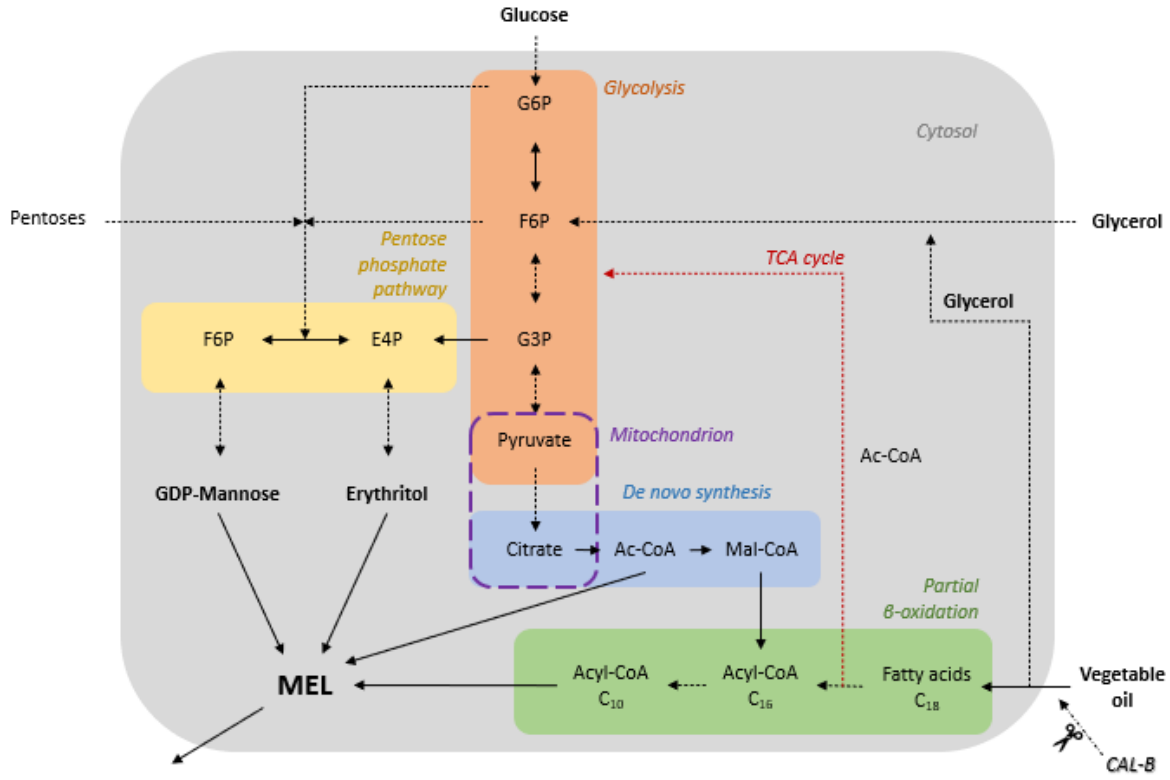

Figure 2.3 Carbon metabolism pathway for MELs production from D-glucose, glycerol, and Vegetable oil. Abbreviations: G6P – glucose-6-phosphate; F6P – fructose-6-phosphate; G3P – glyceraldehyde-3- phosphate; E4P – erythrose-4-phosphate; F6P – fructose-6-phosphate; Ac-CoA – Acetyl-CoA; Mal-CoA – Malonyl-CoA.

MELs production from different substrates can be described as follows: Glucose enters the glycolysis pathway to generate pyruvate, which through the *de novo* synthesis pathway can yield acyl-CoA. Glycerol can act as a substitute substrate, albeit energetically a less efficient

one, and can enter the gluconeogenesis pathway. Vegetable oils are easily converted to acyl-CoA through partial β-oxidation, also generating acetyl-CoA groups which directly incorporate into the tricarboxylic acid (TCA) cycle. Meanwhile, the glycerol released in the process of oil hydrolysis enters the gluconeogenesis pathway.

A conclusion can be made that both hydrophilic and hydrophobic substrates individually can be metabolised into the complete biosurfactant molecule, as is widely proven by reported data for fermentations with both groups of substrates as sole carbon sources. (Kitamoto et al. 1990; Morita et al. 2007b) Reports indicate that higher yields can be obtained with vegetable oils, when an intermittent feed strategy is used, to prevent negative impact by the substrate on the cell. (Rau, Nguyen, Roeper, et al. 2005)

As glycerol can more efficiently generate the hydrophilic mannosylerythritol moiety, and vegetable oils can be used with higher efficiency to generate the hydrophobic moiety, a balance between the substrate should be made to insure higher yields of MELs. However, additional investigation on the effects of substrate presence on the cell, substrate inhibition and the induction of the genes encoding enzymes for specific metabolic pathways is required to establish improved MELs production strategies.

2.5.3 Substrates for MELs production

An overview is given below of hydrophilic (carbohydrates, glycerol) and hydrophobic (vegetable oils and hydrocarbons) substrates reported in literature to be used for MELs production. Additionally, special focus is put on the scarce reporting of complex substrates, biomass materials and their derivatives, as well as waste materials from other branches of industry. Those are regarded as the most promising carbon sources to enable the transition to sustainable industrial-scale production of this biosurfactant.

2.5.3.1 Hydrophilic substrates

Some of the earlies uses of carbohydrates to produce MELs can be traced back to the years after the discovery of yeast strains capable of producing significant amounts of the biosurfactant. MELs production using sugars as a carbon source: including hexoses (Morita et al. 2007b), pentoses (Faria, M. V. Santos, et al. 2014), and disaccharides (Morita et al. 2009a) has been reported.

Results for MELs production using hexoses (glucose) and pentoses (xylose) within our research group (Faria, M. V. Santos, et al. 2014) indicate that low titres of MELs can be achieved with these sugars. In general, the use of carbohydrates as sole sources of carbon rarely generates more than 10-15 $g.L^{-1}$ of MELs at the end of the fermentation, with product to substrate yields rarely exceeding 0.1 $g.g^{-1}$. This is probably due to the carbon being spent on cell growth and maintenance, and not processed along the MELs generation metabolic pathway. As for disaccharides, Morita et al. (Morita et al. 2009a) report production of MELs using sucrose and *M. antarctica* and *Ustilago spp*. fungi as a working microorganism.

Some studies aimed to improve MELs production by performing feeds of mannose and erythritol directly to the cells during fermentation. However, as mannose is only found in small quantities in some fruits and algae (Herman 1971), and erythritol is a biotechnological product used as a food additive. (Carly and Fickers 2018) Therefore, despite their academic merit, these reports have little prospect to have relevance in large-scale production.

Glycerol is a potential substrate for production of MELs, as it easily enters the metabolic pathway through the gluconeogenesis process. Glycerol is easily metabolised by yeasts and is a cheap and convenient carbon source (as storage is simplified for liquid substrates). Indeed, glycerol was found to be an inducer for glycolipid production in other microorganisms. (Ortega

Ramirez, Kwan, and Li 2020) Morita et al. (Morita et al. 2007a) report that a titre of 16.3 $g.L^{-1}$ of MELs was obtained using intermittent feeds of glycerol. Liu et al. (Liu, Koh, and Ji 2011) used crude glycerol, a valuable by-product of the biodiesel industry, to achieve a yield of 0.134 and a product titre of 32.1 $g.L^{-1}$ after 8 days of fermentation. This crude glycerol is actually cheaper than the synthetic precursor of chemically-synthesized glycerol – epichlorohydrin, making the production of glycerol of petrochemical origin not profitable. Due to this fact, 90% of glycerol available on the market is of biological origin, making it a promising sustainable substrate. (Nicol, Marchand, and Lubitz 2012)

2.5.3.2 Hydrophobic substrates

Hydrophobic substrates used to produce MELs can be separated into two distinct groups – vegetable oils and carbohydrates. Facilitated by CAL-B, oils are hydrolysed into free fatty acids, which enter the chain-shortening pathway and are incorporated into the MEL molecule in the form of Acyl-CoA. Alkanes, however, are hydroxylated into alcohols prior to metabolization. As *M. antarcticus* successfully metabolizes alkanes into MELs, it is assumed it possesses an alkane monooxygenase in its enzymatic complex. (Kitamoto, Ikegami, et al. 2001) Van Beilen et al. (van Beilen, Wubbolts, and Witholt 1994) report that this step is rate-limiting for alkane-utilizing yeasts.

Production of MELs using vegetable oils gives very high product to substrate yields, ranging from 0.5 to 0.9 $g.g^{-1}$. (Adamczak and Bednarski 2000; Rau, Nguyen, Roeper, et al. 2005) Better results are obtained with multiple smaller feeds, while large individual feeds of vegetable oil should be avoided as they usually reduce yields and remain partially unmetabolized in the broth at the end of the fermentation.

The use of vegetable oils to produce MELs in an industrial scale is unsustainable for several reason. An unhealthy competition with the food sector can be created, threatening to drive vegetable oil prices up and hinder is availability of an important food source. Also, residual vegetable oil present in the broth at the end of the fermentation is difficult to separate from the product. When MELs are removed from the broth by extraction with ethyl acetate, vegetable oils and MELs are extracted together as they have similar polarities. There are separation methods for removing lipids from MELs using multiple extractions using different solvents, but such separation protocols are complex and inefficient, increasing downstream costs and product losses. (Rau, La, et al. 2005)

Some of the highest MELs yield values reported for non-lipid substrates come from the use of alkanes as the main carbon source. Kitamoto et al. (Kitamoto, Ikegami, et al. 2001) report product to substrate yields of 0.87 $g.g^{-1}$ with *M. antarcticus* T-34 resting cells after 7 days of fermentation using intermittent feeds of short and medium chain alkanes. However, four weeks of fermentation were needed to obtain high MELs titres of 140 $g.L^{-1}$, probably due to previously mentioned rate limitations for alkane consumption. Although these studies are of interest for developing in-situ bioremediation processes, the use of alkanes as a carbon source is extremely unsustainable as they are a non-renewable, usually obtained from petroleum crude and thus are unfit to produce MELs in a larger scale.

High MELs yields were obtained using vegetable oil derivatives, such as biodiesel. Rau et al. (Rau, Nguyen, Schulz, et al. 2005) obtained yields of ~0.5 $g.g^{-1}$ using rapeseed oil methyl esters. As this biofuel can be produced from lipid-based waste materials, it poses as a more promising substrate compared to vegetable oils in terms of its sustainability.

2.5.3.3 Complex substrates – biomass and waste materials

Most biological waste, including agricultural and industrial waste, has a significant content of compounds which not only act as a simple carbon sources, but contains valuable nutrients which could be used in bioproduction. These materials have often a very low direct value, and their management (collection, transportation and processing) is an economic burden to the producers. (Obi, Ugwuishiwu, and Nwakaire 2016) Improper waste management and accumulation in the environment of biological waste is also a great cause of pollution. Their introduction in fragile ecosystems can affect biodiversity and cause proliferation of certain species, as is the case with eutrophication. (Dodds and Smith 2016) Moreover, as these wastes biodegrade, they release greenhouse gasses (CO_2, methane) and cause harm to the environment. Some of such waste materials and industrial by-products are already used as raw material for the production of biofuels, vitamins, antibiotics and other chemicals. (Sadh, Duhan, and Duhan 2018) However, the extent of their use does not match the rates at which they are produced and there is the need to find additional opportunities for their use within a logic of circular economy.

Carbohydrate-based complex substrates include those with free sugar, as well as polysaccharides. Morita et al. (T. Morita et al. 2011) report sugarcane juice as a promising carbon source for MELs production, supplemented with urea, albeit with low productivity. Coconut water, rich in sucrose and invertase, was successfully used to produce MELs in low titres of 3.85. (Madihalli, Sudhakar, and Doble 2020) Bhangale et al. (Bhangale et al. 2013) used honey waste in fermentation in a semi-synthetic medium. As for polysaccharide-based raw materials, reports from our group (Faria, M. Santos, et al. 2014) show that thermically treated and enzymatically hydrolysed wheat straw can be used as a substrate for MELs production. However, as was discussed previously, all sugar-based raw materials provided low titres of MELs, ranging from 2.5-12 $g.L^{-1}$.

As for lipid-based waste materials, they provide better results in terms of process performance. Niu et al. (Niu et al. 2019) produced MELs using waste cooking oil, which is produced in significant amounts in the food processing industry. Soap stock, a low-value waste material often used for biodiesel production, can be used for fermentations to produce MELs and facilitates fast generation of the biosurfactant due to high level of degradation of the lipids and significant presence of free fatty acids. (Dzięgielewska and Adamczak 2013a)

Results from our laboratory show that the combination of these two groups of carbon sources gives best results for MELs production. (Nascimento 2017) Efforts should be made to define feed strategies using the right balance of hydrophilic and hydrophobic waste materials as substrate. Medium optimization would ensure high yields and titres of MELs and would signify a step towards the development of a sustainable large-scale process for this biosurfactant.

2.5.4 Producing microorganisms

MELs are produced by microorganisms from the *Ustilaginaceae* family: yeasts of the *Moesziomyces* genus and the *Ustilago* smut fungi[d]. Moesziomyces yeasts taxonomy was adjusted in 2017 (Kruse et al. 2017), as there were discrepancies in scientific reports that used various names for the same species of yeasts. Thus, based on the phylogenetic data from this report, the standardized taxonomy for the yeasts used in this thesis is:

- *Moesziomyces antarcticus* – previously reported as *Pseudozyma antarctica, Candida antarctica, Vanrija antarctica*

[d] *Ustilago maydis*, a parasitic smut fungus growing on corn ears has been a part of the Central American cuisine since Aztec times, known under the Nahuatl name *Huitlacoche*. The fungus from the infected cobs is collected and prepared as a delicacy, with ritualistic and medicinal significance. Recent research is showing its nutraceutical potential, as it is a source of many bioactive compounds. The contribution of MELs in this dishes sensory and medicinal properties still remains to be proven. (Beas F. et al. 2011)

- *Moesziomyces bullatus* – previously reported as *Moesziomyces aphidis, Pseudozyma aphidis, Pseudozyma rugulosa*[e]

Moesziomyces species such as *M. antarcticus* (Kitamoto et al. 1990; Kitamoto, Ikegami, et al. 2001) and *M. bullatus* (Dzięgielewska and Adamczak 2013a) are the working microorganisms in most reports where significant yields of MELs is reported. These strains have strong productive potentials, can grow efficiently in submerse cultivation conditions and have strong extracellular enzymatic secretion potential.

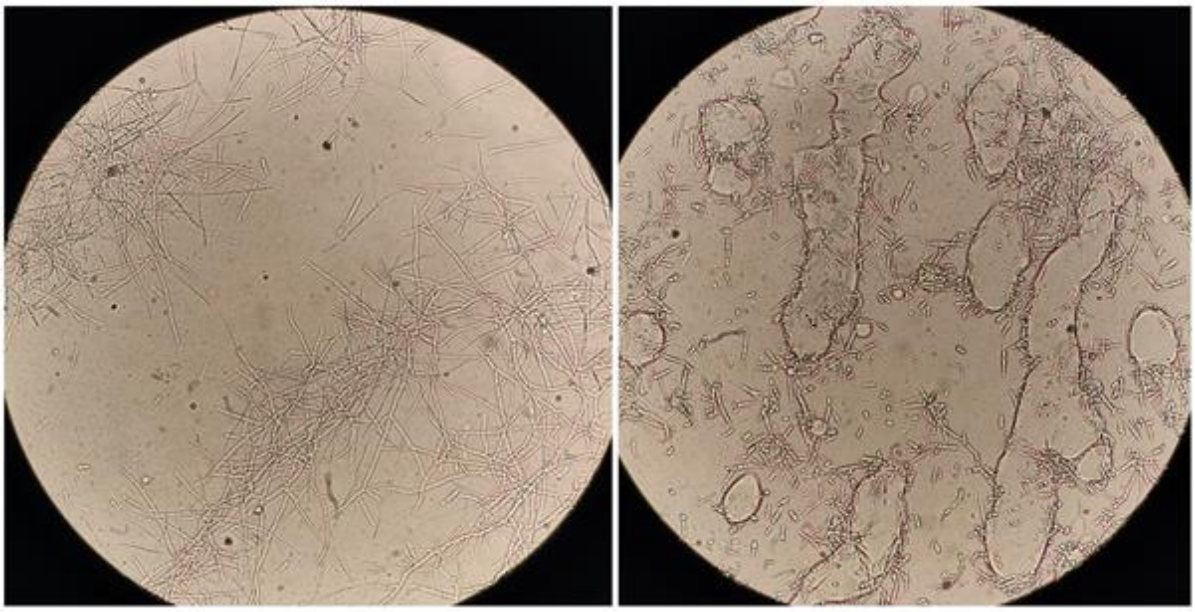

Figure 2.4 Optical microscope images of *M. antarcticus* PYCC 5048 (left) and *M. bullatus* PYCC 5535 (right) after 96h of growth. 500x magnification. Cultivation conditions: 40 g/l of glucose (for *M. antarcticus*); 40 g/l of glucose and 20 g/l of waste frying oil (for *M. bullatus*, oil droplets on the slide and lipid bodies within cells are visible)

Moesziomyces cells have variable shape: they can be fusoid, ovoidal, and cylindrical, as well as to present hyphae and pseudohyphae development. (Boekhout 2011) The microscopic images of the two strains used in this thesis (Figure 2.4) shows the differences in their

[e] Rarely, when these obsolete names are used in this thesis, it is to keep equivalency with literature sources regarding MELs production.

physiology. *M. antarcticus* cells more often form hyphae and pseudohyphae, while *M. bullatus* cells have smaller, oblong, and detached cells. Moesziomyces strains produce prominent internal lipid bodies, which were found to be used as an intracellular storage material and to contain mostly triacylglycerols and MELs. (Kitamoto, Nakane, et al. 1992) These lipid bodies are not only form when lipidic substrates are used, but also form in the absence of carbohydrates carbon sources. (Morita et al. 2007b)

M. antarcticus is the producer of a well know lipase CAL-B (*Candida antarctica* lipase B), with a wide range of reported applications. (Lou et al. 2008; Truppo and Hughes 2011). The enzyme participates in a range of lipid hydrolysis and esterification reactions (Subileau, Jan, and Dubreucq 2018), playing an integral part in MELs production when lipid-based substrates are used. Several commercial enzymatic products based on CAL-B exist, such as the immobilised lipase *Novozyme 435* (Ortiz et al. 2019).

The less studied lipase produced by the same microorganism, CAL-A, has great chemo-selectivity for amino acids and esters, which could make it relevant for their production. It is highly thermostable, showing activity up to 90° C (Domínguez de María et al. 2005). This enables its use in catalysis at higher temperatures compared to CAL-B. Indeed, CAL-A retains 100% activity at 70 ° C, while CAL-B loses 85% if its activity at the same temperature. (Kirk and Christensen 2002) However, CAL-A's activity seems to be highly calcium dependant. (Anderson, Larsson, and Kirk 1998) The role of CAL-A in MELs production is insufficiently explored. Most synthetic and semi-synthetic substrates used in literature sources do not include calcium. The presence of calcium in some raw materials used for MELs production, such as cheese whey (Dzięgielewska and Adamczak 2013a) and cassava wastewater (Andrade et al. 2017a), could have contributed to higher biosurfactant yields by facilitating substrate consumption upon CAL-A activity.

Faria et al. (Faria et al. 2015) reported on the *M. antarcticus* PYCC 5048^{T} and *M. bullatus* PYCC 5535^{T} strains abilities to metabolize lignocellulosic materials by producing xylanolytic enzymes, without cellulolytic enzymes present.

Some strains of *M. antarcticus* were reported (Shinozaki et al. 2013) to secrete cutinases that are able to degrade biodegradable plastics - poly(butylene succinate), poly(butylene succinate-co-adipate), poly(ε-caprolactone), and poly(lactic acid).

Considering typical quantitative metrics used to assess efficiency, such as yields, productivity and titres, yeast strains show superiority over the *Ustilago* fungi for MELs production. Still, there might me unexplored pathways of solid-state production using these microorganisms, which could make them relevant for industrial scale biosurfactant production. *Ustilago maydis*, one of the MELs producing strains (Liu et al. 2011), has been extensively cultivated in the solid state fermentation systems to produce itaconic acid, (Rafi et al. 2014) which is normally less applicable for yeast cultures. The advantages of solid state fermentations over submerged cultivation (easier aeration without foaming, lower operational costs) can be used to provide a potentially more sustainable process for MELs production in the future. (Costa et al. 2018; Mitchell et al. 2011)

Interestingly, another producing strain was discovered belonging to the yeast genus *Kurtzmanomyces* (Kakugawa et al. 2002), belonging to the family *Chionosphaeraceae*, indicating the possible existence of other undiscovered MELs producing microorganisms.

2.5.5 Production conditions and effectiveness

There is many reports providing details on MELs production in shake flasks. However, the number of studies reporting MELs production in Bioreactors, a requirement for industrial MELs production, is limited. Those works are presented on Table 2.1. by decreasing order of productivity values. Most fermentations are performed at similar temperatures, ranging from 25 to 30 °C. As for agitation and aeration, they were usually reported to be set up in a cascade, to maintain the DO (dissolved oxygen) in the broth at a fixed value. This ranges from 20% (Kim et al, 2006), up to 50% (Adamczak and Bednarski 2000). Agitation speeds range from 150 to 750 rpm, which should not present a problem for yeast cultures.

Table 2.1: Literature reports of MELs production in bioreactors

Feed strategy and working volume	MELs ($g.L^{-1}$)	Residual lipids ($g.L^{-1}$)	MELs purity (%)	Productivity ($g.L^{-1}.h^{-1}$)	Yield ($g_{MEL}.g_{substrate}^{-1}$)	Fermentation duration	Substrate	Source
Fed-batch; 3L/5L	120.5	n/a	n/a	0.63	n/a	10 days	Glucose, RSO FFA	(Dzięgielewska and Adamczak 2013b)
Fed-batch; 30L/72L	165	n/a	n/a	0.58	0.93[f]	12 days	Glucose, SBO	(Rau, Nguyen, Roeper, et al. 2005)
Fed-batch; 2L/5L	95	20	82	0.48	0.475	8.3 days	Glucose, SBO	(Kim et al. 2006)
Batch; 2L/5L	45	n/a	n/a	0.31	0.5625	6 days	SBO	(Adamczak and Bednarski 2000)
Fed-batch; 1.5L/2L	32	n/a	n/a	0.17	0.25	12 days	Crude glycerol	(Liu et al. 2011)
Fed-batch; 10L/15L	69	17% (w/w)	51	0.16	0.51	10.9 days	RSO	(Goossens et al. 2016)
Batch; 1L/5L	25.1	n/a	n/a	0.15	0.13	7 days	Sugarcane juice, urea	(T. Morita et al. 2011)
Batch; 3L/5L	1.26	n/a	n/a	n/a	n/a	3.5 days	Cassava wastewater	(Andrade et al. 2017a)

[f] The reason for this unreasonably high yield is that the authors did not consider in the calculations the soybean oil added for foam control.

A fed-batch strategy was used in most of these sources, with periodic additions of substrate. Rau et al. (Rau, Nguyen, Roeper, et al. 2005) report the highest titre of MELs in a bioreactor, reaching 165 g.L-1, with a very high productivity. However, the yield that is reported is unreasonably high, and upon a more careful examination of the report, it can be concluded that the SBO additions for foaming control were not considered for productivity calculations. Other sources (Adamczak and Bednarski 2000; Goossens et al. 2016; Kim et al. 2006) report that high titres were achieved using multiple feeds of vegetable oil.

Only a few reports give information on MELs produced from waste materials in bioreactors. Dzięliewska and Adamczak (Dzięgielewska and Adamczak 2013a) achieved very high titres of MELs using rapeseed oil, previously broken down into FFA using commercial lipases, supplemented with fresh biomass containing resting cells. However, the authors do not report yields obtained and residual unconsumed substrate. Morita et al. (T. Morita et al. 2011) successfully used sugarcane juice supplemented with urea to produce MEL-B.

Most of the studies, as it is evident from the information presented in Table 2.1, do not provide full information on MELs production efficiency. Most of the literature sources do not report substrate to MELs conversion efficiency, omitted the level of residual lipids presented on the end of the fermentation and the purity of the collected MELs. MELs titre, yield and/or productivity are usually reported. Those parameters give valuable information about the fermentation efficacy, but they are not enough to construct a more thorough mass balance needed for upscaling the process and planning production in an industrial scale.

2.5.6 Formation of MEL-rich beads

MELs have low water solubility due to the low polarity of the molecule. During fermentation, the MELs are positioned intracellularly, adsorbed to the cell surface, or form extracellular multimolecular organizations, such as micelles or lamellar structures. With the increase in biosurfactant concentration in the broth, MELs start to agglomerate, along with lipids, into beads of varying physical properties in accordance with their composition.

Only a few literature sources give this phenomenon significant attention and attempt to analyse their composition. Rau et al. (Rau, Nguyen, Roeper, et al. 2005) hypothesised that the beads presence indicated the fast progression of the fermentation and intensified MELs production, with the minimum concentration of MELs needed for their appearance to be 40 $g.L^{-1}$. In a fermentation with *M. bullatus*, the beads were found to contain 60% of MELs, with the remaining fraction containing lipids (free fatty acids and triacylglycerols). The same group (Rau, La, et al. 2005) explored the possibility of separating this MELs enriched phase by heating the broth, letting the beads sediment in a viscous liquid phase, and removing the aqueous phase by decantation.

More recently, Shen et al. (Shen et al. 2019), also in cultures using *M. bullatus*, noted that those beads are present, only small quantities of MELs remain in the broth (less than 2 $g.L^{-1}$). Goossens et al. (Goossens et al. 2016) found that after the addition of more lipid-based substrate, the beads dissolve, only to re-appear later in the fermentation.

Obviously, the appearance of these beads is not only dependant on the concentration of MELs, but it seems that other lipids also play a role in their formation. Fatty acids are always present in a significant fraction, while triacylglycerols have a disruptive effect, as their addition causes

bead disappearance. The beads were reported to have colour varying from yellow to brown, which is possibly related to their purity.

Conclusions made after research within our group found a correlation between the physical properties of MELs beads and their composition. Beads with higher purity were clear and usually had a darker orange/brown colour, while the presence of triacylglycerols caused the beads to have a yellowish-green hue. The bead colour would change as the fermentation progresses, oils hydrolysed and more MELs produced. A significant presence of triacylglycerols would result in floating beads, while their absence in their composition would cause the beads of higher purity to sediment. Beads with more contaminants, especially containing triacylglycerols, would be opaque, due to higher presence of incorporated biomass. Similarly, more pure beads tended to be larger in size, although their general shape and size also depended on the mode of mixing. For cultures in shake flasks and incubated in orbital shakers, late-stage fermentation broth would have a singular large bead (often several cm in diameter), while fermentations in bioreactors resulted in smaller beads (Ø2-5mm).

Interestingly, in most scientific reports regarding this phenomenon *M. bullatus* is used, although in-house better results for MELs production are usually obtained with *M. antarcticus*. One possible explanation is that there is a difference in surface properties of these cells, namely surface hydrophobicity. As it can be observed in Figure 2.4, M. bullatus forms smaller, individual cells. Cell differentiation into these small cells is in general related to lower surface hydrophobicity (Min, Neiman, and Konopka 2020), and it can be concluded that the lack of a hydrophobic surface onto which MELs could adsorb results in the formation of agglomerates of this significantly hydrophobic biosurfactant.

The formation of beads rich in MELs offers a potential for more efficient product harvesting, as, when those structures are present, the product is highly concentrated and contained within such fraction. Due to this, their processing – product extraction and purification, is simplified and has lower requirements for solvents and energy. Gaining knowledge about the underlying reasons for the formation of these beads would enable induction of their appearance, and a more sustainable process overall.

2.5.7 Downstream processing

The resulting broth obtained at the end of the fermentation includes a complex mixture of MELs, residual substrate (including non-metabolised lipids), biomass and enzymes, all suspended in water in a relatively low concentration. Several pathways for collecting MELs from the fermentation broth have been developed, with varying effectiveness and complexity. Most commonly MELs are removed from the broth by liquid-liquid extraction, using organic solvents such as tert-butyl methyl ester (MTBE), ethyl-acetate and hexane. Although single solvent extraction techniques enable the recovery and reuse of the solvent at the end of the process, they are non-selective and result in low product purity. This occurs due to similar polarity of the biosurfactant and residual lipids. While, obtained MELs/lipids mixture have low value, extra downstream process steps represent additional costs.

Rau et al. (Rau, La, et al. 2005) tested several downstream processing strategies to obtain MELs with high purity and low loss of product. Heating the crude MELs enabled their precipitation as a solid mass, resulting in a product with 87% (w/w) purity, with 7% of losses. As an alternative, a multi-solvent downstream technique was proposed. This process included multiple extractions using MTBE, methanol, n-hexane, cyclohexane, and water at varying

ratios and resulted in a product with 100% purity, albeit with 92% losses. Solvent loss and mixtures of solvents that require high energy costs for processing make this process unacceptable in an industrial setting, as it would significantly affect overall production costs.
Other downstream techniques include adsorption on resins (Rau, La, et al. 2005) and silica gel columns (Morita et al. 2007b), with the latter technique providing a 100% pure product with half of the MELs lost in the process.

Filtration seems to be a promising method of separation of MELs from impurities present in the broth. Although reports of this in literature are extremely scarce, Andrade et al. (Andrade et al. 2017) reported the use of ultrafiltration for separating the crude product from extracellular enzymes in the lyophilised foam collected from the bioreactor. However, filtration using membranes with smaller molecular weight cut off, i.e., nanofiltration, could be used to separate MELs from lipidic impurities based on molecular size, regardless the similar polarity of the two components that makes sustainable solvent extraction challenging. Nanofiltration could enable collection of the lipids in pure form, allowing to reuse them as a substrate in subsequent fermentations cycle.

Inefficient and costly downstream strategies are, according with some authors, the main driver of production costs for emerging bioproducts. (Campos et al. 2013) In such case, to develop those production process stage would overcome one of the major hurdles for cost reduction and large-scale of biosurfactants production and subsequent adoption of their use by many industries. In the case of rhamnolipids, a recent review claims that up to 80% of total production cost is allocated to downstream processing, as an economically convincing method was still not developed. (Sekhon Randhawa and Rahman 2014)
Research efforts should focus on simple extraction and purification techniques, which do not require the use of expensive materials or have a high energy cost. Use of solvent mixtures

should be avoided, as it hinders solvent reuse. Finally, effective methods of separating MELs from residual lipids would shift the focus of the process from obtaining high yields, favouring high titters instead. Such a purification technique, if sufficiently cheap, would result in a highly productive process for MELs, securing it position as a relevant player on the biosurfactant market.

2.5.8 Applications of MELs

To have a more sustainable process, novel applications that fit the biosurfactant particular features should be developed, as they will create value to the biosurfactant and encourage further efforts on process improvement. Bioproducts are often positioned as directly substitutes of the currently used chemical products, matching bioproducts and petroleum chemical driven 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.

As discussed before, the intended application can determine how the production process will be designed – in order to satisfy the desired properties of the product. In the case of MELs, these properties would include purity (as well as which types of impurities are acceptable), scale (whether the product is intended to be used as a bulk or specialty chemical), as well as the product price.

As an example, MELs intended to be used for cosmetic purposes might be acceptable if they would contain residual lipids, however solvent presence would be detrimental to the quality of the product. Similarly, medical-grade MELs would require exhaustive downstream processing, the cost of which would be justified by the hefty price of such a product. Finally, when MELs are used as a bulk chemical, as is the case with bioremediation, it would have a lower price and less strict purity requirements, dictating which unit operations could be reasonably used in the production process.

In most cases, many other biosurfactants of microbial origin with similar properties could be used as an alternative to MELs. However, one of the most significant properties that establishes MELs as advantageous is its extremely low critical micelle concentration (CMC). These values, as well as the value of surface tension at CMC, is presented in Table 2.2 for some biosurfactants.

Table 2.2 Critical micelle concentration and surface tension at CMC for some common biosurfactants

Biosurfactant	**CMC (M)**	**CMC ($g.L^{-1}$)**	**Surface tension (mN/m)**	**Reference**
MELs	3.6×10^{-6}	2.4×10^{-3}	25.3	(Morita et al. 2009b)
Trehalose lipids	5.9×10^{-6}	5.0×10^{-3}	28.6	(Tuleva et al. 2008)
Sophorolipids	9.91×10^{-5}	7.0×10^{-2}	40	(Hirata et al. 2009)
Surfactin	3.38×10^{-5}	3.5×10^{-2}	28.3	(Hirata et al. 2009)
Rhamnolipids	4.62×10^{-5}	3.0×10^{-2}	30	(Costa et al. 2010)

Some reports for MELs applications include experimental results, tested *in vivo* or in realistic scenarios. Other reports give result of experiments where MELs application was tested *in vitro* or in a simulated context, or specific property of MELs is quantified, and then a suggested application is implied based on these findings, with further testing needed. The latter case is more common, and this is understandable, as for some specific applications the economic and logistical requirements needed for obtaining results of the intended performance of MELs are too high, or out of scope for scientific groups with limited resources. In order not to exclude these valuable early-stage observations, these "implied" applications will be as well discussed in the following pages.

2.5.8.1 Cosmetic and medical applications

Due to MELs lack of toxicity and positive interaction with the human body, many cosmetic and medical applications were proposed. For their beneficial interactions with skin and hair, several applications of MELs in the field of cosmetics are reported. Yamamoto et al. (Yamamoto et al. 2012) tested MELs on participant's skin, and found it had great moisturizing effects, while Choi (CHOI 2018) patented a ceramide-based skin care product containing MELs in its formulation indented for reducing skin wrinkling. Bae et al. (Bae et al. 2019) observed that MELs inhibit melanogenesis in human melanocytes and a skin-equivalent, opening the possibility of the development of a skin-whitening product, while Bae et al. (Bae et al. 2019) report MELs potential to be used for treating UVA irradiation damage to skin, based on in-vitro tests. Two reports from groups of scientists lead by Morita T. (Morita, Kitagawa, Yamamoto, Sogabe, et al. 2010; Morita, Kitagawa, Yamamoto, Suzuki, et al. 2010) tested MELs applications in the field of trichology, its interaction with hair and hair-growth cells. They report *in vivo* results of

MELs potential use for hair damage repair, as well as stimulation of fibroblasts and papilla cells, critical elements of hair development.

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 reports (Fan et al. 2016; Isoda et al. 1997; Zhao et al. 2001) state MELs possible anticancerogenic applications, based on their ability to damage cancer cells and cause their differentiation. Isoda et al. (Isoda et al. 1999) report that MELs induce neurite outgrowth, opening the possibility of applications for neural damage repair. Morita et al. (Y. 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). Finally, MELs were observed to prevent biofilm formation, with possible use for medical implant and equipment treatment. (Ceresa et al. 2020) Due to its interactions with Ca^{2+} and MAP kinases, MELs were proposed to be applied in dentistry, in preventing inflammation which can lead to necrosis of dental pulp. (Patil, Ishrat, and Chaurasia 2021)

Due to the complexity and pre-requirements needed for *in vivo* tests for medical applications, only one of the reports in medical applications for MELs 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.

2.5.8.2 Agricultural and other 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* f. sp. *tritici* strain T-10 for some plants with hydrophobic leaf surfaces (Yoshida et al. 2015). Finally, crude MELs extract was tested for its ability to form nanoparticles with silver, which proved to have activity against mosquito larvae and pupae. (Ga'al et al. 2021)

Other applications were proposed based on some of the many specific properties MELs have. 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, Ikegami, et al. 2001) MELs have possible applications in the petrochemical industry, and showed to be a promising agent for Enhanced Oil Recovery (EOR), especially due to maintaining stability and activity under extreme temperatures and pH values. (Andrade, C. J. ; Barros, F. F. C. ; Pastore 2015) This property was used to show that MELs were able to prevent ice particle growth, making MELs a promising ice agglomeration control agent. (Kitamoto, Yanagishita, et al. 2001)

The diversity of applications in which MELs excel can indicate that there are many more undiscovered opportunities for this biosurfactant to enter the market. Focus should be put on MELs exceptional properties which give them an advantage over chemical agents and other biosurfactant. All this opens possibilities of MELs use not only as a substitute for existing

agents, but the development of novel ways where multiple features of this biomolecule can be utilized simultaneously.

2.6 Towards sustainable large-scale production of MELs

For a product based on MELs to be competitive on the market with other biosurfactants and with surfactants in general, MELs manufacturing costs need to drop significantly. To achieve such goal, several cost-driven factors within all MELs production stages need to be optimized. Namely, it would be critical to find novel low-cost substrates for MELs production, increase the efficiency of the bioconversion process itself and develop cheap and efficient downstream pathways for obtaining purified MELs. However, to ensure that these solutions will be viable in the long term in our changing world facing uncertainty in many aspects, sustainability has to be considered. The process has to 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 final product formulation itself should have low ecotoxicity should 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 lucrative. 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 has to be developed in a socially responsible way. The downstream treatment of the product should be in line with specific requirements for various applications depending on societal needs; and strive to achieving a final product which is safe for human health. Importantly, substrate selection should take into consideration resource security and avoid unethical competition with the food sector. 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.

Figure 2.5 illustrates the relevant parameters which would need to be improved to achieve a sustainable large-scale production process for MELs. It is highlighted how each of these parameters influences the economic, environmental, or social aspect of sustainability.

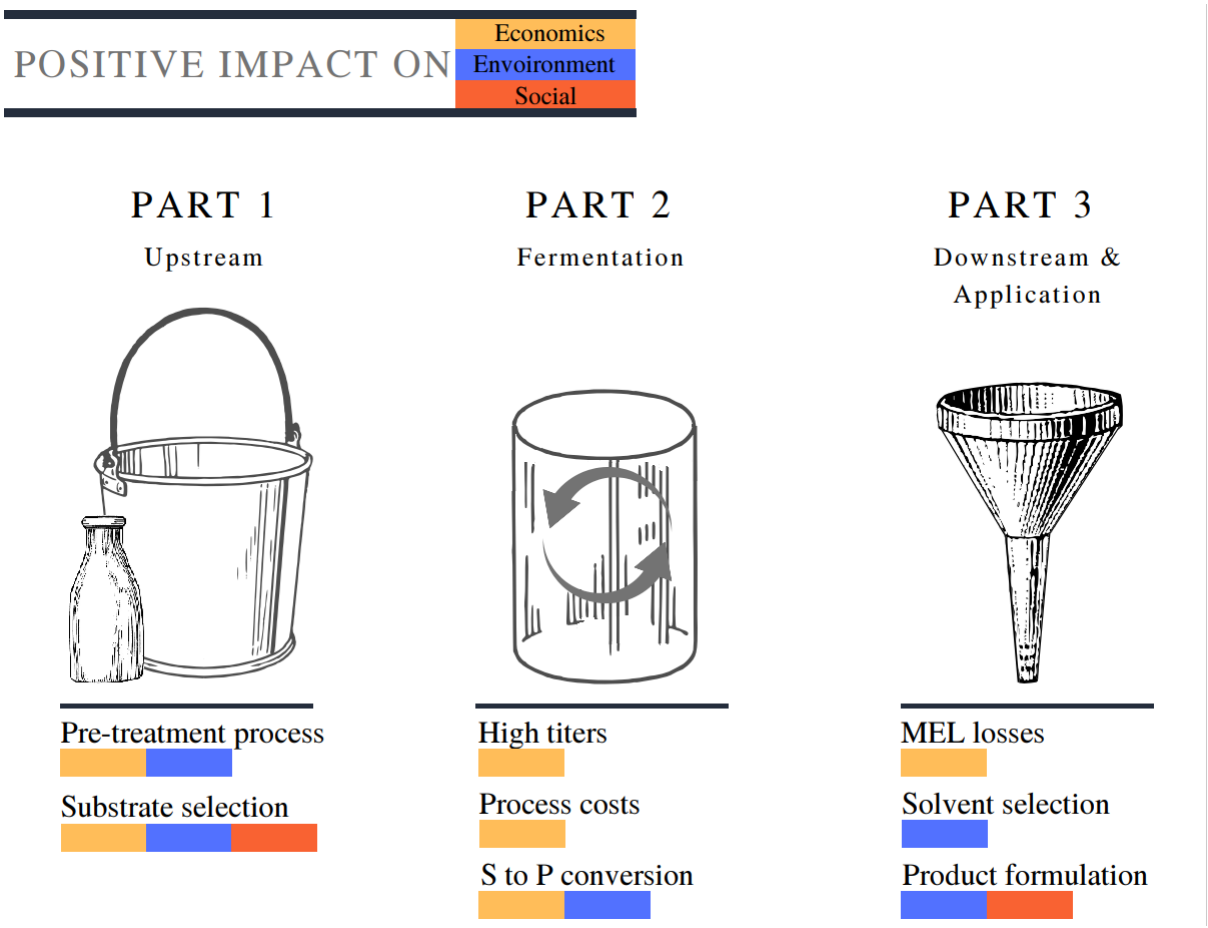

Figure 2.5 Overview of improvements needed for large-scale sustainable production of MELs, with overview of each of their impacts on distinct aspects of sustainability

Finally, the key parameter defining the economy of the process in all of its segments is the intended application of MELs, which creates value to the product. For high-end applications (medical, cosmetic), where lesser amounts of MELs of great purity are needed, production costs and downstream processing expenses can justify higher production costs. The priorities for process development in this scenario shift from reaching high titres and using cheap substrates, towards avoiding residual substrates and reaching high MELs purity. 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 designing a process of high productivity and low energy and mass intensity. 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.

Many pathways towards a large-scale MELs production process are possible, including the use of various substrates, microorganisms, and downstream processing operations. However, the development of novel applications should be performed in parallel with efforts to develop the production process. The decision on final product purity depends on the nature of the intended application and will affect most of the decision-making when designing the production process. Furthermore, the way the MELs would be used, as a bulk chemical or a specialty agent used in relatively small quantities, would determine the economic value of the product. This would affect the selection of substrate and downstream processing technique, with the ultimate goal of developing a fully sustainable MELs production pathway.

2.6.1 Disparity of analytical methods and data representation in literature sources

Different analytical methods are used for quantification and characterization of MEL. The discrepancies between such methods accuracy and the common omission of data, critical to obtain a clear picture of process efficiency, prevents a proper comparison between different process options reported on the literature.

The different analytical methods developed to characterize MELs vary in complexity and precision. High pressure liquid chromatography, with Evaporative Light Scattering Detectors (HPLC-ELSD) can quantify MELs directly, and distinguish between different MELs types. (Goossens et al. 2016) This method gives the most complete information on MELs concentrations and provides data on residual hydrophobic nutrients.

Alternatively, the fatty acids composing the hydrophobic moiety of the molecule can be esterified with methanol and analysed with gas chromatography. This method relies on the fact that MELs molecules contain short fatty acid chains (C8-C14), while most hydrophobic carbon sources contain longer fatty acid chains (> C16). (Faria, M. Santos, et al. 2014) Still, as some working strains produce MELs with longer fatty acid chains, along with the possibility that certain fatty acids can be present, without being incorporated in the substrate or the product, makes this method limited to some yeast strains. Despite the availability of the previously mentioned methods for MELs characterization, many literature sources report data obtained by unreliable measurements of MELs concentration.

The Anthrone method is a well-studied spectrophotometric method sometimes used for determining glycolipid concentrations, including MELs. (Hodge and Hofreiter 1962) This method rely on the reaction between the anthrone reagent and the carbohydrate moiety of the biosurfactant, resulting in a measurable coloration. This method, in spite of being used in scientific reports for determining MELs levels in the broth (Kim et al. 2006; Madihalli et al. 2020), is flawed due to its imprecision. Namely, not only responds positively to other carbohydrates present in the broth react (originating from the substrate or of metabolic origin), but also for other glycolipids and poly-carbohydrates, leading to overestimation of MELs (Loewus 1952), and incoherent mass balances of the bioconversion process.

Another unreliable method often used is the gravimetrical method, which relies on performing a liquid-liquid extraction with the broth, washing the extract with various solvents, drying it, and measuring its mass. In these reports an often groundless assumption is made that the total collected mass consists solely of MELs. (Adamczak and Bednarski 2000; Dzięgielewska and Adamczak 2013a) The result is an unconvincingly high yield, and, again, the inability to construct a sound mass balance of the process.

Finally, complete information regarding the fermentation efficiency is rarely presented. This would 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 impairs a reliable and critically comparison between different studies and production approaches.

This issue is especially prominent in sources reporting production of MELs at the bioreactor scale. Only 25% of the studies presented in Table 2.1 report the amount of residual substrate that was not used and remain at the end of the fermentation. This information enables us to have an insight into final product purity, process effectiveness and potential downstream processing difficulties - all important aspect of the process.

In the joint effort to develop a sustainable industrial process, transparent, reliable, and comprehensive data representation should be imperative.

This lack of stringency in literature regarding production of biosurfactants in general is recognised by Twigg et al. (Twigg et al. 2021), who called for increased scrutiny in reporting. The authors criticize the use of unreliable analytical methods, referring to issues with the orcinol and anthrone colorimetric assays, and the imprecise, confusing, and misleading use of process-defining terms (titre and yield; productivity and volumetric productivity; purity and uniformity).

2.6.2 Substrate selection for sustainable production of MELs

The first step in developing 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, 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 effects on the MELs producing microorganism metabolism, other matters affect the decision-making regarding the selection of an adequate substrate.

As it is evident from the previously discussed metabolic pathways for MELs production, both hydrophilic and hydrophobic carbon sources can be used as substrates. However, the selection of the substrate to 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 would satisfy most of the following criteria:

- Metabolic compatibility – the substrate should be easily consumed by the microorganism, with the eventual application of simple pretreatment processes.
- Favourable for MELs production - it should enter the metabolic pathway in a way which optimally prompts MELs production and cell growth/maintenance.
- Non-inhibitory in higher concentrations - to enable a simplified feed strategy and achieve high MELs titres, the cells should not be affected negatively by the presence of the substrate in a higher concentration.
- Exhaustive – it should not merely be a carbon source, but should be complex and provide more nutrients (minerals, growth factors, etc) to reduce the need for addition of mineral salts and reliance on supplementary substrates to a minimum
- Economically profitable - The cost of the substrate should not affect drastically the overall costs of production.

- Non-competing with food markets - for economic and strategic reasons, the raw material should be neither used for food or feed production.
- Ubiquitous - the substrate should be widely available, with possibility of production on various soils and in different climates.
- Renewable - to achieve high process sustainability, the substrate should be fully renewable, with a minimal carbon footprint.
- Low ecological impact - substrate production and pretreatment should not generate toxic waste harmful for the environment or require consumption of copious amounts of fresh water and energy, as well as unsustainable chemicals. Also, preferable substrates are the ones that are undesirable by-product or waste from another industrial process.
- Non-seasonal - the raw material should be obtainable throughout the year, to achieve higher production efficiency. Alternatively, it should be non-perishable and easily stored for extended periods of time.

As no single substrate would satisfy all these criteria, trade-offs will be made considering the different advantages of using one substrate over another. Such decision-making process should be done without compromising the overall sustainability of the process. Recently there has been an active effort to update many of the existing well-developed biotechnological processes towards the use of alternative substrates, with the aim of increasing overall process sustainability. The use of low value feedstock can reduce upstream costs and avoid unwanted competition with the food supply market. Those usually include biomass-based residues (agricultural waste, wood processing shavings, or forest residues), or unwanted by-products and intermediate goods from various industries (crude glycerol, cheese whey, paper pulp, or sugarcane bagasse and molasses).

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 has been reported (see section 2.5.3.3). Interestingly, some of the highest productivities reported come from fermentations with residue-based substrates, as previously discussed (Section 2.5.3.3).

2.6.3 Considerations regarding upscaling MELs production in bioreactors

To achieve sufficient high yields, the bioconversion step of the process require high oxygen supply, which in bioreactors is achieved thorough mixing and intense aeration. 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 (Kim et al. 2006; Rau, Nguyen, Roeper, et al. 2005) state that vegetable oil addition was used in the first days of fermentation to avoid foam formation. In some cases, these feeds are overlooked, and not considered when calculating the mass balance and product to substrate yields. As the foam fraction contains significant amounts of MELs, harvesting MELs from the generated foam can present an opportunity to efficiently recover MELs from the system. Andrade et al. (Andrade et al. 2017a) report that foam created during fermentation was collected and processed, which proved to be an effective way to collect MELs.

Other strategies to control excessive foam formation successfully evaluated, such as the addition of synthetic foam-controlling agents or the use of oxygen-enriched air. However, the use of petrochemical-based compounds which remain in the effluents or product tend to decrease the overall sustainability of the process, driving up the process costs and have the risk to contaminate final product. Overall, focus should be put on using feed strategies that include

addition of lipid-based substrates in key time points when foam formation is expected or exploring alternative mixing and aeration methods and innovative bioreactor setups.

Chapter 3

3 Towards Mannosylerythritol lipids (MELs) for bioremediation: Effects of NaCl on *M. antarcticus* physiology and biosurfactant and lipid production; Ecotoxicity of MELs

3.1 Abstract

Mannosylerythritol lipids (MELs) are a group of biosurfactants with a wide range of potential applications, due to their excellent tensioactive properties, biocompatibility, and biodegradability. One of the envisioned uses for MELs is for bioremediation in marine and terrestrial environments. However, knowledge regarding their ecotoxicity is limited. Ultimately, current costs of production for this biosurfactant are too high to make it competitive in the surfactant market. In an effort to facilitate the use of MELs for marine bioremediation purposes, production using sea water in medium formulation was explored. Cells were exposed to various levels of NaCl during fermentation, and the effects of increased salinity on the cells and their performance was monitored. Also, cells were briefly exposed to an osmotic shock by introducing pure NaCl into the broth, to understand their physiological response. Concentration of NaCl of 10% (w/w) was found to inhibit growth completely, while the culture kept some productive capacities at lover salinity levels. Although the overall effect of NaCl in the medium was negative, cells produced more lipases in these stress conditions. Furthermore, the changes triggered by the osmotic shock caused changes in cell surface and affected their hydrophobicity, reducing levels of MELs adsorbed to the cells, which in turn led to an increase in formation of MELs-rich beads. Marine-level salinity (3.5%) was found to be sufficient to enable production of MELs in unsterile conditions and inhibited an introduced bacterial contaminant. Finally, toxicity levels of MELs to a model marine organism were lower than other biosurfactants, especially in supernatant form.

3.2 Introduction

Moesziomyces antarcticus (former *Candida antarctica* and *Pseudozyma antarctica*) is a yeast strain well known as a Mannosylerythritol lipids (MELs) producer. MELs are an amphiphilic molecule and a potent biosurfactant, with a wide array of possible industrial applications ranging from cleaning and bioremediation (Marchant and Banat 2012), to cosmetic and therapeutic (Rodrigues et al. 2006). Lowering cultivation and downstream costs is still needed to enable large-scale production of MELs.

Moesziomyces antarcticus is also a potent producer of extracellular enzymes, mainly lipases. The main lipase produced is *Candida antarctica* lipase B (CAL-B), which has a crucial industrial role in hydrolysis of lipidic materials (Velonia et al. 2005). The lipase production by the yeast is crucial for the consolidated production of MELs from lipid-based substrates.

Biotechnological production has a high consumption of fresh water. Sea water is, however, an abundant resource, which could be utilized in biotechnological production (it is used already for algae cultivation), if the 3.5% of salt present in the medium, as well as marine microbiological contaminants, do not affect the working microorganism's performance. One of the main issues with this concept is that sea water negatively effects stainless steel bioreactors, by corroding the surfaces in contact with water over time. (Compere Chantal 1997) Alternative materials for bioreactor production should be considered in designing the equipment in a production line of this kind.

In this study, the ability of *M. antarcticus* to produce lipases and MELs in media with NaCl present in the growth medium is estimated, as this could contribute to the use of sea water in the production process, thus increasing the overall sustainability of the process. NaCl, sea water or wastewater with high salinity (such as that generated in the process of producing drinking

water from seawater by reverse osmosis and some food processing facilities) could be also used as a basis for the growth medium.

Furthermore, this would possibly enable non-sterile fermentation conditions to be used, as the osmotic stress could deter most common contaminants in the fermentation step, from the Escherichia and Bacillus genera, which lack the ability to survive in hypertonic conditions. (Carlucci and Pramer 1959)

The effect of sodium chloride in various concentrations on yeasts is studied and documented in detail in literature. Decades of research on this topic focused mostly on baker's yeast (*Saccharomyces cerevisiae*), as well as infectious species, such as those of *Candida*, *Cryptococcus*, etc. For these genera, literature sources report that NaCl causes physical changes as a result of the yeasts reaction to the stressor. NaCl is commonly used to induce an osmotic stress response, activating a mitogen-activated protein (MAP) kinase cascade, the high-osmolarity glycerol (HOG) pathway, as an effort of the cell to equalize the transmembrane osmotic pressure (Hohmann 2002b). This effect is fast and fully reversible, meaning that the cell can facilitate release and metabolization of unneeded glycerol in of the cell after adaptation or further change in the medium.

The aim of this work was to explore a new perspective for MELs production process to compete with other industrially produced biosurfactants. The overall goal was to stimulate beneficial physical changes in the microorganism, which were reported for other yeasts, to simplify downstream processing, decrease the needs for fresh water during production, as well as to enable growth and MELs production in non-sterile conditions. To fully study the interactions of *M. antarcticus* and NaCl, and to study the physiological effects it has on the microorganism, both short exposures to osmotic stress (addition of pure NaCl), and constant stress (with NaCl present in the media from the very beginning of the fermentation) were examined.

The continuous extensive use of petroleum products results in their accidental release in marine and terrestrial ecosystems during their use and transportation. (Zhang et al. 2019) Their presence causes extensive environmental harm, and a search for alternative to invasive remediation efforts turned towards more eco-friendly alternatives. Bioremediation of oil spills is performed by introducing microorganisms that are able to degrade the spilled hydrocarbons, as well as chemicals of biological origin which facilitate their natural degradation. (Prince and Atlas 2005) *M. antarcticus* is known to metabolize hydrocarbons (Kitamoto, Ikegami, et al. 2001), and MELs were suggested as a potential bioremediation chemical which accelerates petroleum degradation by marine microorganisms. (Yu et al. 2015) With this in mind, the ability of *M. antarcticus* to metabolize hydrocarbons and crude oil into MELs was evaluated. Before being applied in the field of bioremediation, the potential toxic effect of MELs on living organisms must be evaluated. In this work, MELs ecotoxicity are evaluated for the first time, by testing its inhibitory effects on the marine organism used as a marine ecotoxicity model, *Artemia franciscana*.

3.3 Materials and methods

3.3.1 Microorganisms and maintenance

Moesziomyces yeast strain *M. antarcticus PYCC 5048^T* (CBS 5955) was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal, and maintained following a previously established protocol (Santos et al. 2019). Strains were plated on YMA (yeast extract (t.a., Oxoid LTD) 3 g L^{-1}, malt extract (t.a., Oxoid LTD) 3 g L^{-1}, peptone (t.a., BDH) 5 g L^{-1}, D-glucose (p.a., Fisher Chemicals) 10 g L^{-1} and agar (JMVP) 20 g L^{-1}) and incubated for 3 days at 30 °C. Cultures were kept at 4 °C and renewed every week and stored at −80 °C in 20% (w/v) glycerol (≥99.5%, JMGdS) to be recovered when necessary.

Escherichia strain *E. coli DH5-α* was used to induce contamination when testing the ability of *M. antarcticus* to grow in non-sterile conditions. A tube with 5 ml of Luria-Bertani broth (Nzytech) was inoculated with 10 µL of cryopreserved stock (with 17.5% glycerol), containing approx. $5x10^8$ cells/ml. The tubes were incubated for 24 h, at 37 °C and 250 rpm. A sample amount containing approx. $1x10^5$ of cells was used to simulate contamination. This strain was acquired from the iBB laboratory strain collection.

3.3.2 Media and cultivation conditions

Erlenmeyer flasks were used for preparation of inoculum and batch fermentation. The previously described media and conditions were used (Santos et al. 2019). Inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of medium containing 3 g L^{-1} $NaNO_3$ (p.a., PanReac AppliChem), 0.3 g L^{-1} $MgSO_4$ ((≥99.5%, Panreac AppliChem), 0.3 g L^{-1} KH_2PO_4 ((≥99.5%, Chem-Lab NV), 1 g L^{-1} yeast extract, 40 g L^{-1} D-glucose, and incubated at 27 °C, 250 rpm, for 48 h (orbital incubator, AraLab). Batch cultivations were performed in Erlenmeyer flasks containing 1/5 working volume of mineral (0.3 g L^{-1} $MgSO_4$, 0.3 g L^{-1} KH_2PO_4, 1 g L^{-1} yeast extract) supplemented with 40 g L^{-1} D-glucose. The experiment started by transferring 10% (v/v) inoculum, corresponding to approx. 0.6 g L^{-1} of cell dry weight (CDW), followed by incubation at 27 °C, at 250 rpm. with the addition of 20 g.L^{-1} of soybean oil (refined, OliSoja) on day 4.

In certain conditions, NaCl (≥99.5%, Sigma-Aldrich) was added to the media as a stress agent, initially or subsequently, in amounts of 1%, 3.5%, 5%, 7.5% and 10% (w/w). Periodical samples were collected to quantify biomass (CDW), monosaccharides, lipids, MELs, as well as protein content and lipase activity.

For tests with hydrocarbons, light crude oil was used, obtained from Troll field, Norway, kindly provided by SINTEF as well as a hydrocarbons mixture of n-dodecane, n-tetradecane, and n-hexadecane (≥99.0%, Arcos Organics) in equal mass ratios. Hydrocarbon substrates were added in 5 $g.L^{-1}$ concentration on day 4, into the culture grown on the previously described media with an initial addition of 40 $g.L^{-1}$ of glucose. These tests were performed with fresh water (0% NaCl) and marine-level saline water (3.5%).

3.3.3 Biomass content and viable cell determination

Cell growth was quantified by cell dry weight (CDW), using a previously described protocol. (Santos et al. 2019) CDW was determined from 1 mL culture broth by centrifugation at 10 000 rpm for 10 min (Sartorius 1-15P centrifuge), washing with deionized water (twice) and drying at 60 °C for 48 h. Supernatant from the centrifuged samples was collected and used for various analyses.

To determine the viable cells content, samples were diluted and plated on YMA, and formed colonies were counted.

3.3.4 Exposure to NaCl as stress agent, and release of intracellular glycerol

When indicated, cells were cultivated with no NaCl present, which was added later in order to cause osmotic stress in the established cultures of *M. antarcticus*. For these experiments, 5 ml samples of fermentation broth were placed in test tubes with pure NaCl (ITW Reagents, 99.5% purity), using concentrations (w/v) of 1%, 3.5%, 5%, and 7.5%, and slowly mixed on a rotating mixer at 12 rpm (IKA Loopster mixer), in order to prevent physiological damage to the culture, for 30 and 120 min. In order to release intracellular glycerol, a method reported in literature

was applied (Petelenz-Kurdziel et al. 2013). Namely, after the required time of exposure to NaCl, tubes were placed in boiling water for 10 min, centrifuged for 3 min at 4000 rpm (Heraeus Sepatech Labofuge 200 centrifuge), to remove solid cell debris, and the supernatant was used for analysis.

3.3.5 Analysis of fermentable sugars, nitrates, and glycerol concentrations

In the collected sample supernatants, monosaccharides, nitrates, and glycerol were quantified in a high-performance liquid chromatography (HPLC) system (VWR Hitachi, Darmstadt, Germany) equipped with a RI detector (L-2490, VWR Hitachi, Darmstadt, Germany), UV-detector (L-2420, VWR Hitachi, Darmstadt, Germany) and a Rezex^{TM} RHM-Monosaccharide H+ (8%) column (300 mm × 7.8 mm, Phenomenex), at 65 °C. Milli-Q water was used as mobile phase at 0.5 mL.min^{-1}.

3.3.6 Analysis of MELs and Lipid concentrations

MELs concentrations in the samples were calculated based on the results acquired by Gas chromatography of methyl-esters, as previously described (Santos et al. 2019). The fatty-acid composition of biological samples was determined by methanolysis and GC-FID analysis of methyl esters. Pure methanol (20 mL) (HPLC grade, VWR Chemicals) was cooled down to 0 °C and 1 mL acetyl chloride (p.a., Sigma-Aldrich) was added to generate a water-free HCl/methanol solution. Culture broth samples (3 mL) were freeze-dried (lyophilizer, Martin Christ GmbH), weight and mixed with 2 mL HCl/methanol solution and incubated for 1 h at 80 °C (Memmert BM400 incubator) for transesterification into methyl esters. Heptanoic acid (p.a., Sigma-Aldrich) was used as internal standard. The resulting product was extracted with

hexane (1 mL) (HPLC grade, Fisher Chemicals) and 1 µL of the organic phase was injected in a GC system (Hewlett-Packard, HP5890), equipped with an FID detector and an Agilent HP Ultra2 capillary column (L 50 m × I.D. 0.32 mm, df 0.52 µm). The oven was programmed to an initial temperature of 140 °C and three temperature gradients were defined: 140 to 170 °C at 15 °C min^{-1}, 170 to 210 °C at 40 °C min^{-1} and 210 to 310 °C at 50 °C min^{-1}. A final time of 3 min at 310 °C was defined. Carrier gas was used with a split of 1/25. MEL were quantified through the amount of C8, C10 and C12 fatty acids 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 quantification of glycolipids based on a specific moiety was previously described by Faria et al. (Faria, M. V. Santos, et al. 2014)

All lipids and lipid derivatives (free fatty acids, mono-, di- and triacylglycerols) are represented as lipids in graphs.

3.3.7 Analysis of crude oil and hydrocarbon concentrations

To extract the hydrocarbons from the fermentation broth, n-hexane (≥95%, Fisher Chemical) was used. After 12 days of fermentation, 0.1 mL of pristane (10 $g.L^{-1}$ in n-hexane) (≥98.0%, Sigma-Aldrich) was added as an internal standard to quantify extraction losses. Then, the total content of the flask was transferred to a separation vessel and a total 50 mL of hexane was added. Another 25 mL of hexane was used to rinse each flask and then added to the vessel. The contents were mixed vigorously for 1 minute, left to separate and the top organic layer was removed. This hexane phase was evaporated on a rotavapor, corrected to 50 ml, and 1 ml sample was used for analysis.

Samples were analysed by gas chromatography (Hewlett-Packard, HP5890). Initial oven temperature was 60°C and held for 2 min. Temperature was then increased at a rate of 6°C/min

until reaching 310°C, where it was kept for 5 min. Injector and detector temperature was 300°C and 310°C, respectively. Purge was set to turn on after 0,66 min. Injected sample volume was 1 μL. Calibration curves were made for both crude oil and alkane mixture using a series of dilutions (5 $g.L^{-1}$, 2 $g.L^{-1}$, 1 $g.L^{-1}$, 0.5 $g.L^{-1}$, 0.2 $g.L^{-1}$ in n-hexane) with 5-α-androstane (HPLC grade, Sigma-Aldrich) as a secondary external standard. A relative response factor (RRF) was then calculated using Equation 3.1.

$$RRF = \frac{A_{std} \times C_{andr}}{A_{andr} \times C_{std}}$$

Equation 3.1 RRF equation for GC analysis of hydrocarbons

For the crude oil samples, automatic integration of peaks was used with a minimum area to height ratio of 1 from 6 min to 50 min of the GC-FID spectrum, from which the area of the internal standard peaks (5-α-androstane, pristane) was subtracted. For the alkane mixture, the three hydrocarbon areas were individually integrated. Based on the initial and measured concentrations of the standards, final values were corrected to account for losses in extraction and measurement errors.

3.3.8 Extracellular protein and lipase activity analysis

Extracellular protein content was assessed using The Thermo Scientific™ Pierce™ BCA Protein Assay, in a 96 well microplate (with absorption values measures using a microplate reader, MultiScan Go, Thermo Scientific), with albumin as standard. All runs were done in triplicates.

The enzymatic assays were performed based on a method acquired from literature (Gomes et al. 2011), in a 96 well microplate, with p-nitrophenyl butyrate (≥98%, Sigma-Aldrich) was as substrate. One unit (U) of lipase activity is defined as the amount of enzyme releasing 1 μmol p-nitrophenol per minute.

3.3.9 Ecotoxicity experimental setup and protocol

For testing marine organism ecotoxic effects of MELs, Artemia Toxicity Screening Test for Estuarine and Marine Waters ARTOXKIT M kit was used, following the standard protocol. (Artoxkit, 1990) In a 24-multiwell plate 10 spores were added in each plate, and incubated at 25°C for 30 h, with exposure to light with a minimum of 3000-4000 lux. The hatching starts after about 18-20 hours, and after 30 hours most of the larvae will have moulted into the desired instar II-III stage. The larvae are then transferred from the hatching medium to a rinsing well containing 1 ml of the test solution, thus exposing the larvae to the appropriate test solution before they enter the actual test well and minimizing dilution of the test solution during transfer. Then, ten larvae taken from the respective rinsing well are exposed in triplicate to 1 ml of each concentration of the test sample in the remaining wells. This bioassay design is based on one control and five increasing toxicant concentrations, each with 3 replicates of 10 animals. The incubation is carried out in the dark at 25 °C and after 24 hours the dead larvae in each test well are counted. In the end the % mortality and, for the definitive tests, the median lethal concentration (LC50) are calculated. Tests were performed in triplicate, with marine-level saline water (3.5% of NaCl).

To compare MELs to other biosurfactants, sophorolipids (provided by Holiferm, UK) and rhamnolipids (90% purity, Agae Technologies, US) were used.

3.4 Results and discussion

Moesziomyces antarcticus culture was exposed to various levels of NaCl, in different groups of experiments to observe and describe the various effects of saline stress on the microorganism. Results are grouped in four major sections, according to the experimental aim. In the first group of experiments, *M. antarcticus* was exposed to NaCl constantly during the whole fermentation. The goal of these experiments is to establish the level of tolerance to osmotic stress of *M. antarcticus*, and NaCl levels at which it is still able to produce MELs and CAL-B. This opens the possibility of using saltwater as a substitute for freshwater in the formulation of the growth medium.

The second group of experiments consists of short exposures to salt, to study the rapid changes in physiology of the culture, in order to gain knowledge that can be used in engineering alternative production processes.

In the third set of experiments, *M. antarcticus* was grown in various non-sterile conditions, in the presence of NaCl.

Finally, to enable the use of MELs for oil spill bioremediation in marine and terrestrial environments, production of MELs in the presence of NaCl with hydrocarbons as a carbon source was estimated, as well as ecotoxic (and phytotoxic, Appendix 10.2) effects of the biosurfactant.

3.4.1 Effects of continuous exposure to NaCl on *M. antarcticus*

To simplify the way in which this large set of data is presented, focus is given only on the relation of the data obtained with salt presence in the media compared to the blank cultures (with 0% NaCl). Thus, key values is presented in ratios (%), indicating how the corresponding level of NaCl in the medium deviated the values for each parameter compared to the blank, as seen in Figure 3.1, while the complete profiles over cultivation time are presented in the Appendix, Figure 10.1.

The results of this study of the effect of NaCl on *M. antarcticus* biomass growth (Figure 3.1) are consistent with to the effects reported in literature for *S. cerevisiae* (Wei, Tanner, and Malaney 1982), indicating that the increased levels of NaCl in the medium affect *M. antarcticus* by negatively affecting biomass development, and extending the lag-period. All cultures performed worse than the blank (with 0% NaCl) in terms of DCW, except the culture with 1% NaCl, which was better than the blank by roughly 20%.

The flasks containing 10% NaCl in the growth medium had no detectable changes in any of the measured parameters, and no observable growth of biomass occurred, meaning that the salt level was high enough to stop all metabolic activity of the cells added with the inoculum (as seen in Figure 3.2). This concentration of salt was not used for other experiments, and these results were thus excluded from the remaining part of the paper.

3.4.1.1 Effect on metabolism: extracellular proteins and lipase production, and free fatty acid (FFA) consumption

One of the main preconditions for MELs production is that there is a sufficient amount of active lipases. They have a key importance in MELs production, as they play a role in the consumption of lipid-based substrates, which is one of the major steps in the biotransformation chain towards MELs generation.

Results for extracellular protein and lipase activity (Figure 3.1), as well as the specific activity of the lipases calculated from these two values (Appendix, Figure 10.1), show some difference among the various growth conditions. Although increasing levels of salt in the media decreased the amount of extracellular protein produced, the activity of the lipases in these flasks was higher than the blank, with the cultures grown in presence of 3.5 and 5% NaCl performing over 40% better than the blank. This high lipase activity in flasks with less total protein resulted in a higher specific activity of lipases in flasks with higher levels of NaCl present in the media.

At first, these results could seem surprising, since growth and basic metabolic activity of the cells was hindered by salt presence in the media. However, it is common for yeast and fungal strains to react to stressors with stimulated extracellular protein secretion (Heilmann et al. 2013). Furthermore, osmotic stress causes a drop in gene expression for proteins which have a role in amino acid metabolism, cell wall maintenance, nucleosome structure, DNA synthesis, and nucleotide metabolism (Hohmann 2002b), as they play a key role in cell proliferation. In hyperosmotic stress conditions, the cells tend to promote production of osmoregulatory and other enzymes which can increase survival rates under such conditions.

This can be the explanation to the unexpectedly high lipase secretion. Although the overall metabolism of the strain might be affected, the increase of lipase production could be a cell response to the stress caused by increased osmolarity.

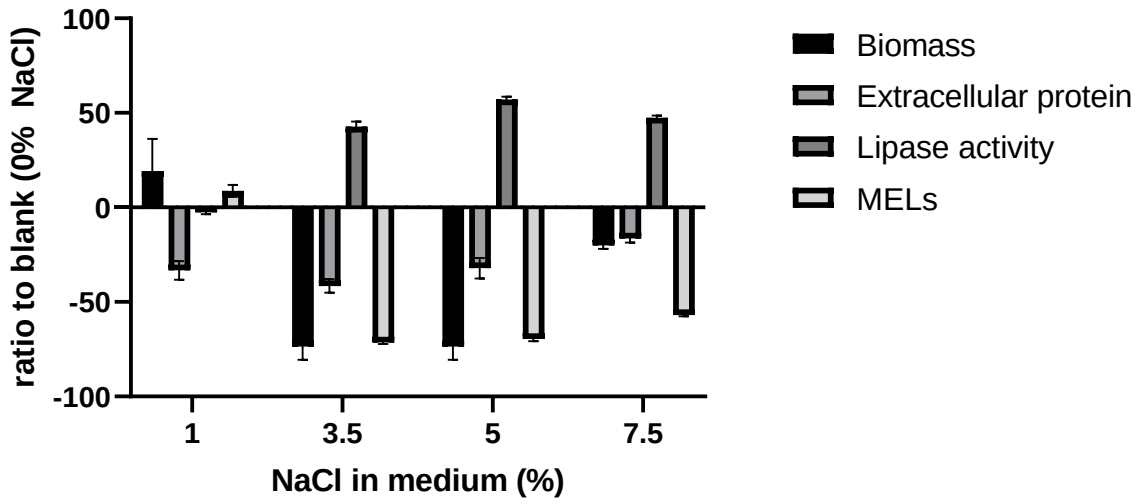

Figure 3.1: Values of relevant parameters in flasks containing various concentrations of NaCl on day 14, compared to the blank (with 0% NaCl)

This is additionally proven by data from figure showing free fatty acid profiles over time (Appendix, Figure 10.1). What can be observed is that, although 20 $g.L^{-1}$ of oil was added on day 4, cultures with higher NaCl concentrations in the medium had higher free fatty acid concentrations in samples collected in later days. This indicates that, although the metabolism of lipids was compromised, the hydrolytic capacities of the cell were still high, breaking down more lipids than they were able to consume.

Thus, the use of seawater or NaCl rich wastewater for CAL-B production seems to be a promising new production process, which would cut down freshwater consumption and the overall sustainability of the process.

3.4.1.2 Effect of NaCl in growth medium on MELs production

Finally, to determine whether production of MELs in an industrial scale with growth media containing salt is promising, we must examine NaCl effect on MELs production. Profiles of MELs produced by cells exposed to different levels of salt during growth (Appendix, Figure

10.1) show high amounts of MELs present in the liquid fraction of the collected cultivation broth for flasks with 0% and 1% of NaCl, with the latter over performing the blank by roughly 10%. Based on these values alone, it can be concluded that the cultivations with higher NaCl concentration underperformed significantly.

However, due to poor water solubility, MELs which are not adsorbed to cells has the tendency to form solid beads in the fermentation broth (Figure 3.2A), which prevents homogeneous sampling, and reliable results to be obtained from the liquid fraction of the broth alone. These beads can be harvested using a rough filtration step, for instance with a sieve. They are composed mainly of MELs, however significant content of free fatty acids and biomass is present. During a fermentation with increase titers of MELs, agglomerations form and eventually dissipate. Potential reasons for this will be examined in other chapters of this thesis.

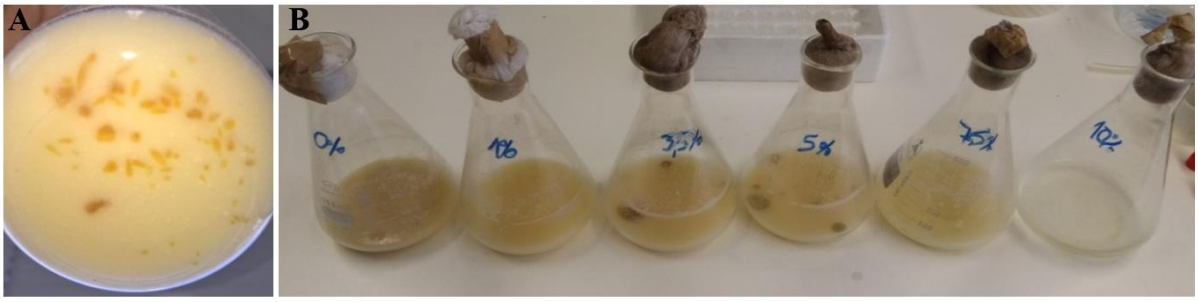

Figure 3.2: A - MEL-rich beads formed during later stages of fermentation; B - Flasks with different concentrations of NaCl in the fermentation media, after 14 days of fermentation (with the MELs beads present in flasks with 3.5%, 5% and 7.5%).

Flask with 3.5%, 5% and 7.5% contained significant amounts of solid MELs beads (as seen in Figure 3.2B). These beads were recovered using a metal strainer (opening diameter ~1 mm) and extracted with ethyl-acetate two times. MELs and lipid concentrations were then determined for the collected extracts, and their composition was analyzed (Figure 3.3).

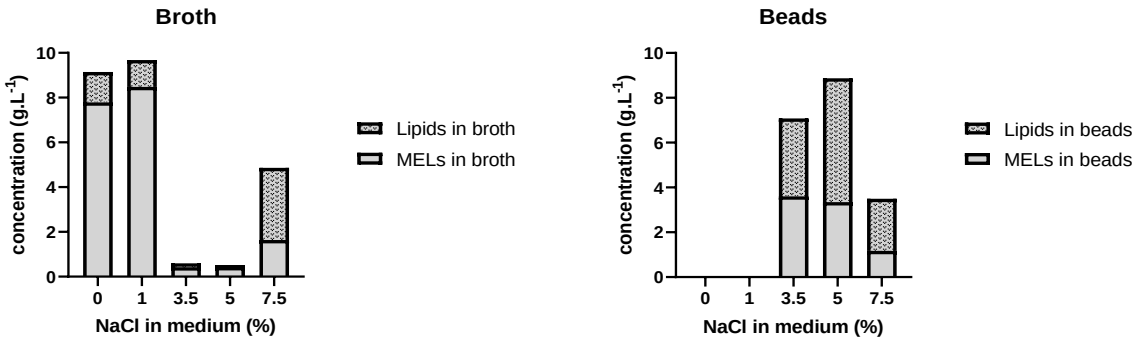

Figure 3.3: Distribution of MELs and fatty acids (FA) in the liquid (broth) and solid fraction (beads) collected from the flasks after 14 days of fermentation.

These results correspond to values for MELs profiles in cultures with various NaCl levels (Appendix, Figure 10.1), but some variance is present due to inhomogeneous sampling in the presence of solids.

Flasks with 0% and 1% of NaCl developed these MELs beads earlier, on day 8, and they disappeared on day 10. However, this bead appearance does not depend solely on the profile of MELs in the broth. Based on MELs and lipid profiles (Appendix, Figure 10.1), as well as data from Figure 3.3, it seems that free fatty acids play an integral part in the formation of these beads, as they are present in the beads in a significant quantity. Alternatively, due to cell surface changes due to aging, cells tend to have more MELs adsorbed on their surface in later stages of fermentation.

Table 3.1 Composition of MELs beads collected from fermentation flasks of conditions with 3.5, 5 and 7.5% (w/w) NaCl content in the media, at day 14.

Component (%)		NaCl content in media		
		3.5%	**5%**	**7.5%**
Wet content		39.7	23.6	38.8
Dry content *	**MEL**	27.6	34.5	56.8
	Fatty Acids	33.1	20.1	23.4
	Biomass**	39.3	45.4	34.5

* Dry content was expressed as dry mass ratio determined after drying the total pellet sample for 48h at 60 °C.

** All dry content components, excluding lipids, fatty acids, and MEL, were considered biomass, including cells and cell debris.

3.4.2 Intracellular glycerol accumulation by *M. antarcticus* as a response to hyperosmotic stress

Two main stress response mechanisms observed in *S. cerevisiae* and other yeasts are the increase of intracellular glycerol levels and tendency of cells to flocculate. A study of changes in intracellular glycerol levels as a reaction to osmotic stress caused by NaCl and sorbitol (another commonly used osmolyte) in *Saccharomyces cerevisiae* and *Pichia guillierondii* (Nasser and El-Moghaz 2010) reported continuous increase of glycerol levels for up to 40 hours after stress agent addition.

To test if *M. antarcticus* manifests these responses, a set of flasks were prepared, in order to be sacrificed at the end of the fermentation, and stress response could be quantified as a relative increase in intracellular glycerol concentration.

Intracellular glycerol concentration was measured after exposure of *M. antarcticus* to different amounts of salt for 30 and 120 min (Figure 3.4). Intracellular glycerol accumulation was higher in *M. antarcticus* samples exposed to higher concentrations of salt, while such accumulation ceases after some time, dropping significantly by the end of two hours of exposure. Either the cells start to adapt to the osmotic stress, recover, and release some of the produced glycerol, or the cells response mechanisms are exhausted and the high-osmolarity glycerol (HOG) pathway is reversed. The reaction was similar in all cases, with a glycerol spike in the first sample measured and a later drop, except for the flask with 1%, where cells responded slower, continuously accumulating glycerol over the course of 2 hours.

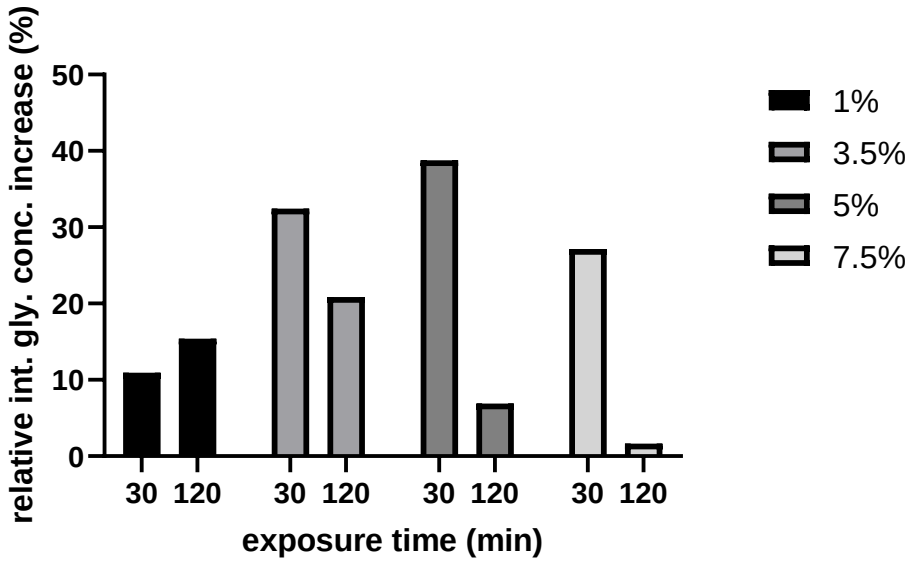

Figure 3.4: Increase of intracellular glycerol concentration for samples collected on day 14, which were exposed to 1%, 3.5%, 5%, 7.5% (w/w) of salt for 30 min and 2h, relative to initial concentration before exposure.

To test the difference between continuous exposure to NaCl, and brief stressor exposure, a set of flasks were incubated. The first group, the blanks, had no salt present in the media, while the others had 3.5% of NaCl. On certain days, flasks were collected and sacrificed, in order to determine intracellular glycerol concentration, increase relative to the blank (Figure 3.5).

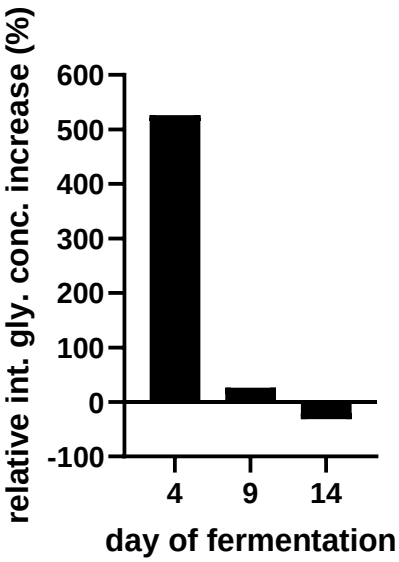

Figure 3.5: Change of intracellular glycerol concentration in flasks with 3.5% NaCl present in the media relative to blank (0% NaCl).

What can be observed is that, initially, the culture growing in the presence of NaCl had significantly higher intracellular glycerol concentrations. However, in later stages of the fermentation, probably due to slower growth and compromised metabolic activity due to constant exposure to the stressor, the culture with salt present had less accumulated glycerol inside the cells. This indicates that NaCl presence negatively influences the basic metabolism of the yeast, since it weakens the ability of the cell to respond to osmotic stress with time.

3.4.2.1 Physiological changes in *M. antarcticus* due to hyperosmotic stress

Salts are used in standard analytical techniques to measure cell hydrophobicity, such as in the Salt Aggregation Test (SAT). (Rozgonyi et al. 1985)

In order to determine what effect does a brief exposure to NaCl have on the physiology of *M. antarcticus* cells, a test was performed with a sample of the fermentation broth that was mixed in with salt for 30 minutes, with photomicrographs of cells before and after exposure (Figure 3.6). It can be observed that a short exposure to hyperosmotic stress caused rapid cell flocculation.

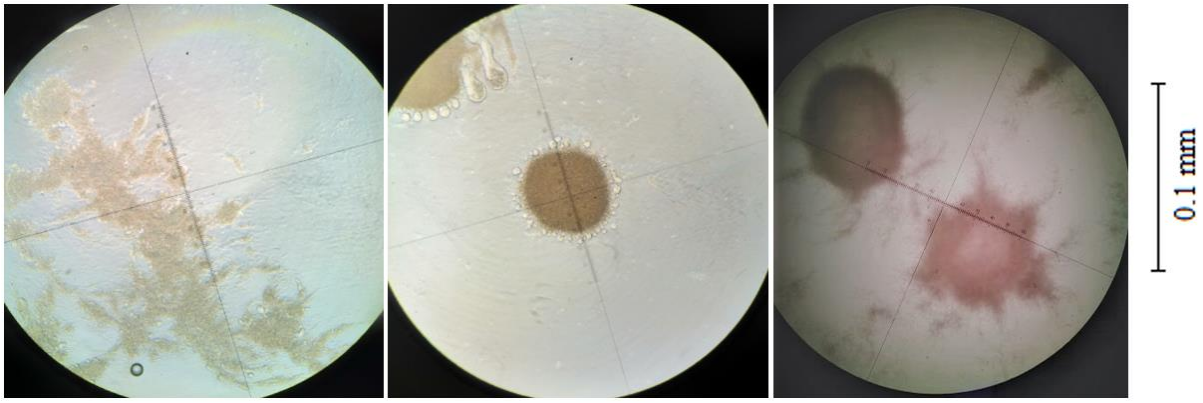

Figure 3.6: Morphology of *M. antarcticus* cells before (left) and after 30 minutes of exposure to 5% NaCl (middle). Results after a further exposure to the culture exposed to NaCl to 50mM EDTA for 30 minutes (right)

On the other hand, the fact that NaCl causes disruption of floccules of brewer's yeast has been reported in literature (MILL 1964), due to antagonistic effect of Na ions compared to Ca ions, and this phenomenon is widely used in analytical practice for deflocculation prior to analysis (Singh et al. 2015). Namely, sodium ions tend to substitute calcium, which normally enables forming ionic bridges and causes cell flocculation with great efficiency. (Stratford 1989)

However, this was not observed with *M. antarcticus*, as the floccules did not dissipate completely after exposure to EDTA, which should inhibit the effect of the ions on the cells. This means that the cells aggregate not due to salt bridges alone, but due other additional stimuli. This was proven to be true by observing that these cell floccules remain unchanged when the culture was exposed for EDTA 50mM for 30 minutes.

3.4.3 Non-sterile MELs production

In order to test the ability of *M. antarcticus* to grow in media with NaCl in non-sterile conditions, a set of flasks with 3.5% NaCl was prepared and incubated for 14 days. Initially, growth medium and flasks were sterilized for all runs, to secure that the initial conditions were identical among different runs. Over the experiment, three different conditions were followed:

i. flask fermentations and sampling were performed in sterile conditions,
ii. fermentations prepared in sterile conditions, sampling/handling in non-sterile conditions
iii. fermentations prepared in sterile conditions, sampling/handling in non-sterile conditions, with an induced contamination using ~10e5 E. coli cells after 24h of fermentation per flask.

This contaminant was selected since it is ubiquitous, mildly resistant to salt, and proved to be a common contaminant. Samples were collected over time, and biomass (CDW) and viable cell number were determined by counting colony forming units on Petri plates (Figure 3.7).

It can be observed that there is no significant difference in biomass concentration and viable cell number among the different conditions tested. The presence of the intentionally introduced, or any other contaminant, did not compromise the *M. antarcticus* cultivation.

Viable cells were determined over time by counting the number of colony forming units on a plate after incubation. This allowed to clarify both the composition of the biomass (yeast or bacteria cells) and how the added contaminant affected yeast cells viability (Figure 3.7). Petri plates with samples from all flasks showed only colonies of *M. antarcticus*, with the set of flasks with the induced contamination not showing any increase of contaminant colonies number over time, i.e., the contaminant culture did not develop.

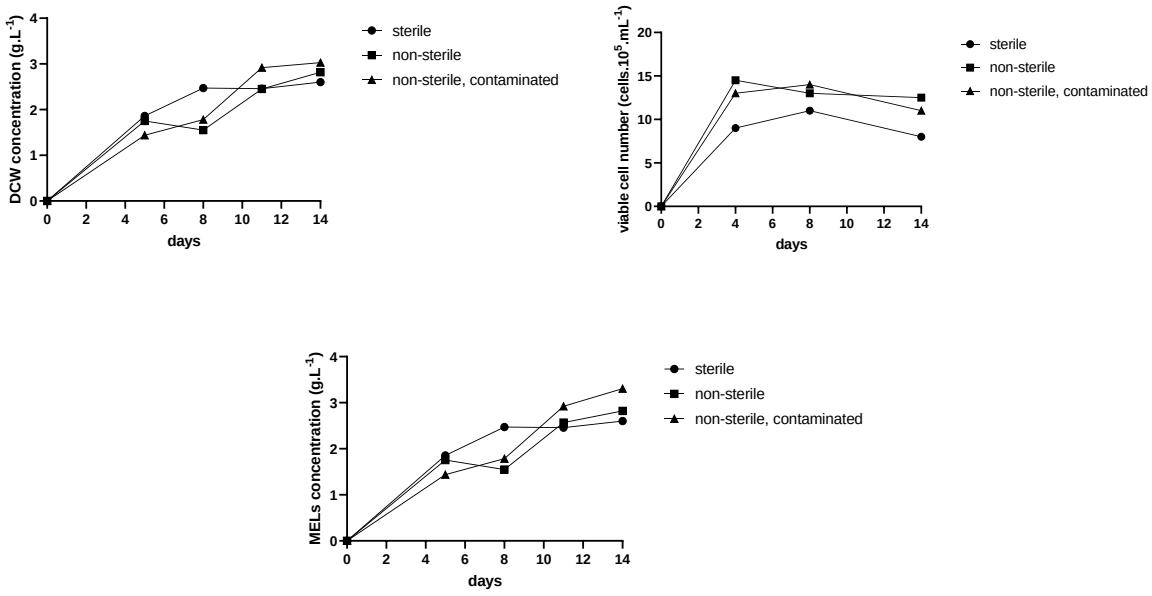

Figure 3.7: Cell biomass, MELs profiles, and CFU for cultures in three explored sterility conditions

Furthermore, MELs production was not affected. These parameters indicate that the presence of the contaminant has not affected the performance of *M. antarcticus*, and the culture was successfully grown in non-sterile conditions.

In order to test if the contaminant microorganism, *E. coli DH5-α*, was able to grow in these conditions without competition with the yeast, another group of flasks was prepared and its biomass profile over time was determined. These flasks were inoculated with the same number

of cells which were used to contaminate other flasks. The contaminant was unable to grow significantly in the medium containing 3.5% NaCl, which means that sea water level of salt is enough to enable non-sterile conditions to be used in the MELs production process, by impeding the metabolism of most common contaminating microorganisms enough to prevent them from compromising the main fermentation.

To determine if the presence of NaCl was indeed the reason for the inhibition of the contaminant, a blank for this experiment was performed with the same setup as non-sterile culture with introduced contamination, although no NaCl was added (NaCl 0%). The contaminant outperformed *M. antarcticus* in terms of growth and inhibited the yeast from developing. This proved that the NaCl is the key factor enabling *M. antarcticus* to grow in the presence of non-osmotolerant contaminants.

3.4.4 Crude oil and hydrocarbon tests

To estimate the dual marine bioremediation effect - the hydrocarbon-consuming microorganism and the petroleum-dispersive properties of the biosurfactant, *M. antarcticus* was grown on media with and without NaCl present following the previously described protocol, where the vegetable oil was substituted by 5 $g.L^{-1}$ of crude oil and a mix of linear alkanes and added on day 4. The residual MELs, FFAs, crude oil and alkanes were extracted from the fermentation broth and their concentration were analysed. The results for residual hydrocarbons are presented in Figure 3.8.

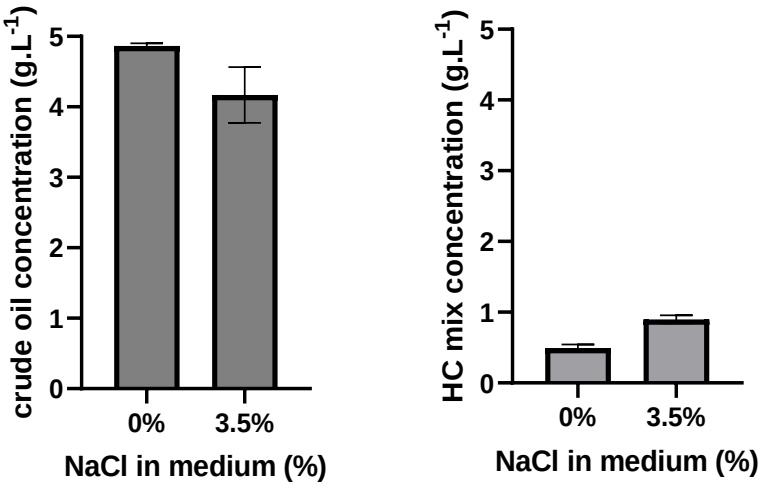

Figure 3.8: Concentration of crude oil (determined by GC) in medium containing % and 3.5% NaCl after 12 days, with a feed of 5 $g.L^{-1}$ of crude oil on day 4 (left); Concentration of the mix of hydrocarbons in medium containing 0% and 3.5% NaCl after 12 days, with a feed of 5 $g.L^{-1}$ of the hydrocarbon mix on day 4

Although it seems that the consumption of hydrocarbons was affected by salt presence, ANOVA analysis did not show a statistic difference between the consumption of both hydrocarbons for cultures cultivated in media with 0% and 3.5% of NaCl. These results indicate that the presence of salt did not inhibit the cultures metabolism. Results for MELs and FFA concentrations extracted from flasks after 12 days of cultivation are presented in Figure 3.9.

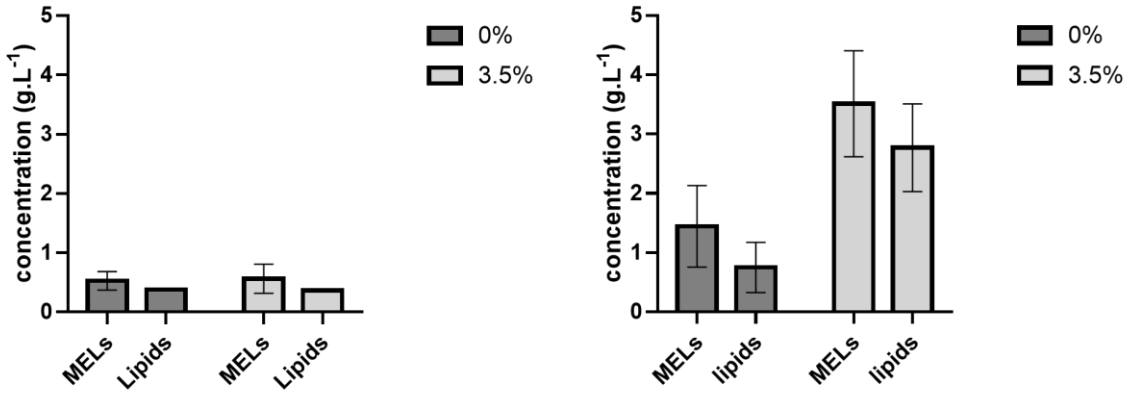

Figure 3.9: Left - Concentration of MELs and FFA (determined by GC) in medium containing 0% and 3.5% NaCl after 12 days, with a feed of 5 $g.L^{-1}$ of crude oil on day 4; Right - Concentration of MELs and FFA (determined by GC) in medium containing 0% and 3.5% NaCl after 12 day with a feed of 5 $g.L^{-1}$ of a mixture of alkanes on day 4.

For crude oil, MELs and lipid concentration is similar between the two sets of flasks, and salt presence did not inhibit MELs production. However, based on data regarding crude oil consumption from Figure 3.8, it can be concluded that the small amounts of MELs and lipids produced probably originates from the glucose added initially to the medium. However, for the mix of linear alkanes, MELs production is more significant. This is understandable, as impurities present in crude oil, such as aromatic compounds, asphaltenes and heavy metals, which are not present in the alkane mix, have inhibitory effects on the culture. Furthermore, the culture grown with alkanes in the presence of NaCl was more productive, indicating a possible stimulating effect of NaCl on the cultures ability to metabolize alkanes. To further study toxicity effects of the hydrocarbons on the cells, colony forming unit concentration was determined, and presented in Figure 3.10.

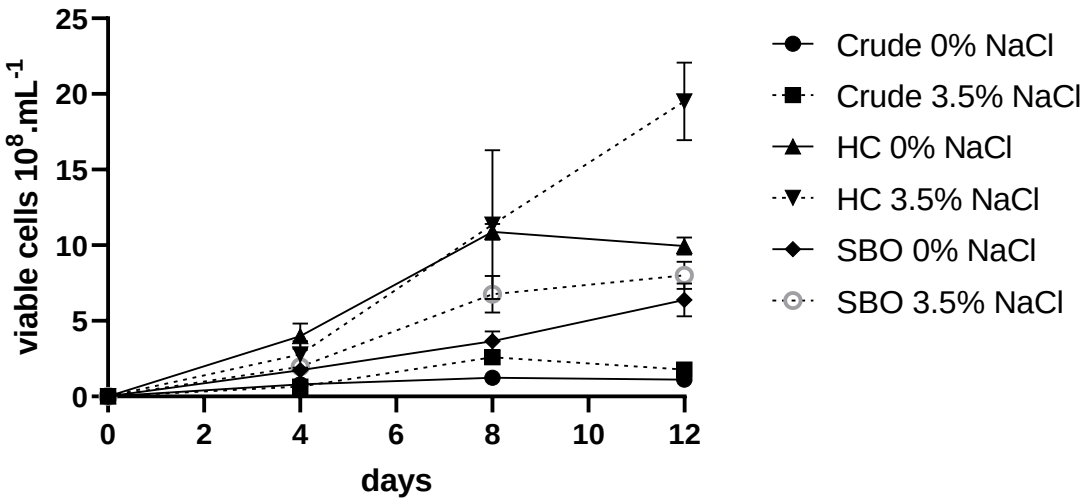

Figure 3.10: Concentration of CFUs for cultures with 40 g.L^{-1} of glucose at day 0 and crude oil (5 g.L^{-1}), alkane mix (5 g.L^{-1}), and SBO (20 g.L^{-1}) added on day 4, in media containing 0% (full lines) and 3.5% NaCl (dashed lines)

Hydrocarbons showed to have effects on cell proliferation. This is especially evident in cultures cultivated with NaCl present. It seems that stress, caused by salinity and moderate hydrocarbon toxicity, had a positive effect on cell proliferation. The effect was, however, opposite when crude oil was used, and the intense toxicity inhibited culture development. Although stress normally negatively affects yeast cell proliferation, Tamás and Hohmann (Tamás and Hohmann 2007) report that cell proliferation and regulation of protein kinase A are tightly connected in *S. cerevisiae*. Although induction of cell proliferation caused by osmotic and toxic stress in *M. antarcticus* seems evident from the presented data, this requires further investigation.

3.4.5 Ecotoxicity of MELs

MELs potential role in bioremediation is already hypothesised in literature. (Yu et al. 2015) Here, we established that *M. antarcticus* has the potential to produce MELs in the presence of

marine level of NaCl. However, before using MELs for bioremediation, ecotoxicity concerns need to be addressed. In order to estimate MELs toxic effects in a marine setting, the model organism for marine ecotoxicity, the brine shrimp *Artemia franciscana* was used. Cysts were immersed in solutions of several compounds with a range of concentrations, and IC50 values were estimated based on cyst mortality. MELs were tested in their crude form, as extracted from the fermentation broth, as well as using a cell-free supernatant containing 2.32 g. L^{-1} of MELs. To compare its performance with other glycolipid biosurfactants, tests were also performed using sophorolipids and rhamnolipids in crude form. Pure marine salt water and potassium dichromate were used as the negative and positive blank, respectively. Data for IC50 values is presented in Table 3.2.

Table 3.2: IC50 values (with standard deviation) for various compounds for brine shrimp *Artemia franciscana*.

Compound	**IC50 (mg.L^{-1})**	**St. dev.**
$K_2Cr_2O_7$	30.88	4.13
MELs	512.70	66.81
Supernatant	1383.00	52.84
Rhamnolipids	316.30	47.98
Sophorolipids	327.10	20.05

Among tested glycolipid biosurfactants, MELs outperformed sophorolipids and rhamnolipids, whose toxicity values match those reported in literature (Delbeke 2016; Sobrinho et al. 2013). Interestingly, the performance of MELs in their crude and supernatant form differs greatly. There are two possible reasons for this to occur. First, residual solvent present in the crude MELs, which is extracted from the fermentation broth, could affect the brine shrimp, and

increase its' mortality rate. Alternatively, MELs in supernatant could form multimolecular structures with more ease, and thus effectively reduce their "availability" to the shrimp and have reduced interactions with them.

The detailed graphs, indicating mortality rate at each tested concentration in the definitive tests, are presented in Figure 3.11.

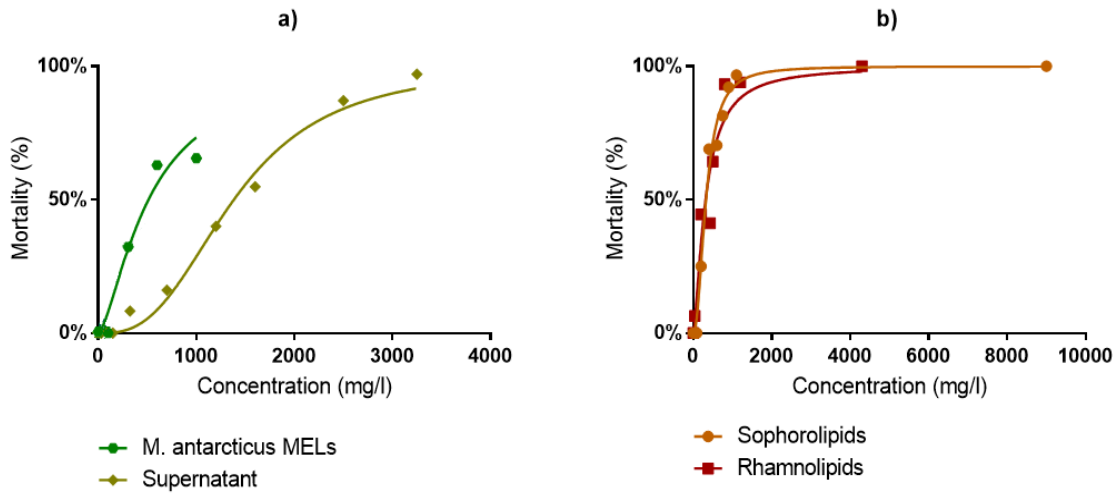

Figure 3.11 Artemia franciscana toxicity definitive tests of different OSRA. a) *M. antarcticus* MELs and the supernatant; b) sophorolipids and rhamnolipids.

Although phytotoxicity is out of scope for the marine context of this chapter, preliminary tests were performed using lettuce seeds as a model for phytotoxicity, and these results are presented in Appendix 10.2.

3.5 Conclusions

M. antarcticus was grown in media with different concentrations of NaCl present, biological samples collected at different stages of the fermentation were exposed to various amounts of osmotic stressor (NaCl) and different tests and analysis were performed in order to determine cell reactions.

Firstly, it can be concluded that NaCl presence in the growth medium negatively affects growth and basic metabolism (such as sugar and lipid consumption) of the microorganism, which indirectly affects MELs production.

One of the aspects that was less affected was lipase production and activity since the combination of slower metabolism and increased lipase secretion due to stress kept the results similar for most levels of NaCl present. This could make cultivation of *M. antarcticus* in seawater (or a partial mixture with seawater) for CAL-B enzyme production a promising technology which should be researched further.

Osmotic stress responses, such as intracellular glycerol accumulation and surface changes which cause agglomeration, reported for *Saccharomyces cerevisiae* and other yeasts, are also seemingly observed in *M. antarcticus*. Garay-Arroyo et al. (Garay-Arroyo et al. 2000) report that certain proteins accumulate at the surface of the cell as a response to osmotic stress, resulting in increase in agglomeration probably due to a change in cell hydrophobicity. Other sources (Ibstedt et al. 2014) report as well that cells exposed to hyperosmotic stress have changes in surface proteins similar to those that occur due to aging and other stressful effects. These changes cause the cells to flocculate, which was observed in *M. antarcticus* as well. These conclusions contribute to the understanding of the strain, and open possibilities for further research which could contribute to increases in MELs and CAL-B yields in biotechnological production in an industrial scale.

Presence of NaCl in the medium promoted formation of MEL-rich beads. Physiological effects of NaCl probably caused surface changes in the cells, and MELs adhesion to the cell walls was lowered, which promoted bead formation. These finds are important since they give us a better understanding of the mechanisms causing these beads to form, and later disappear. Controlling

and promoting bead formation would be beneficial from an industrial process aspect, as this would simplify the downstream process, which remains one of the main difficulties in MELs production. Namely, treating small amounts of MELs beads, rather than the complete volume of broth from the reactor, can decrease the need for solvents used in MELs extraction. This decreases the environmental impact of the process and reduces downstream costs.

MELs was produced in non-sterile conditions in water with 3.5% NaCl (equivalent to seawater salt levels), as well with a frequent contaminating microorganism present which inhibited the yeast development when no NaCl was present. This could enable production in unsterile conditions, thus contribute to the final goal of lowering production costs of CAL-B and MELs, increasing the sustainability of the production process, and making MELs a more competitive biosurfactant in the market.

As MELs are hypothesised to be used for marine bioremediation applications, it was of interest to explore *M. antarcticus*' ability to metabolise hydrocarbons and produce MELs successfully in saline water. Although crude oil showed to be toxic to the culture, MELs were successfully produced from a mix of linear alkanes. Production was higher in the culture with 3.5% of NaCl. Osmotic stress was observed to promote cell proliferation, which is unusual compared to data from literature relating osmotic stress with proliferation arrest in other yeasts. (Tamás and Hohmann 2007)

Finally, ecotoxicity of the biosurfactant was explored. Marine ecotoxicity to the model marine organisms *Artemia franciscana* was tested with MELs, which was compared to other biosurfactants. Here, MELs proved to be less toxic than the other compounds, with an even lower toxicity observed in MELs in supernatant form. These results indicate that MELs can be used safely for marine oil spill bioremediation.

Chapter 4

4 Production of Mannosylerythritol lipids (MEL) from vegetable oils: Exploring lipase application on substrate pretreatment

4.1 Abstract

An important feature of the production of mannosylerythritol lipids (MELs), a group of potent biosurfactants, are the significant losses that occur during downstream processing. The main difficulty in this step is the separation of MELs from residual triacylglycerols, which are used as a carbon source. To overcome this issue, triacylglycerol feeds can be limited, and MELs titres thus lowered, which affects the overall efficiency of the production process. Here, alternative feed strategies are explored: CAL-B, an autochthonous lipase produced by *Moesziomyces antarcticus* PYCC 5048^{T} in a separate fermentation, is used to partially break down the vegetable oil used in the feeds into free fatty acids (FFA) or fatty acid alcohol esters (FAAE). These were then used as substrate feeds for MELs production, and relevant parameters were monitored. The results showed that the lipase produced by *M. antarcticus* PYCC 5048^{T} can generate fatty acids and alcohol esters, which were then consumed by the yeasts to produce MEL, increasing titres (12.68 g.L^{-1} for FFA; 12.26 g.L^{-1} for FAME) if compared to conventional feeding strategies using oil as carbon source (7.45 g.L^{-1}). Prehydrolysed feeds also caused faster appearance of orange beads rich in MEL, which could be removed multiple times over cultivation, together with multiple pretreated substrate feeds, representing a more efficient process for MELs production and downstream, and increasing productivity of MELs in bead form by 2-3 times.

4.2 Introduction

Mannosylerythritol lipids (MELs) are a group of extracellular glycolipids known for their tensioactive versatility, which enables their use as surfactants. Carbohydrate and lipid-based substrates are used for the production of MELs as carbon sources, as well as glycerol, alkanes and other substrates, with varying influence on the production of this secondary metabolite. (Kruse et al. 2017) High titers of MELs are obtained using vegetable oils as substrates for *Moesziomyces antarcticus*. (Rau, Nguyen, Schulz, et al. 2005)

However, significant fractions of the bio produced MELs are then lost during downstream due to difficult separation from residual triacylglycerols, as they form various stable MEL/water/oil systems. (Worakitkanchanakul et al. 2008) As the current separation techniques for MELs recovery are unsustainable and/or expensive, and are inefficient at disrupting these stable supramolecular structures, achieving good titers of MELs with high levels of residual triacylglycerols is unreasonable. Triacylglycerol levels during fermentation are seldom reported and titers are often presented rather than yields and bioconversion efficiency. Focus needs to be put on achieving a commercially competitive product, with an efficient downstream processing treatment.

Moesziomyces antarcticus is also able to produce lipases, which break down triacylglycerols that comprise the vegetable oil. *Candida antarctica* lipase B (CAL-B) (outmoded name for *M. antarcticus*), a widely available commercially produced enzyme, is often used for generation of fatty acids, either in the presence of water, with the goal of producing diacylglycerols (Meng et al. 2014), or in the presence of alcohols, for alcohol ester production. Immobilized enzymes are used preferably, as their reuse lowers process costs when using commercial CAL-B. The

enzymatic reaction temperature reported ranges from 38-60 °C, with reaction times of 36-72h and pH 7.5. (Ramesh, Harini, and Fadnavis 2015; SreeHarsha et al. 2019)

CAL-B utilization in biodiesel production has been reported. Ognjanovic et al. (Ognjanovic, Bezbradica, and Knezevic-Jugovic 2009) reported high conversion rates of sunflower oil to methyl esters (FAME), in a packed-bed reactor with immobilized enzymes. In a different work (Bharathiraja et al. 2016) CAL-B was immobilized in sodium-alginate beads prior to use in a transesterification reaction of oil of microalgal origin, with best conversion rates attained at 48°C.

The use of commercially available fatty acid alcohol esters (obtained from a reaction of transesterification with chemical catalysts) for biosurfactant production have been reported. Kitamoto *et al* (Kitamoto, Ikegami, et al. 2001) report that *M. antarcticus* (*Candida antarctica*) resting cells produced MELs from methyl esters as a substrate, after 7 days of fermentation with a productivity of 0.43-0.62 g/g of substrate, depending on the fatty acid chain length. Rau et al. (Rau, Nguyen, Schulz, et al. 2005) reported that another species of the same genus, *Moesziomyces bullatus* (*Pseudozyma aphidis*) produced MELs from biodiesel (rapeseed oil-derived methyl esters) with a productivity of 0.5 g/g of substrate, performing slightly poorer than rapeseed oil and soybean oil, with the productivity of 0.68 and 0.62 g/g of substrate, respectively. The productivity of sophorolipids was increased in *Starmerella bombicola* cultivation in the presence of chemically produced methyl esters derived from soybean oil, on a glycerol-based substrate, compared to the case when triacylglycerols were used.

Considering these two features of *M. antarcticus* cultivations- the ability to produce MELs and lipase activity - native lipases were used to pretreat the supernatant in a way which elevates the constraints present during normal fermentation, namely oil hydrolysis as the supposed

bottleneck in the bioconversion process. The aim of this work was to develop a pathway for faster and more efficient MELs production, with less residual triacylglycerols and residual lipids at the end of the fermentation. Native lipases produced by the culture, in the form of lipase-rich extracellular crude extracts from *M. antarcticus* cultivations, were used for hydrolysis of vegetable oils to render fatty acids and transesterification of vegetable oils using alcohols to the production of fatty acid alcohol esters. The products obtained from this pretreatment of vegetable oil were used as a substrate in subsequent *M. antarcticus* cultivation, with the aim of facilitating MELs production (Figure 4.1).

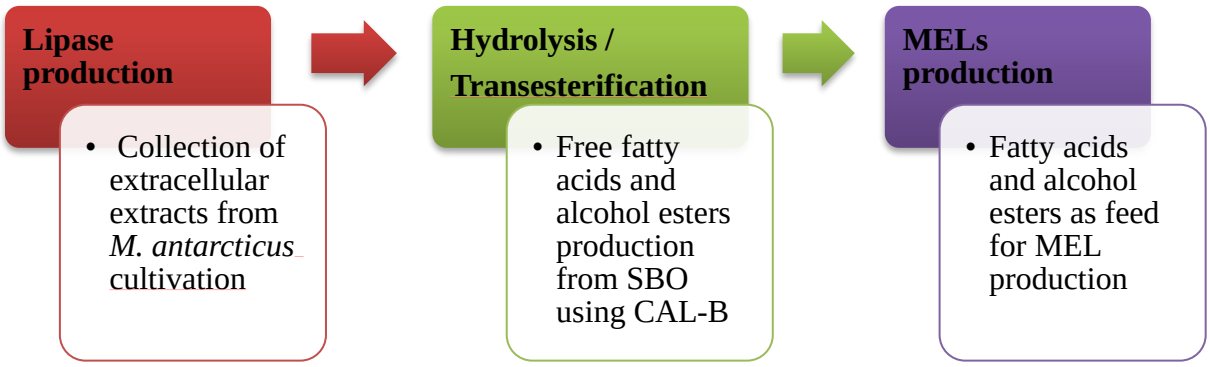

Figure 4.1 Overview of the process steps towards the use of pre-treated lipids for MELs production

4.3 Materials and methods

4.3.1 Microorganisms and maintenance

Moesziomyces yeast strain *M. antarcticus PYCC 5048^T* (CBS 5955) was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal, and maintained following a previously established protocol (Santos et al. 2019). Strains were plated on YMA (yeast extract (t.a., Oxoid LTD) 3 g L^{-1}, malt extract (t.a., Oxoid LTD) 3 g L^{-1},

peptone (t.a., BDH) 5 g L^{-1}, D-glucose (p.a., Fisher Chemicals) 10 g L^{-1} and agar (JMVP) 20 g L^{-1}) and incubated for 3 days at 30 °C. Cultures were kept at 4 °C and renewed every week and stored at −80 °C in 20% (w/v) glycerol (≥99.5%, JMGdS).

4.3.2 Media and cultivation conditions

Erlenmeyer flasks were used for preparation of inoculum and batch fermentation. Inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of medium containing 3 g L^{-1} $NaNO_3$ (p.a., PanReac AppliChem), 0.3 g L^{-1} $MgSO_4$ ((≥99.5%, Panreac AppliChem), 0.3 g L^{-1} KH_2PO_4 ((≥99.5%, Chem-Lab NV), 1 g L^{-1} yeast extract, 40 g L^{-1} D-glucose, and incubated at 27 °C, 250 rpm, for 48 h (orbital incubator, AraLab). Batch cultivations were performed in Erlenmeyer flasks containing 1/5 working volume of mineral (0.3 g L^{-1} $MgSO_4$, 0.3 g L^{-1} KH_2PO_4, 1 g L^{-1} yeast extract) supplemented with 40 g L^{-1} D-glucose. The experiment started by transferring 10% (v/v) inoculum, corresponding to approx. 0.6 g L^{-1} of cell dry weight (CDW), followed by incubation at 27 °C, at 250 rpm.

These flasks were incubated for 14 days, or longer in the case of cultures with multiple bead harvests. Soybean oil (SBO), partially hydrolyzed soybean oil, and soybean oil enriched with methyl, ethyl and butyl esters were added to the cultures after 96 h (on day 4). Periodical samples were collected to quantify biomass (CDW), monosaccharides, fatty acids mono-, di- and triacylglycerols, MELs, as well as protein content and lipase activity.

4.3.3 SBO hydrolysis with CAL-B: generation of fatty acids and alcohol esters

Cultivation conditions described in previous section were followed to produce lipases from *M. antarcticus*: an Erlenmeyer flask containing 40 g.L^{-1} of glucose, with or without the addition of

soybean oil at day 4 were used, as well as with an additional of 40 $g.L^{-1}$ of glucose added. After establishing the best feed strategy for optimal lipase production, that fermentation broth was collected, centrifuged for 8 min at 8000 rpm and the lipase-rich supernatant was collected and used for hydrolysis. To find the best hydrolysis strategy, the supernatant was mixed with oil in different ratios, pH was corrected with 1M NaOH and kept at various temperatures, according to the Box-Behnken with three factors DOE (Figure 4.2). The flasks with the supernatant and SBO mix were kept on orbital shakers for 72h, with occasional sample collection to determine the profiles of lipid degradation product and lipase activity. Box-Behnken design results were analyzed with the Design Expert 11 software, and 3D projections of the results were performed.

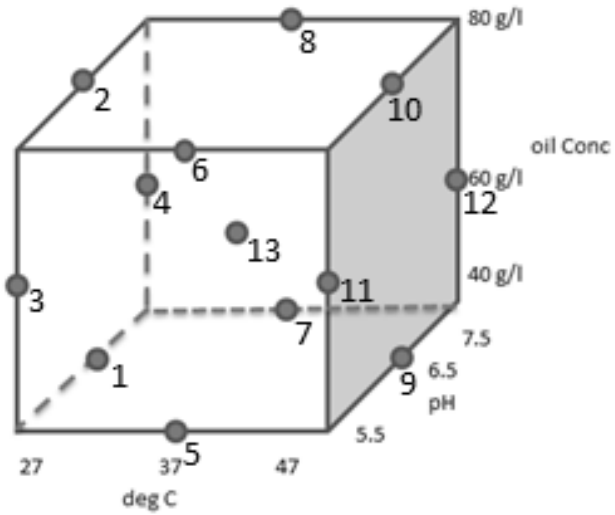

Figure 4.2 Box-Behnken with three factors, with variation of temperature - 40, 60 and 80 °C, pH - 5.5 (unmodified), 6.5 and 7.5, and oil concentration - 40, 60 and 80 $g.L^{-1}$.

As a comparison, commercially available free CAL-B enzymes (Novozym® 435, Novozymes, Denmark) were used by diluting to the appropriate unit of enzyme activity per volume corresponding to the one in the best selected conditions with supernatant (3 U/ml), or in their original, concentrated form, following protocol from literature. This protocol is based on the one reported by Yang et al. (Yang, Sohn, and Kim 2009) Namely, the appropriate amount of sterile Milli-Q water was added, to enable hydrolysis of lipids and its derivatives. As conversion

into alcohol-esters was poor with the lipase-rich supernatant, commercially available immobilized enzymes were used for production of alcohol esters, with methanol (HPLC grade, VWR Chemicals), ethanol (96% v/v, Manuel Vieira Lda.) and butanol (99%, JMGS) were added to a 4:1 alcohol to SBO molar ratio. The mixtures were incubated in an incubation box (J.P. Selecta, s.a.) on a magnetic mixer (Ikamag REO, Drehzahl Electornic) at various temperatures, in accordance with the DOE. Half of the alcohol was added at t=0, and the other half 12h after the beginning of the reaction. The immobilized enzymes were hydrated prior to utilization. In some cases, emulsifying agents – Xanthan (p.a., Sigma) and MELs, were added (0.5% w/w of SBO in reaction) to further improve conversion rates.

4.3.4 Biomass content determination, Analysis of fermentable sugars, alcohols, and glycerol concentrations

Cell growth was quantified by cell dry weight (CDW), using a previously described protocol. (Santos et al. 2019) CDW was determined from 1 mL culture broth by centrifugation at 10 000 rpm for 10 min (Sartorius 1-15P centrifuge), washing with deionized water (twice) and drying at 60 °C for 48 h. Supernatant from the centrifuged samples was collected and used for various analyses.

In the collected sample supernatants, monosaccharides, alcohols, and glycerol were quantified in a high-performance liquid chromatography (HPLC) system (VWR Hitachi, Darmstadt, Germany) equipped with a RI detector (L-2490, VWR Hitachi, Darmstadt, Germany), UV-detector (L-2420, VWR Hitachi, Darmstadt, Germany) and a Rezex^{TM} RHM-Monosaccharide H+ (8%) column (300 mm × 7.8 mm, Phenomenex), at 65 °C. Milli-Q water was used as mobile phase at 0.5 mL.min^{-1}.

4.3.5 Analysis of fatty acids, mono-, di-, triacylglycerol, and fatty acid alcohol esters concentrations

The analysis of fatty acids, mono-, di-, triacylglycerols and fatty acid alcohol esters content was performed using a method developed by Badenes et al (Badenes, Lemos, and Cabral 2010). Samples of supernatant (200 μL) were mixed with 1 μL of acetic acid (96%, Arcos Organics) 58.5 mM and 799 μL of n-hexane (HPLC grade, Fisher) and centrifugated at 10000 rpm for 2 minutes. The organic phase was recovered and used for analysis by high-performance liquid chromatography (HPLC) using a ChromolithPerformanceRP-18 end capped (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% (MilliQ) and phase C = n-hexane/2-propanol (4:5, v/v).

4.3.6 Analysis of MELs and lipid concentrations

MELs concentrations in the samples were calculated based on the results acquired by gas chromatography (GC) of methyl-esters, following the protocol elaborated in Chapter 3.

To extract MELs from the total broth, two extractions with ethyl-acetate (p.a., Fisher Chemicals) were performed (in 1:1 v/v ratio). For MELs beads which were formed in some cases, a crude metallic sift (pore diameter ~0.5 mm) was used to separate them from the rest of the broth, and extractions were performed separately. The solvent was evaporated on a rotavapor (Buchi R-3), and the transesterification reaction described above was performed directly on the extracts.

4.3.7 Extracellular protein and lipase activity analysis

Extracellular protein content was estimated using The Thermo Scientific™ Pierce™ BCA Protein Assay. An albumin standard was used for forming the calibration curve. In a microplate, 2 μL of sample supernatant was mixed with 8 ml of milli-Q water (to make a 1:4 dilution), and 200 μL of the working reagent (50:1 mixture of reagents A and B) was added to each well. The plate was incubated for 30 minutes at 37 °C, and absorbance was measured at 562nm on a plate reader (MultiScan Go, Thermo Scientific).

The enzymatic assays were performed as described in Gomes *et al* (Gomes et al. 2011). The substrate used for the enzymatic assays was p-nitrophenyl butyrate (≥98%, Sigma-Aldrich). All enzymatic activities were carried out in a 96 well microplate, and the reaction mixture was composed by: 2.63 mM of p-nitrophenyl butyrate was dissolved in 50 mM acetate buffer (pH 5.2) and 4% of triton-X-100.To initiate the enzymatic assay, 90μL of p-nitrophenyl butyrate 2.63 mM solution and 10 μL of the supernatants was added. Then the reaction mixture was incubated at 37ºC for 15 minutes, and after that, the reaction was stopped by adding 200 μL of acetone. The absorbance was measured at 405 nm in a microplate spectrophotometer (MultiskanTM GO, ThermoFisher Scientific), and the enzymatic activity was determined. One unit (U) of lipase activity is defined as the amount of enzyme releasing 1 μmol p-nitrophenol per minute.

4.4 Results and discussion

4.4.1 Lipase production optimization

Optimal conditions to produce lipases to be used for pretreatment of the soybean oil substrate and the generation of free fatty acids were explored. As the phase of exponential growth ends roughly after 4 days of fermentation, and the initial amount of substrate is mostly consumed, another addition of a carbon source can be performed. This is usually a hydrocarbon or a lipid-based substrate. To test the combination of substrates that results in a high lipase activity in a short time, the following feed strategies were tested:

- Day 0: 40 $g.L^{-1}$ of glucose
- Day 0: 40 $g.L^{-1}$ of glucose; Day 4: 40 $g.L^{-1}$ of glucose
- Day 0: 40 $g.L^{-1}$ of glucose; Day 4: 20 $g.L^{-1}$ of SBO

The results of lipase and specific enzymatic activity for these fermentations are given in Figure 4.3.

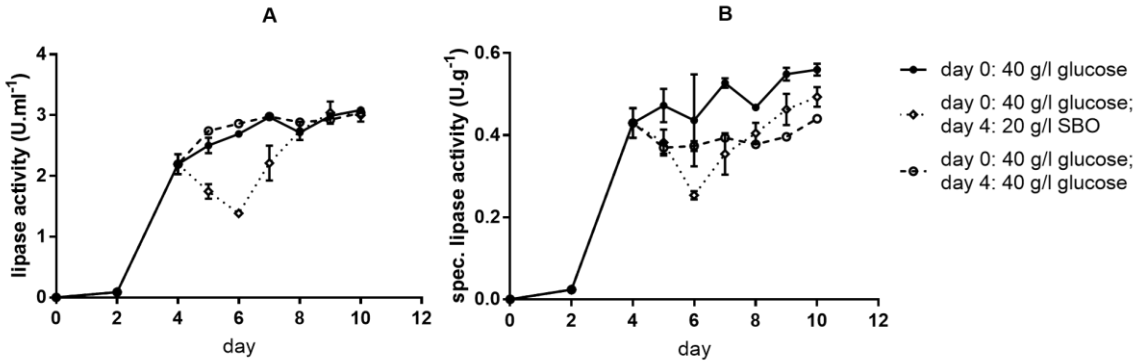

Figure 4.3 : Extracellular lipase activity (A) and specific extracellular lipase activity (as units of lipase activity per unit of mass of extracellular protein) (B) profiles for *M. antarcticus* cultured in D-glucose (40 $g.L^{-1}$) and further addition of carbon source, D-glucose (40 $g.L^{-1}$) or soybean oil (20 $g.L^{-1}$) at day 4

The results showed that cultivations in D-glucose resulted in higher extracellular lipase activity, while it was determined that the addition of soybean oil caused a drop in lipolytic activity. The second feed of D-glucose on day 4 has not affected the lipase activity level significantly, and it also fostered other proteins to be produced, dropping the specific activity of lipases. Thus, for further steps in the experimental plan, supernatant collected at day 7 from cultivations of *M. antarcticus* in 40 $g.L^{-1}$ of D-glucose with no further carbon source added was considered as the best lipase production strategy.

4.4.2 Oil hydrolysis optimization

Lipase-rich supernatant was mixed with vegetable oil to find the best strategy for oil hydrolysis according to the Box-Behnken DOE. Besides the lipase-rich supernatant, a solution of commercial CAL-B in liquid form was used, to test the importance of synergy of lipases with other supernatant components. Enough stock enzyme was used to reach the activity of 3 U/ml of activity, which is the maximum extracellular lipase activity reached in a fermentation with 40 $g.L^{-1}$ of D-glucose after 7 days and no further concentration is considered. To adjust the pH value of the supernatant according to the experimental plan, the pH was corrected using 2M NaOH (as the initial pH of the supernatant was ~5.5). The commercial lipase solution was prepared in 50mM Phosphate buffer at various pH values, ranging from 5.5 to 7.5 (with a slight pH correction for pH 5.5 using 2M HCl).

The enzyme solutions were mixed with soybean oil, and maintained at different conditions, with a three-level variation of parameters, according to the Box-Behnken experimental design (Figure 4.2). The results for enzyme activity, presented as Triacylglycerol to FFA conversion rates, are presented in Figure 4.4.

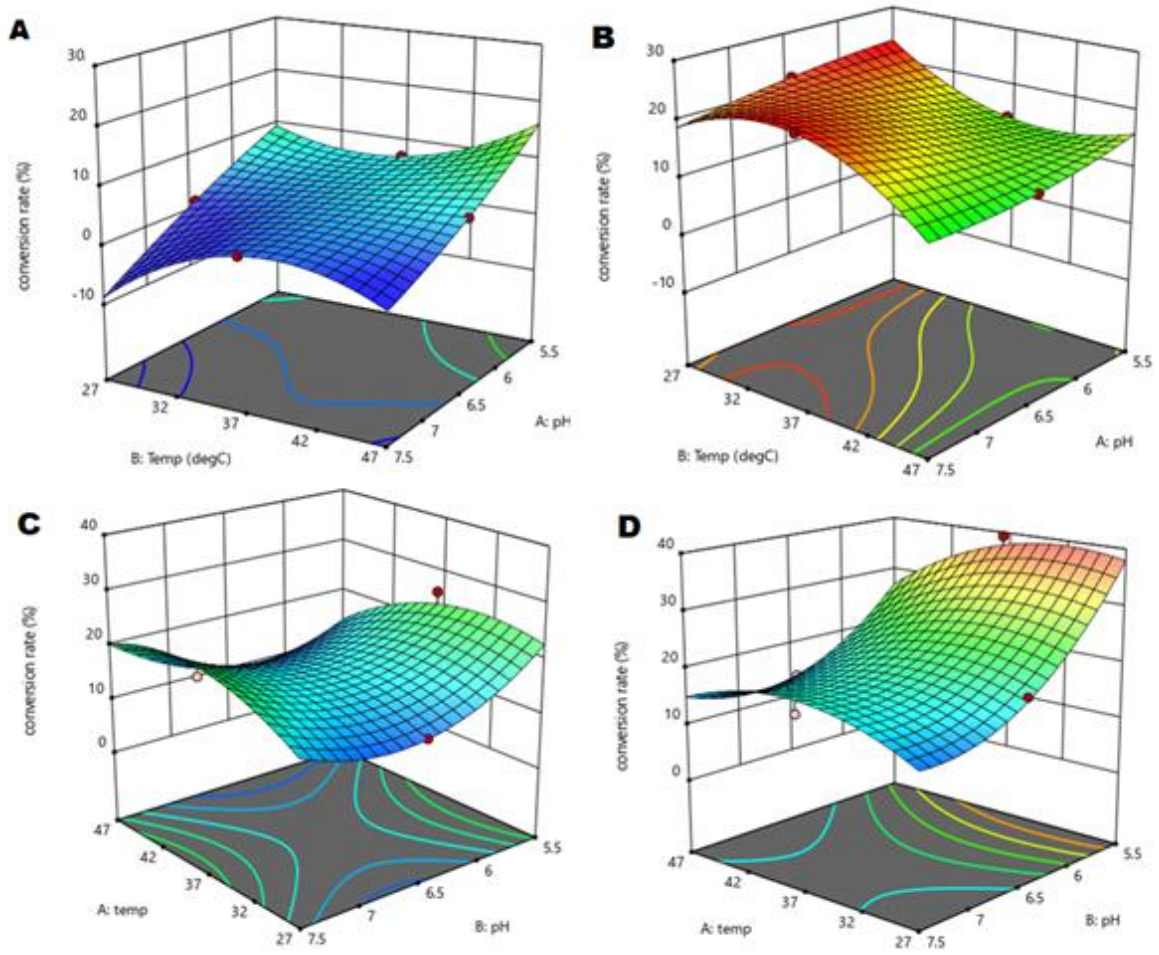

Figure 4.4 Box-Behnken design results for lipase-facilitated oil hydrolysis, presented as conversion rate of oil to fatty acids. A: commercial enzyme, 40 $g.L^{-1}$ of oil; B: commercial enzyme, 80 $g.L^{-1}$ of oil; C: supernatant, 40 $g.L^{-1}$ of oil; D: supernatant, 80 $g.L^{-1}$

Results are expressed precisely as conversion rate of oil to free fatty acids (conv. rate = conc. FFA / initial conc. of oil) since it was observed that monoacylglycerols and diacylglycerols are not present in significant quantities, with a level of <1% and <5% (w/w) in the total lipid mixture, respectively.

Based on the results for the 13 conditions analyzed, it was established that the commercial enzyme in buffer solution would achieve the best conversion rate at 27°C, pH of 5.5 and 80

$g.L^{-1}$ of oil, where 24% conversion rate was projected. However, for the Lipase-rich supernatant (3 U/ml of activity), 39% conversion rate was achieved with the following conditions: 37°C, pH of 5.5 and 80 $g.L^{-1}$ of oil. This improvement of conversion rate when using supernatant can be explained by the positive effect of ions or cofactors on lipases, a phenomenon already reported in literature (Ventura et al. 2012), or by the possible positive contribution of the present MELs as an emulsifier, which facilitates the conversion process, as small amounts of MELs are already present after 4 days of fermentation, when the supernatant is collected.

To establish the positive effect of MELs presence in oil hydrolysis, the biosurfactant in crude form was added to the flasks which were kept in the optimum condition already established. Xanthan, a commonly used emulsifier of microbial origin, was also tested in the same conditions. The results showed that addition of 0.5% of emulsifier, Xanthan and MELs, increased final FFA concentrations, with the former achieving 43% hydrolysis efficiency, while MELs increased the conversion rate to 52%.

Based on these results, for further steps in the experimental plan, the lipase-rich supernatant was used to prepare hydrolyzed oil feeds by mixing it with 80 $g.L^{-1}$ of oil, the addition of 0.5% of MELs (w/w, in relation to SBO), without pH correction (~5.5 pH), and incubated at 37 °C. The same strategy was tested for fatty acid alcohol ester generation. The best condition combination of parameters for fatty acid production was used: 37°C, pH of 5.5 and 80 $g.L^{-1}$ of oil, with addition of 0.5% MEL, with the adequate amount of alcohol added (alcohol: SBO molar ratio 4:1). The idea was that CAL-B would generate free fatty acids from triacylglycerols, with the same enzyme facilitating the formation of esters with the alcohols. However, use of supernatant and alcohol resulted in poor transesterification performance. The results of the content of the lipid fraction after 48h of incubation under these conditions are presented in

Figure 4.5A. It seems that the alcohol presence negatively affected CAL-B's performance, with butanol having the most drastic negative effect observed. This corresponds to findings reported by Banik et al. (Dutta Banik et al. 2016) which find that butanol had inhibitory effects on CAL-B, with the alcohol most probably binding to the active spots of the enzyme. It was also reported that methanol also has negative effects on lipase CAL-B, as Pollardo et al. (Pollardo et al. 2017) reported on methanol toxicity to this enzyme. This can explain how these two alcohols negatively affected the hydrolysis step of the ester generation process. As for the esters themselves, they did not accumulate in significant concentrations, with only 0.5-1.5% of esters in the lipid fraction. HPLC analysis of the aqueous fraction after the transesterification reaction showed some glycerol generation, while a significant amount of alcohols was remaining. These results indicate that another approach should be used for ester generation.

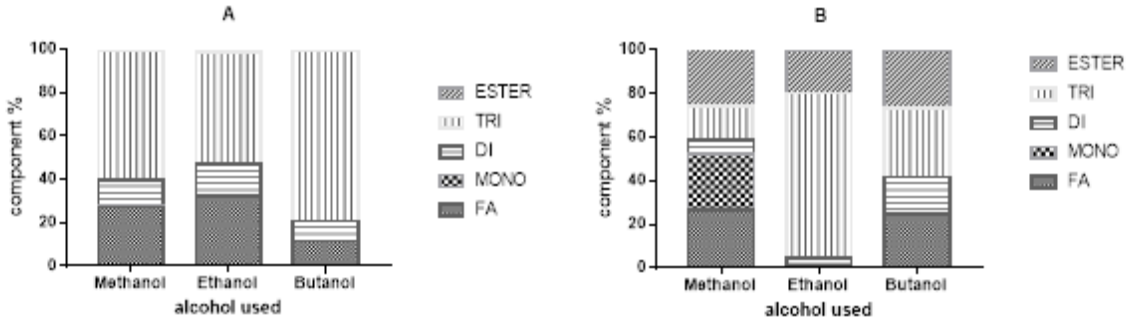

Figure 4.5 A - Composition of the lipid fraction for ester production using supernatant mixed with different alcohols; B - Composition of the lipid fraction for ester production using immobilized commercial CAL-B mixed with different alcohols

To generate FAAE from vegetable oil, an alternative method was followed, using commercially available immobilized CAL-B (Novozym® 435, Novozymes, Denmark) to produce esters, following a modified protocol based on the one developed by Yang et al. (Yang et al. 2009) (see Materials and Methods). If compared to the use of lipase-rich supernatant strategy, here

the generation of esters was generally higher, as shown in Figure 4.5. About 26% (w/w) of FAME (fatty acid methyl esters) and FABE (fatty acids butyl esters) was generated, and 15% (w/w) of FAEE (fatty acids ethyl ester). HPLC results of the polar phase showed exceedingly high concentrations of glycerol, and some residual alcohols, except for butanol. In the case of this alcohol, no residual butanol was present, which could be either due to evaporation or adsorption to the lipase active sites.

Unexpectedly, when immobilized enzymes were used, ethanol ester production was lower than the production of methyl and butyl esters. There are several possible reasons for this to happen, one of which could be an interaction with the acrylic resin of the lipase carrier. In a study about the interaction of ethanol and acrylic resin used for production of dental implants, Sideridou et al. (Sideridou, Karabela, and Bikiaris 2007) report degradation of certain acrylic resin-based matrices in water/ethanol mixes. This could be a possible explanation for the drop of ester production - the alcohol caused deformations on the resin-based carrier, reducing the activity of the immobilized enzyme. However, since significant amounts of fatty acids are produced, the issue might be that the ethyl esters are less stable, and the reverse reaction, from ester to fatty acid and alcohol, occurred in a higher degree than with other alcohols. Nonetheless, immobilized enzymes would be used in further parts of the work to pretreat the soybean oil substrate for fermentation, as they proved to be far superior to lipases from the broth supernatant in terms of esterase production.

Finally, the compositions of the pretreated substrates to be used as feeds for MELs production, are presented in Table 4.1. The nomenclature for those substrates (FFA, FAME, FABE), to be used as carbon source in the following fermentation feeding strategies, is related with the pre-treated soybean oil enriched with the corresponding component, depending on the method and/or alcohol used. Although the conversions were not complete, the amount of

triacylglycerols in the final composition of these substrates is significantly reduced (if compared with untreated soybean oil), which fits within the goals of this work facilitating the lipid-to-MELs conversion process by partially hydrolyzing the substrate.

Table 4.1 Composition of the pre-treated soybean oil to be used as substrate in *M. antarcticus* cultivations (FFA feed prepared with supernatant, esters prepared with immobilized CAL-B), with the relevant component being highlighted.

Component (%)	SBO	FFA	FAME	FAEE	FABE
Free fatty acids	0.4	**51.6**	26.5	28.7	24.5
Monoacylglycerols	0.0	1.6	25.7	0.0	0.0
Diacylglycerols	0.6	13.7	6.3	2.5	17.0
Triacylglycerols	**99.0**	33.1	15.8	54.4	31.9
Esters	/	/	**25.7**	**14.4**	**26.6**

4.4.3 Production of MELs with substrate based on pretreated oil

The pretreated oil rich in fatty acids (FFA), methyl- (FAME), ethyl- (FAEE) and butyl-esters (FABE) were used as carbon source in *M. antarcticus* cultivation for the production of MELs. The outputs of the cultivation of *M. antarcticus* using these pretreated feeds were compared to the use of untreated soybean oil (SBO). These lipid feeds were fed into cultivations at day 4, up to a total lipid concentration of 20 $g.L^{-1}$. The results for profiles of relevant parameters are presented in Figure 4.6.

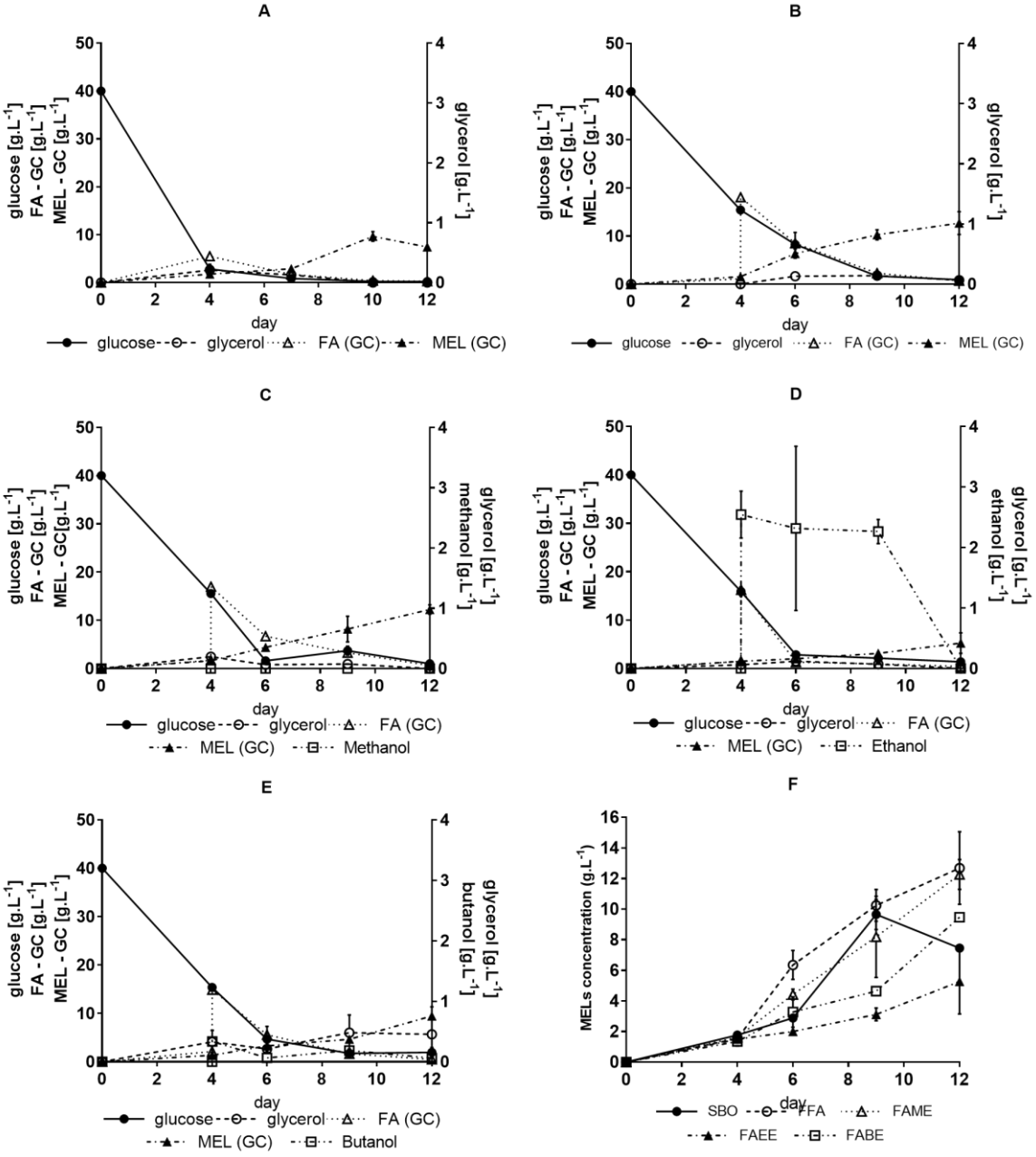

Figure 4.6 Metabolites and MELs profiles for fermentations with feeds of - A: SBO; B: FFA; C: FAME; D: FAEE; E: FABE. F: MELs concentration profiles for all conditions

Firstly, all pretreated feeds enabled MELs production. Most of them performed similarly or better than the untreated SBO, with only the ethyl ester feed resulting in reduced MELs titers. Small amounts of methanol (Figure 4.6C) and butanol (Figure 4.6E) released steadily by ester hydrolysis have not resulted in growth inhibition or apparent obvious negative effects on the

metabolism. However, unlike ethanol, they seemed to be continuously consumed by the microorganism. The ethanol level, however, remained stable and only dropped by day 12, either due to yeast metabolization or evaporation, as seen in Figure 4.6D. Besides the ethanol released by hydrolysis of the esters, some could have been added with the lipid-based feed, since ethanol can dissolve relatively well in oil. Nevertheless, the results show that *M. antarcticus* was able to metabolize esters by hydrolyzing them, and subsequently metabolizing the free fatty acids. In addition, higher MELs production was achieved with methyl ester feeds compared to oil.

The results for this approach, where vegetable oil is partially hydrolyzed prior to fermentation by native lipases, are very promising since availability of FFA in earlier stages of the fermentation seems to stimulate MELs production. MELs profiles indicate that the pre-hydrolysis step effectively reduced the fermentation period and enabled reaching higher MELs concentrations compared to feeds with untreated oil. (Figure 4.6A, B)

Some negative effects of alcohol presence in early stages of the fermentation were observed for butanol, which corresponds to previously observed occurrences in the previous section of this work. The reported methanol toxicity on CAL-B (Pollardo et al. 2017), was not observed in *M. antarcticus* cultivations, as no methanol accumulation was found, suggesting that it was being consumed as it was being released in the transesterification reaction. Furthermore, it seems that methanol stimulated the metabolism of the *M. antarcticus*. Although no literature data was found *on M. antarcticus* interactions with methanol, it can be observed that methanol causes some positive effects on similar yeasts. Li *et al.* (Li et al. 2003) report that methanol, up to 0.5% (v/v) in the growth medium stimulated extracellular protein production in the *Pichia pastoris* yeast. Also, Yasokawa *et al.* (Yasokawa, et al., 2010) report that methanol stimulated gene expression in *Saccharomyces cerevisiae*, mainly contributing to production of integral membrane proteins.

4.4.4 Impact of substrate on MELs-rich bead formation

At some points throughout the fermentation, high variations in MELs results are present between duplicate fermentations, illustrated by the large as error bars (Figure 4.6F). The issue might stem from the fact that beads rich in MELs were formed, which prevent homogenous sampling. This is more prominent in flasks fed with fatty acid-rich oil and fatty acid methyl esters, while soybean oil fed flasks developed these beads with a 48h delay. This was an indication that the feeds which enabled faster MELs bead formation were enabling faster fermentation process progression. The appearance, size, and color of these beads over time in the tested conditions is presented in Figure 4.7

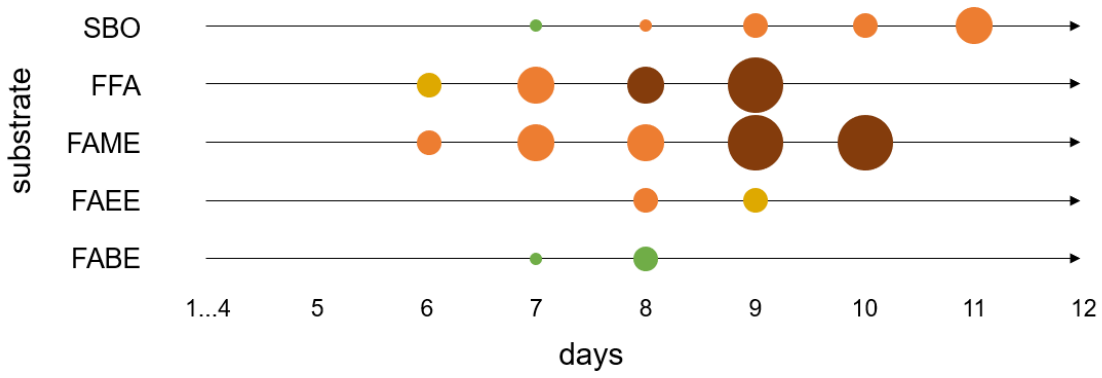

Figure 4.7 Progression of size and colour of MELs beads in flasks fed with various lipid-based substrates. Size (diameter) - Tiny: >1 mm; Small: 1-3 mm; Medium: 3-10 mm; Large <10 mm. Colour of the field indicates the colour of the beads on that day.

Assuming MELs beads formation a measure of fermentation progression, their earlier appearance in flasks with feeds of free fatty acids and methyl-esters indicates that these feeds contribute to improve the productivity of MELs. In the earlier experience within our group, we could conclude that the color of the beads indicates how "mature" they are i.e., how rich in MELs they are. Green and yellow beads tend to have lower MELs levels, and are rich in

triacylglycerols and fatty acids, respectively, while darker orange beads have high MELs concentrations in their extract (>80%). Furthermore, texture of the beads is related to triacylglycerol presence, with very firm beads without any triacylglycerols present, and soft, viscous beads when triacylglycerols are present. Finally, beads floating on the surface of the broth were a result of higher levels of residual triacylglycerols.

The occurrence of these beads in later stages of MELs production, when MELs levels in the broth are high, is already reported in literature. Rau et al (Rau, Nguyen, Roeper, et al. 2005) report that *M. bullatus* produced these beads in a fed-bath reactor and concluded that they are an indicator of increased production of MELs. It is also stated that, with the progression of the fermentation, the beads grew, and changed their color from greenish yellow to orange. In another work Rau *et al* (Rau, Nguyen, Schulz, et al. 2005) reported production of beads in shake flask cultures. However, both reports stated that there were difficulties in gathering these agglomerates due to their softness and viscosity, probably due to high levels of residual oils present in the medium, as well as in the beads. Also, it is stated that these beads formed only when MELs levels exceeded 40 $g.L^{-1}$, which opposes the findings in this paper.

As three sets of flasks developed beads by day 7 (feeds with SBO, FFA and FAME), a set of flasks was prepared with the intention to analyze MELs-rich beads and the bead-free broth separately. Flasks were incubated for 7 days; beads were removed, and the two fractions were analyzed separately. The results on MELs and lipids concentration, and the ratio of triacylglycerols, monoacylglycerols and free fatty acids in the total lipid content in the lipid component of the extract, are presented in Figure 4.8A and Figure 4.8B, respectively.

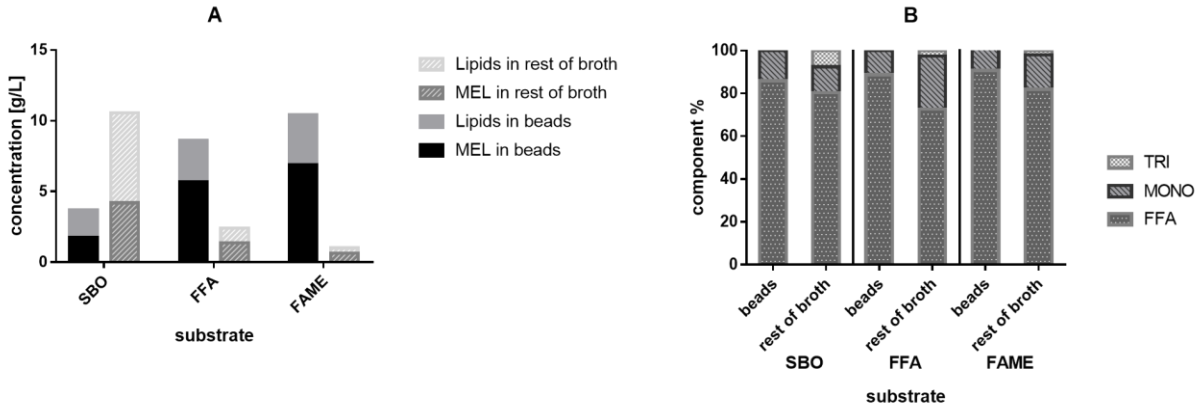

Figure 4.8 Cultivation of *M. antarcticus* PYCC 5048^{T} in 40 g.L-1 of D-glucose and feeding of SBO, FFA or FAME (20 g.L-1) at day 4, at 27ºC, 250 rpm, for 7 days. A) Concentration of MELs present in extracts of MELs beads and bead-free broth; B) Content (in %) of components comprising the lipid fraction from the beads and bead-free broth, triacylglycerols (TRI), monoacylglycerols (MONO) and free fatty-acids (FFA).

From the apparent higher concentration of MELs in bead form when pretreated substrates were used (presented in Figure 4A), as well as lower accumulation of free-fatty acid in the broth, one may suggest we can deduce that the cultures fed with soybean oil were not as developed, in terms of cell maturity and extracellular enzymatic complex, by day 7 as the ones fed with pretreated substrates, fatty acids (FFA) and fatty acid methyl esters (FAME). We can conclude this from the fact that MELs levels were lower, and there was an accumulation of free fatty acids in the medium. Also, an amount of unhydrolyzed triacylglycerols was present in the broth, as seen in Figure 4B. Meanwhile, cultures fed with FFA and FAME were producing more MELs, with lower fatty acid content in the beads and the broth, and extraordinarily little residual triacylglycerols.

4.4.5 Multiple bead harvest approach to production of MELs

As the broth after bead removal still contained viable cells and a strong extracellular enzymatic complex, the possibility of achieving multiple bead "harvests" in one 12-day fermentation was studied. Two sets of flasks were prepared, fed with FFA and FAME substrates, since these two substrates proved to form solid MELs beads after 7 days reliably. On day 7, the beads were removed in sterile conditions, and another 20 $g.L^{-1}$ of the corresponding lipid-based feed was added to the bead-free broth. New beads started forming on day 10, and they matured on day 12, when they were collected. The beads were analyzed using GC, and these results are presented in Figure 4.9. As with previous GC results, the lipid fraction represents total non-MELs lipids.

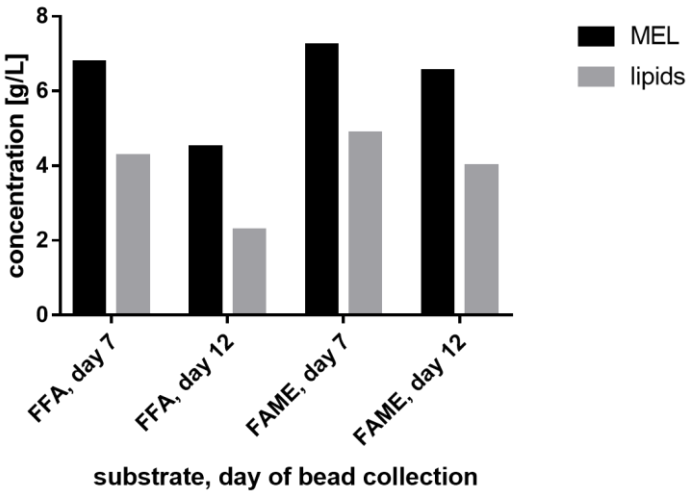

Figure 4.9 GC results for beads collected on day 7 and day 12, for cultures fed with FA and FAME-based substrates.

Based on these results, we can conclude that it is possible to achieve higher MELs yields using these two pretreated substrates. The MELs obtained is in bead form, less solvent is needed for their purification, and their downstream treatment is easier. The remaining broth contained

insignificant amounts of MEL, 2.23 $g.L^{-1}$ and 0.34 $g.L^{-1}$ for cultures fed with FA and FAME, respectively. In conclusion, for cultures fed with FA-based substrate, about 12 $g.L^{-1}$ of beads are collected after 12 days of fermentation, while for cultures fed with FAME-based substrate this value was about 13.5 $g.L^{-1}$.

The possibility of consistent production of MELs in bead form multiple times throughout a single fermentation has a large impact on the development of a production process. After it was established that after harvesting the MEL-rich beads more lipid substrate can be added and the fermentation can continue, a different approach to the production process was tested. After the beads were formed, which were solid, orange, and dense (indicating richness in MELs and lack of triacylglycerols) they were separated under sterile conditions, as described before, and the bead-free broth was returned to a sterile flask, along with another 20 $g.L^{-1}$ of the lipid substrates (FFA and FAME). This process was repeated multiple times, as long as new beads were forming within a reasonable length of time. The beads were analyzed and the results are presented in Figure 4.10.

As it can be seen in the figure, the first MEL-rich set of beads forms for both substrates only after 3 days of the initial lipid addition. After the removal of the bead, the culture proceeds to consume the newly introduced substrate and produce MELs. However, with time, the gaps between the addition of the substrate and development of new beads increases. For FFA, they are increase from the initial 3 days to 6 days, and a total of 4 sets of MEL-rich beads were harvested. On the other hand, cultures fed with the FAME-based substrate, produced beads 5 times (one duplicate produced only 4), and the gaps between bead collection (from the addition of the initial lipid feed) increased from 3, for the first collection, to 7 days for the last collection.

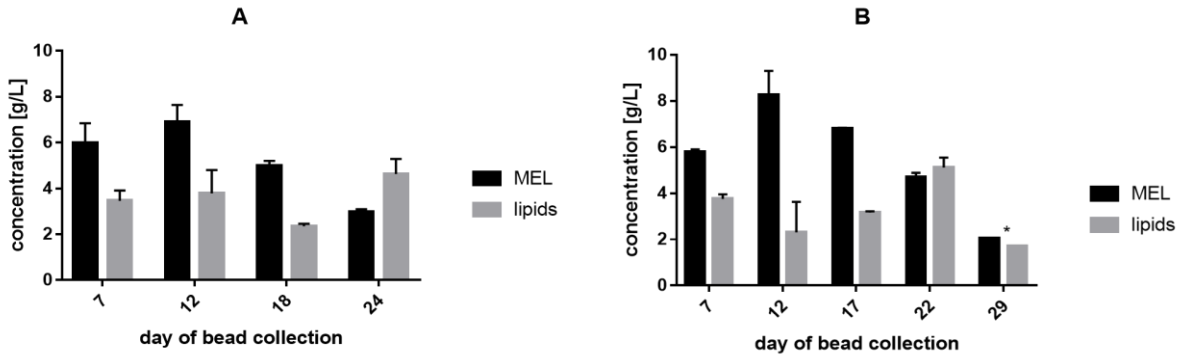

Figure 4.10 GC results for beads collected on multiple days, for cultures fed with FA (Figure 11A) and FAME-based substrate (Figure 11B). Error bar missing for day 29 of fermentation for FAME-fed cultures, since only one duplicate developed MELs beads

One of the most important parameters determining whether a process is efficient or not is yield of unit of product produced per unit of substrate added. In order to compare this parameter for fermentations with a single bead recovery and the ones with multiple harvests, yields have been calculated based on the total dry mass of beads that were collected. Data comparisson of mass of beads collected, yields per unit of lipid substrate added, as well as total yields, is presented in Table 4.2.

Table 4.2 Comparison of data for single and multiple bead recovery approaches in terms of yield. * - only one duplicate developed MELs beads.

Substrate	**FFA**		**FAME**	
Fermentation duration (days)	12	24	12	29
Total glucose added ($g.L^{-1}$)	40	40	40	40
Total lipids added ($g.L^{-1}$)	20	80	20	100
Number of bead harvests	1	4	1	5*
Total mass of MELs in beads recovered ($g.L^{-1}$)	4.49	20.857	6.53	27.572
q_{MEL} **($g_{MEL\ in\ beads}.L^{-1}.day^{-1}$)**	0.374	0.869	0.544	0.951
Y'_{MEL} **($g_{MEL\ in\ beads}.g^{-1}_{lipidic\ substrate\ added}$)**	0.224	0.261	0.326	0.276
Y''_{MEL} **($g_{MEL\ in\ beads}.g^{-1}_{total\ substrate\ added}$)**	0.075	0.174	0.109	0.197

* - only one duplicate developed MELs beads.

qMEL – MEL (present in the form of beads) productivity ($g_{MEL\ in\ beads}.L^{-1}.day^{-1}$).

Y'_{MEL} – MELs yield considering the lipidic substrate added ($g_{MEL\ in\ beads}.g^{-1}_{lipidic\ substrate\ added}$)

Y''_{MEL} - MELs yield considering the initial D-glucose and further lipidic substrate added ($g_{MEL\ in\ beads}.g^{-1}_{total\ substrate\ added}$)

The yield in terms of product to lipid substrate of this process is lower, compared to a fermentation with a single bead recovery on the last day of fermentation, since a significant part of the lipids is spent on cell maintenance, growth, and energy. However, if we take into consideration that only one carbohydrate feed was performed (on day 0) for all fermentations, and we compare yields of MELs per total substrate added, the multiple harvest approach proves to be beneficial, increasing the yield by 130% and 81% for FFA and FAME as substrates, respectively. Also, in terms of productivity (expressed as mass of MELs per volume of broth and total fermentation duration), the multiple harvest approach is more efficient since multiple lag periods and exponential growth phases are avoided.

The increase of the time span between two bead collections (the time it takes for the culture to produce enough MEL for the bead to form) indicates that the conditions in the fermentation worsen with the fermentation progression. This is understandable, since only the lipid-based substrate is added. As stated before, the initial hydrocarbon feed (at day 0) is performed to promote the development of the enzymatic complex of the culture. Furthermore, other key nutrients, nitrates, sulphates, as well as micronutrients present in the yeast extract, are crucial for basic metabolic functions of the culture, and are probably depleted during this extensive fermentation period. At a certain point, the culture's metabolism is in such a bad condition that MELs production drops, as well as the ability to consume lipids (indicated by the increasing lipid levels between bead harvests). Furthermore, older yeast cultures tend to become more hydrophobic, as reported (for *S. cerevisiae*) by Powell et al. (Powell, Quain, and Smart 2003), and we can assume that the reason the beads form more slowly, and even stop forming altogether after a certain point in the fermentation, is due to increasing amounts of MELs adsorbed the cells.

Still, we need to take into consideration the MELs that were remaining in the broth at the end of the fermentation period, which can be extracted using the conventional method (with ethyl acetate). These MELs also contribute the overall process productivity. After new beads stopped forming, analyses were performed by GC to determine MELs concentration in the residual broth. These results are present in Figure 4.11. Also, in Table 4.3, another analysis of yields and productivity was performed, this time with taking into consideration the total MEL (from beads and residual broth) for both the standard fermentation protocol and the extended fermentation with multiple bead harvests.

Table 4.3 Comparison of data in terms of yield and productivity for regular 12-day fermentation, with single extraction of total MEL; and multiple bead recovery approach, with MELs recovery from residual broth.

Substrate	**FFA**		**FAME**	
fermentation duration (days)	12	36	12	36
total glucose added ($g.L^{-1}$)	40	40	40	40
total lipids added ($g.L^{-1}$)	20	80	20	100
number of bead harvests	1	4	1	5
total MELs recovered ($g.L^{-1}$)	11.89	32.353	12.03	70.69
MELs productivity (g of MELs / l day)	0.991	**0.899**	1.003	**1.245**
yield (mass of MELs / mass of lipids added)	0.594	**0.404**	0.602	**0.448**
total yield (mass of MELs / mass of substrate added)	0.198	0.269	0.201	0.320

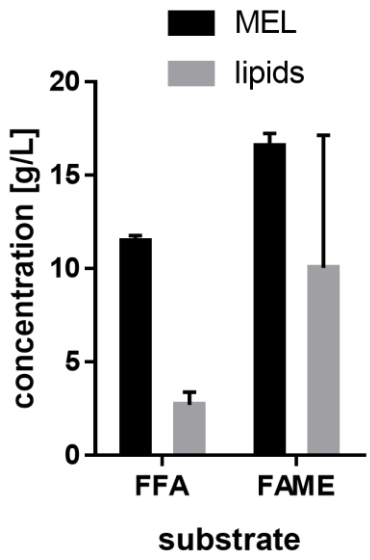

Figure 4.11 GC results for MELs in broth on day 36, after multiple bead collections, for cultures fed with FA and FAME-based substrate.

What can be observed is that large amounts of MELs remained dissolved in the broth, without forming beads. This is probably due to high hydrophobicity of mature cells, as discussed before, causing MELs to adhere to the cell walls. Cultures fed with FFA contained less residual MEL. However, a longer period between the last lipid addition (12 days) was observed. Most of the lipids were consumed, leaving the extract with 80% purity. As for the cultures fed with the methyl-ester based substrate, the flasks had a widely different residual levels of lipids, indicated by the large error bar. One, from which no bead was removed on day 29, contained more residual lipids, which the cells were unable to consume fast enough to produce MELs, so the extract purity was only 48%. The other one, however, contained fewer residual lipids, with extract with 85% purity.

We can consider a production process where beads are removed when they appear, and fresh lipid substrate is added, with the residual broth used for extraction, as an alternative to the conventional process where only the total broth is used for extraction after 12 days of fermentation. Comparing the performance of these processes, as it was done in Table 4.3, we can see that the extended fermentation with multiple MELs bead harvests is beneficial in terms of total yield. If the MEL from the residual broth is extracted when the last set of beads is collected, this would also cause a great increase of MELs productivity.

4.5 Conclusions

Native lipases produced by *M. antarcticus* were used to partially hydrolyze vegetable oil, forming free fatty acids and alcohol esters, which were used for MELs production in the main fermentation. Feeds of pre-hydrolyzed and esterified oil increased MELs production efficiency by the culture, by increasing MELs titers, reducing levels of residual triacylglycerols in the

broth, with the prospect of shortening the fermentation duration. Based on the presented results, with these feeds lipase hydrolysis is no longer the bottleneck of the fermentation.

Furthermore, it was observed that feeds rich in fatty acids and methyl-esters promoted MELs bead generation in earlier stages of the fermentation, with most of the total MELs present being in bead form. As these beads are easy to remove, due to their stiffness caused by high MELs content, they can be collected and treated separately, enabling a simplified downstream and lower need for solvents needed for extraction. Industrial side-streams rich in free fatty acids, such as those obtained from biodiesel production, could be potentially used as feeds or to enrich the feeds used in MELs production fermentations.

Furthermore, multiple "harvests" of beads are achievable, as more substrate can be added after the removal of the beads. This approach increases MELs productivity by almost double, by eliminating multiple lag periods and exponential growth phases. Also, it increases the cost efficiency in a scenario where MELs are produced industrially, since there is less down time - non-productive fermentation time and time spent on culture preparation. However, other micronutrients (which could be possibly added in the form of yeast extract) seem to become depleted over time. Their potential addition to fermentations with multiple bead harvests should be explored, to prevent culture degradation. Also, the culture could be refreshed periodically since cell aging has a possible negative effect on bead formation, due to changes in cell surface. When considering both MELs in bead form, as well as residual MELs in the broth that can be extracted using the conventional extraction method, yields and productivity are even higher.

Overall, this approach including pretreatment of substrate using native lipases - suggests a more efficient and potentially more sustainable alternative for MELs production when compared with the existing processes of using vegetable oils.

Chapter 5

5 Novel process for *in-situ* harvesting of MEL-rich beads from fermentation broth

5.1 Abstract

In the previous chapters, it was shown that when Mannosylerythritol lipids (MELs) are produced by *Moesziomyces* spp. using lipid as substrates, this poorly soluble biosurfactant tends to form beads rich in the product. MELs are normally recovered from the fermentation broth using a liquid-liquid extraction with similar volume ratio of organic solvent to fermentation broth. Therefore, recovering those beads without the use of solvents or reducing solvent intensity would increase process sustainability. In the previous chapters, shake flask studies proved that the fermentation can be continued, after harvesting these beads, by adding more lipidic substrate. To enable this in a bioreactor, without interrupting and jeopardizing the fermentation, a 3D-printed device for bead harvesting was designed, prototyped, and validated in bioreactor operations. This device works on the principle of a modified crossflow macrofiltration, enabling to trap MEL-rich beads in one of its chambers, as fermentation broth with cells is recirculated through the device from and back to the bioreactor. This new device can be installed in any bioreactor and does not create a danger of contamination as it is a closed system. The device design and operation parameters were set to avoid cell damage by shear stress. The device was tested in a bioreactor with 4 L working volume, using D-glucose (at the beginning of the fermentation) and waste fried oil (WFO) as the secondary carbon source. The fermentation was operated with 20 $g.L^{-1}$ feeds of WFO, added on day 0 and day 4 and day 7. The first harvest of beads took place 7 days after the fermentation started, yielding a total of 12 $g.L^{-1}$ in MELs (i.e., g of MELs in the beads divided per broth volume) with 65% (w/w) purity. Such MELs were produced from the first two feeds of 20 $g.L^{-1}$ WFO, added on day 0 and day 4. After another 4 days (i.e., at day 13 of culture), a second harvest of beads was carried out yielded, this time with 8 $g.L^{-1}$ in MELs at 70% purity. Such MELs were produced from the 20 $g.L^{-1}$ of WFO fed on day 7, added just after the first bead retrieval. Further processes optimizations are required to secure good and continued culture performance. Still, the

proposed process setup enables higher MELs productivity, as it avoids time consuming multiple exponential growth phases and reduces bioreactor preparation operational costs.

5.2 Introduction

Mannosylerythritol lipids (MELs) are a group of extracellular glycolipid surfactants, produced by using carbohydrate- and lipid-based substrates as carbon sources, as well as glycerol, alkanes, among other substrates. The selection of substrate has an influence on MELs productivity and characteristics (Günther et al. 2015). High titers of MELs are obtained using vegetable oils as substrates for *Moesziomyces* strains. (Rau, La, et al. 2005)

MELs tends to form beads in the broth (Goossens et al. 2016), presumably due to the low solubility of this biosurfactant. However, these beads appear and disappear unpredictably, and there is a lack of understanding of the underlying variables that cause this. These beads contain varying levels of lipids, biomass, and water. The appearance of these beads is favourable, as they can be collected and processed instead of extracting MELs from the complete fermentation broth with solvent intensive methods such as liquid-liquid extraction. This simplifies downstream processing and requires lower quantities of organic solvents to be used.

MELs are found in the following states in the fermentation broth:

- Free MELs - dissolved in liquid (normally at low levels). These levels rarely exceed few grams per litter, which can be determined by measuring MELs in the supernatant obtained by the removal of cells and beads after centrifugation
- MELs in beads – MELs agglomerated into beads of varying consistency, colour, and size based on their composition and mixing regime (the beads are typically larger in shake flasks incubated in orbital shakers than in bioreactors with more turbulent mixing obtained by action of immersed impellers)
- Intracellular MELs – usually present in relatively low levels, as intracellular storage materials in lipid bodies, which are mostly made out of lipids. (Leu et al. 2020)

- Pericellular – surrounding the cell, forming a certain pericellular matrix, or adsorbed on the surface of the cell (absorption onto producer cells was reported for other biosurfactants (Zhong et al. 2007)

As this chapter is focused on the phenomenon of bead formation, an overview will be made of the observations made so far regarding factors that influence their appearance and properties. Then, the process design will be presented which includes *in-situ* harvesting of beads; a novel harvesting device design and respective prototype will be presented. The functionality of the prototype will then be validated in a bioreactor fermentation, for proof of concept.

5.3 Materials and methods

5.3.1 Microorganisms and maintenance

Moesziomyces bullatus PYCC 5535^{T} (CBS 6821) was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal. Strains were plated on YMA (yeast extract (t.a., Oxoid LTD) 3 g L^{-1}, malt extract (t.a., Oxoid LTD) 3 g L^{-1}, peptone (t.a., BDH) 5 g L^{-1}, D-glucose (p.a., Fisher Chemicals) 10 g L^{-1} and agar (JMVP) 20 g L^{-1}) and incubated for 3 days at 30 °C. Cultures were kept at 4 °C and renewed every week and stored at −80 °C in 20% (w/v) glycerol (≥99.5%, JMGdS)

5.3.2 Media and cultivation conditions

Erlenmeyer flasks were used for preparation of inoculum. Inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of medium containing 3 g L^{-1} $NaNO_3$ (p.a.,

PanReac AppliChem), 0.3 g L^{-1} $MgSO_4$ ((≥99.5%, Panreac AppliChem), 0.3 g L^{-1} KH_2PO_4 ((≥99.5%, Chem-Lab NV), 1 g L^{-1} yeast extract, 40 g L^{-1} D-glucose, and incubated at 27 °C, 250 rpm, for 48 h (orbital incubator, AraLab). Main fermentation was performed in a 5 L bioreactor (BIOFLO 3000, New Brunswick Scientific, USA) with 4 L working volume containing mineral medium (0.3 g L^{-1} $MgSO_4$, 0.3 g L^{-1} KH_2PO_4, 1 g L^{-1} yeast extract) supplemented with 40 g L^{-1} D-glucose. The experiment started by transferring 10% (v/v) inoculum, corresponding to approx. 0.6 g L^{-1} of cell dry weight (CDW), followed by incubation at 27 °C and 1 vvm aeration with filtered air. An agitation cascade system was set up to maintain DO level at 20, by varying agitation between 150-700 rpm. The lipid substrate – waste fried oil (WFO) (obtained from potato frying, provided by McDonalds) was initially added to the broth at a concentration of 20 $g.L^{-1}$, with additional feeds on day 3, and after every bead removal (day 7 and 11).

The lipid substrate – waste fried oil (WFO) was initially added to the broth at a concentration of 20 $g.L^{-1}$, with additional feeds on day 3, and after every bead removal.

5.3.3 Construction of the bead separation device

The device was printed using the MakerBot Replicator 2 3D printer, with 1.75mm Ø Acrylonitrile Butadiene Styrene (ABS) filament. The extruder temperature was set to 210 °C, while the platform was heated to 110 °C. After the pieces were printed, metal rods of 1mm Ø were inserted to form a grid. The openings on both sides were covered with plexiglass plates, to enable visibility inside the device. All the connections were closed with ABS dissolved in acetone (1:1 w/w ratio), which formed a glue which would dry after 24h. The inside surfaces were coated with PDMS (), to increase resistance to solvents of the plastic device.

5.3.4 Growth and biomass determination

Cell growth was quantified by cell dry weight (CDW), using a previously described protocol. (Santos et al. 2019) CDW was determined from 1 mL culture broth by centrifugation at 10 000 rpm for 10 min (Sartorius 1-15P centrifuge), washing with deionized water (twice) and drying at 60 °C for 48 h. Supernatant from the centrifuged samples was collected and used for various analyses.

To determine the viable cells content, samples were diluted and plated on YMA, and formed colonies were counted.

5.3.5 Analysis of fermentable sugars and nitrates

Monosaccharides and nitrates were quantified in the collected sample supernatants using a high-performance liquid chromatography (HPLC) system (VWR Hitachi, Darmstadt, Germany) equipped with a RI detector (L-2490, VWR Hitachi, Darmstadt, Germany), UV-detector (L-2420, VWR Hitachi, Darmstadt, Germany) and a RezexTM RHM-Monosaccharide H+ (8%) column (300 mm × 7.8 mm, Phenomenex), at 65 °C. Milli-Q water was used as mobile phase at 0.5 mL.min-1.

5.3.6 Gas chromatography (GC) analysis

MELs concentrations in the samples were calculated based on the results acquired by Gas chromatography of methyl-esters, as previously described (Santos et al. 2019). The fatty-acid composition of biological samples was determined by methanolysis and GC-FID analysis of methyl esters. Pure methanol (20 mL) (HPLC grade, VWR Chemicals) was cooled down to 0

°C and 1 mL acetyl chloride (p.a., Sigma-Aldrich) was added to generate a water-free HCl/methanol solution. Culture broth samples (3 mL) were freeze-dried (lyophilizer, Martin Christ GmbH), weight and mixed with 2 mL HCl/methanol solution and incubated for 1 h at 80 °C (Memmert BM400 incubator) for transesterification into methyl esters. Heptanoic acid (p.a., Sigma-Aldrich) was used as internal standard. The resulting product was extracted with hexane (1 mL) (HPLC grade, Fisher Chemicals) and 1 µL of the organic phase was injected in a GC system (Hewlett-Packard, HP5890), equipped with an FID detector and an Agilent HP Ultra2 capillary column (L 50 m × I.D. 0.32 mm, df 0.52 µm). The oven was programmed to an initial temperature of 140 °C and three temperature gradients were defined: 140 to 170 °C at 15 °C min^{-1}, 170 to 210 °C at 40 °C min^{-1} and 210 to 310 °C at 50 °C min^{-1}. A final time of 3 min at 310 °C was defined. Carrier gas was used with a split of 1/25. MEL were quantified through the amount of C8, C10 and C12 fatty acids 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 quantification of glycolipids based on a specific moiety was previously described by Faria et al. (Faria, M. V. Santos, et al. 2014)

All lipids and lipid derivatives (free fatty acids, mono-, di- and triacylglycerols) are represented as lipids in graphs.

5.3.7 Analysis of fatty acids, mono-, di-, triacylglycerol, and fatty acid alcohol esters concentrations

The analysis of fatty acids, mono-, di-, triacylglycerols and fatty acid alcohol esters content was performed using a method developed by Badenes et al [11]. Samples of supernatant (200 µL) were mixed with 1 µL of acetic acid 58.5 mM and 799 µL of n-hexane and centrifugated at 10000 rpm for 2 min. The organic phase was recovered and analyzed by high-performance

liquid chromatography (HPLC) using a ChromolithPerformanceRP-18 end capped (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).

5.4 Results and discussion

5.4.1 Overview of factors affecting beads formation

The factors that influence the presence of beads in the broth are not well understood. One of the first reports mentioning beads formation during MELs production fermentations was authored by Rau et al. (Rau, Nguyen, Roeper, et al. 2005). In this report, it is hypothesised that beads formation is an "indicator of enhanced MELs production", and it was observed that beads formation occurs when titres of MELs are above 40 $g.L^{-1}$. However, as beads appear at various concentrations of MELs, and disappear in later stages of the fermentation without a drop in the concentration of the biosurfactant, MELs concentration cannot be the only parameter influencing their formation. Namely, if the MELs production is performed rapidly, beads will form in early stages of the fermentations (before day 7-8), despite the relatively low MELs concentration. When beads are present, most of the MELs are in bead form, and the bead-free broth contains low levels of the biosurfactant.

In the 12- to 14-day fermentations performed in the previous chapters, the beads usually appear at day 7-9 and disappear after day 10-11. The fermentation is usually not stopped when beads formation is observed, as the levels of unmetabolized lipids is still high, and longer fermentation cultures increases both MEL titres and purity.

M. bullatus presents more beads than *M. antarcticus* in similar fermentation conditions, despite the generally lower MELs productivity obtained with the former microorganism. As one of the main differences between these two microorganisms is their physiology (as pictured in Figure 2.4), there might be a relationship between culture organization into small single cells and bead formation. *Moesziomyces bullatus* cells might detach from each other after reproduction (and in this way avoid filamentous growth) due to the low cell hydrophobicity (Brito et al. 2020), and this same property might prevent formation of pericellular MELs structures (surrounding cells and absorbed onto them). Finally, lowering cell hydrophobicity causes beads to appear more (as suggested in Chapter 3).

In the previous chapters was studied (i) the effect of osmotic stress on cell surface properties and (ii) the effect of the pretreatment of lipids fed as carbon source for MELs production. Such studies provided insights on possible feed strategies to enable the highest yields of MELs-rich beads. A brief follow-up study, using two different microorganisms - *M. antarcticus* and *M. bullatus*, made within our team allowed us to conclude that, for *M. bullatus*, an initial addition of oil can be made, which enables bead formation after 5-6 days. By applying pretreated lipid feeds, mainly fatty acid methyl esters, this period was made even shorter.

A series of fermentations were carried out, with different working microorganisms, media, substrate, and feed strategies (Table 5.1). Flasks of 250 m, with a working volume of 50 ml, were incubated at 27°C for 12 days, on an orbital shaker with 250 rpm. Bead appearance and progression was visually controlled, and changes in color and size (a rough estimation of bead diameter) was tracked.

Table 5.1 Bead presence and appearance for different experimental setups: working microorganisms, substrates (SBO - soybean oil, FFA - free fatty acid, FAME - fatty acid methyl esters, as used in Chapter 4) and feed strategy (day of lipid substrate addition). Color of field

indicates the color of beads on certain day, and sizes expressed as: T - tiny - <1 mm; S - small - 2-5mm; M - medium - 5-10 mm; L - large - >10 mm.

Microorganism	Substrate	Day of addition	day											
			1	2	3	4	5	6	7	8	9	10	11	12
M. antarcticus	SBO	4							T	T	S	S	M	
M. antarcticus	FFA	4						S	M	M	L			
M. antarcticus	FAME	4						S	M	M	L	L		
M. bullatus	SBO	4							S	M	M	L		
M. bullatus	SBO	0				S	M	L	L	L	S			
M. bullatus	FAME	0			S	M	L	L	M					

In the previous chapters, it was studied the effect of osmotic stress on cell surface properties (chapter 3) and the effect of the pretreatment of lipid feeds of MEL production (chapter 4). This chapter includes a study the effect of feed strategies on MEL-rich beads yields, using two different microorganisms - *M. antarcticus* and *M. bullatus*. As stated before, within our team, preliminary studies suggested that, for *M. bullatus* cultures, an initial addition of oil enables bead formation after 5-6 days. This is strategy is less successful in promoting significant formation of MEL-rich beads when *M. antarcticus* is used. Moreover, applying pretreated lipid feeds, mainly fatty acid methyl esters, this period was made even shorter.

A more comprehensive study on the effects of calcium presence in the growth medium which would stimulate CAL-A activity for both *M. antarcticus* and *M. bullatus*, is presented in Appendix 10.2.

In Chapter 4, the results show that multiple "harvests" of beads are possible in a single shake flask fermentation, by collecting the beads when they are fully developed (large, dark orange colour), and the fermentation can continue by adding more hydrophobic substrate. This approach increases MELs productivity by almost double, by eliminating multiple lag phase periods and exponential growth phases for biomass build up. Also, it potentially decreases MEL production costs in a scenario where MEL is produced industrially, since there is less down time, i.e., non-productive fermentation time and time spent on culture preparation. However, other micronutrients (added through the yeast extract included on initial culture medium composition) seem to become depleted over time and should be added to prevent culture degradation. When considering the residual MEL left on the broth, after MEL-rich beads harvesting, yields and productivity are even higher with the multiple harvest approach than the single stage feed batch culture. MEL production behaves as of a secondary product, thus adding feeds of the hydrophobic substrate promotes mainly MEL production, while the initial addition of the hydrophilic substrate is spent on cell proliferation. Residual MEL left on the fermentation broth, after beads harvesting, can be used as an aqueous solution product (after centrifugation for cell removal) or upon recovery using the conventional extraction method.

5.4.2 Designing and prototyping a device for *in-situ* harvesting of MEL-rich beads from a bioreactor

Concept: In the case of shake flask experiments previously reported on chapter 4, the beads were removed using straining, while the bead-free broth was transferred to a clean flask and more lipidic substrate added for further MEL production.

Collecting MEL-rich beads from a bioreactor without disturbing the fermentation is more challenging. The beads have to be physically removed from the culture at the right culture time,

with minimal losses of broth; since they would dissolve as fermentation progresses and broth properties change. Moreover, it is crucial to secure sterility of the culture and avoiding invasive bead collection strategies which would lead to contamination. For this purpose, a 3D printed device was designed (Figure 5.1). This device is an external macrofiltration vessel, with grid openings of 1 mm, able to collect beads when they reach a sufficient large size, and allowing small beads, biomass, and the remaining liquid fermentation broth to pass through the grid gaps and return to the bioreactor. The dimensions of the grid gaps and flow rates used were selected with the intention to avoid cell death due to shear stress.

Prototyping: The device was extruded by 3D fused deposition modelling of ABS, and then coated with PDMS for cytocompatibility, solvent resistance and impermeabilization. The prototype device prepared for an initial proof of concept assessment is able to collect 100 ml of beads under sterile conditions. Such dimension is adequate to be coupled with our bioreactors of 1.5L to 4L working volume, depending on the amounts of MEL-rich beads formed. However, this device is fully scalable.

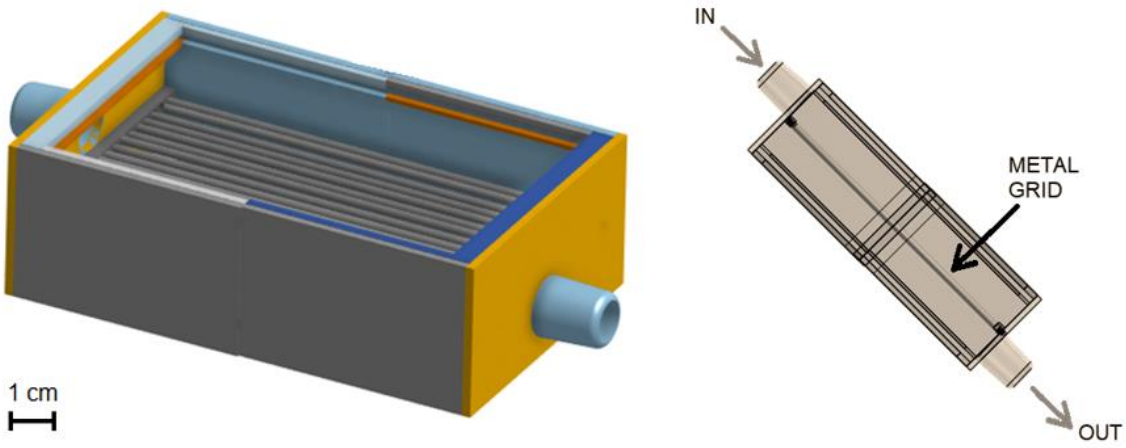

Figure 5.1 Left: 3D printed macrofiltration device. Top cover removed for visibility of the interior. Right: Principle of operation of the device. The device is placed at an angle, in order to enable gravitational forces to aid separation, reducing hydrodynamic stress on the beads which could deform them.

Operation settings: The device is operated by pumping the fermentation broth with MEL beads out of the bioreactor through the device and device grids, where MEL-rich beads remained trapped within the top chamber. Broth and small beads pass through the device and are returned to the bioreactor. The tubes collecting the beads into the device and connecting the device to the bioreactor were selected to provide sufficient large diameter (Ø ~1.2 mm) to allow large MEL beads pass. A peristaltic pump, place after the device and before the bioreactor, is used to pull the liquid fraction already depleted o the large beads. After the large beads have been separated, the device can be opened to recover them (which in this case would result in a relatively pure solvent-free product), or a solvent such as an alcohol can be pumped into the device through a separate tubing system, to dissolve the beads. Different solvents can be used for this purpose. For MEL liquid-liquid solvent extractions usually is used ethyl acetate. However, for this particular operation, methanol, a polar solvent of low boiling point, was selected to prevent interactions with the plastic device and facilitate solvent recovery. After

these operations, the device is ready for the next cycle of bead collection. In the current work a prototype was manufactured and used with materials easy to extrude and apply at laboratory scale. However, industrial applications of this device would require more robust materials to be used. The presence of methanol has negative impact on steel and titanium used on connections, and alternative materials should be considered for large scale production. The presence of trace amounts of methanol, carried out from the device to the bioreactor, is not an issue, as methanol can be metabolized, and it has a simulative effect on the cell metabolism (as described in chapter 4). The process diagram is presented in Figure 5.2.

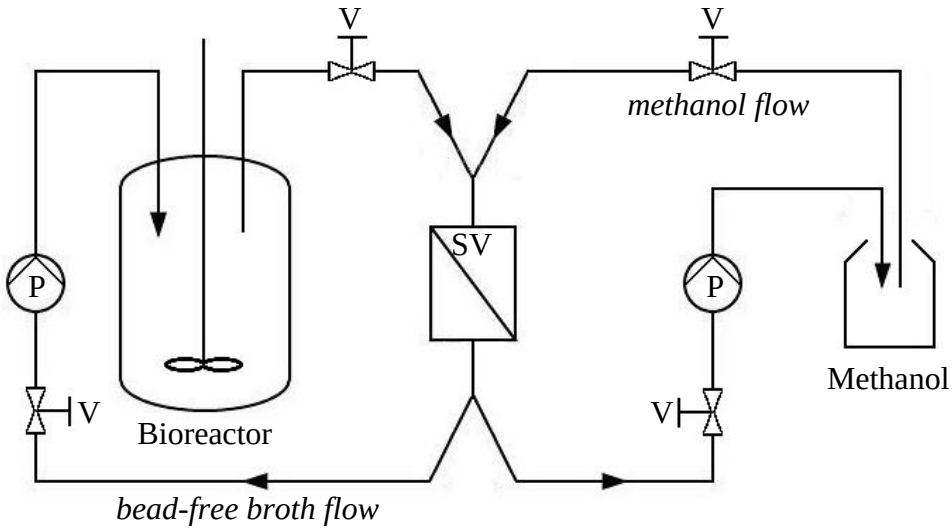

Figure 5.2 Diagram of proposed MEL production process, with bead separation and recirculation of bead-free broth. SV - separation vessel; V - valve; P - pump (peristaltic).

5.4.3 Validation of the prototype device at laboratory scale: in-situ MEL-rich beads harvesting from a bioreactor

The fermentation was carried out in the bioreactor with 4 L working volume, using D-glucose as initial carbon source. An initial feed of WFO was added at day 0, with an additional one at

day 3. *Moesziomyces bullatus* was used as the working microorganism, as it is more productive in terms of bead formation. The progression of bead development is given in Table 5.2.

Table 5.2 Progression of MELs bead development during fermentation in bioreactor. Avg. diameter of beads: S - > 2mm; M - 2-8 mm; L < 8 mm. Colours of field indicate bead colour. * - day of addition of WFO; † - day of bead removal

Day	0-2	3	4-5	6	7	8-9	10	11	12-16
Beads	/	S*	/	M	L*†	/	S	M*†	/

Beads were appearing faster in the bioreactor assay than in shake flasks assays, where it took one day longer for them to form after the addition of the oil. In the bioreactor, the beads were oblong, and uniform in shape and size, which was not the case for the shake flasks, where the beads had larger diversity in size and shape. The faster formation of the beads might be due to the better control of parameters and better supply of oxygen in the bioreactor, while the uniformity among the beads might be due to more severe agitation with the impellers. Samples were periodically taken from the bioreactor and analysed for key parameters. These profiles are shown in Figure 5.3.

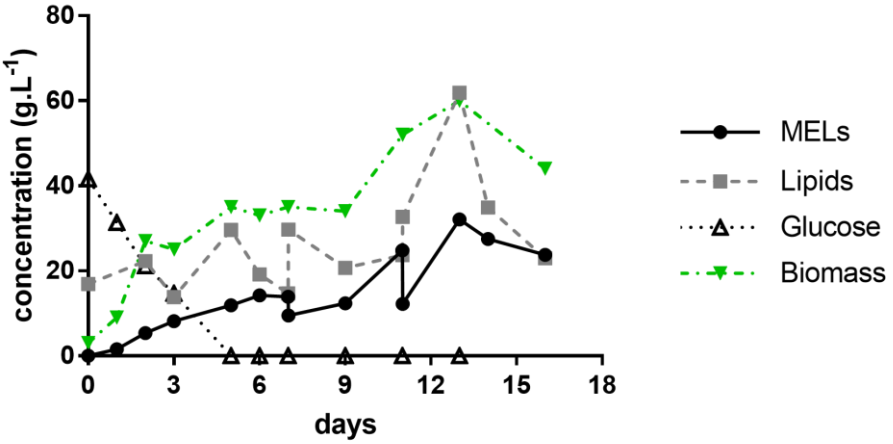

Figure 5.3 Profiles of key fermentation parameters in bioreactor. WFO addition on days 0, 3, 7, and 11. Bead retrieval on days 7 and 11. MELs quantification in this figure is underestimated as it results from a fermentation broth heterogeneous sampling, typically without including larger MEL-rich beads.

D-Glucose was consumed in the first few days and enabled the formation of a significant amount of biomass. Although the concentration of lipids remained higher than the concentrations of MELs estimations, one should consider that precise estimation of MEL concentration is challenging when beads are present. The estimation of MEL concentration samples of the fermentation broth, that were analysed to form the profile in Figure 5.3, include contributions from the diluted, intra- and pericellular MELs, as well as MEL in some smaller beads. For a more precise estimation of MEL concentration in the fermentation, the bioreactor would have to be stopped and the whole fermentation broth would have to be retrieved and analyse, including the beads present at such time. With the designed device, this is possible without disturbing the flow of the fermentation, as shown in Figure 5.4. The whole recovery procedure took 10 minutes to remove about > 80% (rough estimation) of the beads from the 4-litre working volume. The flowrate of solution through the device was roughly 1.4 $L.min^{-1}$, and

3.5 volumes of the fermentation broth was recirculated through the device to retrieve the beads. On the days on which the beads were collected, their contents were analysed.

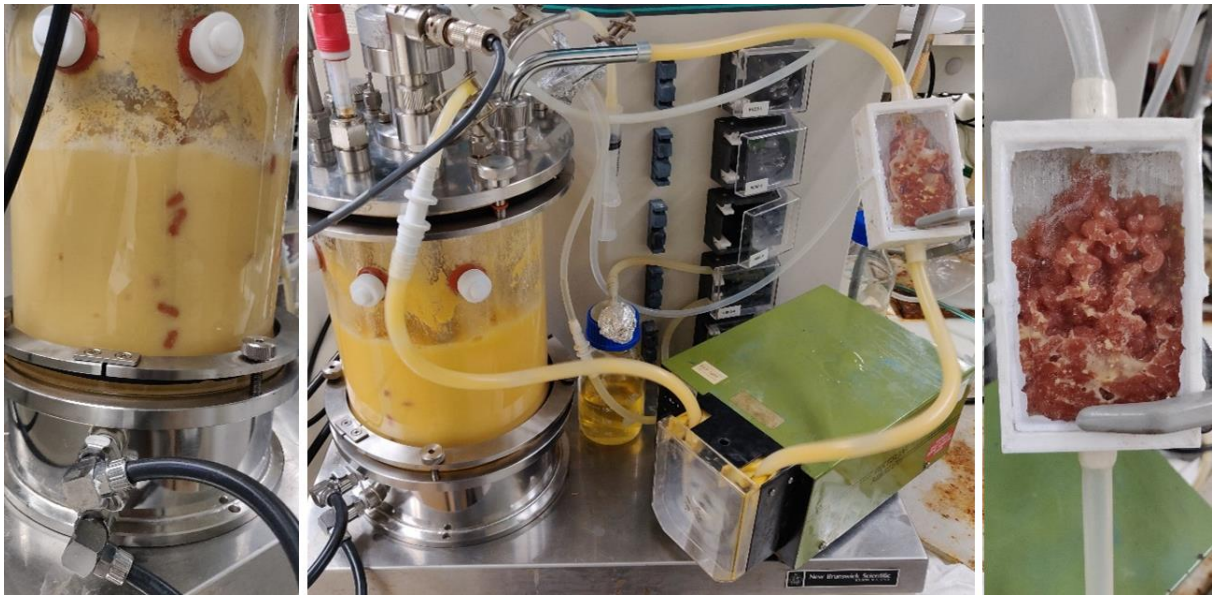

Figure 5.4 Left: Fermentation broth on day 7 (beads visible); Middle: Setup for bead collection with bead separation device; Right: Separation device. Beads forming a solid mass on front side of grid, while broth circulates freely back to the reactor.

The mass of collected beads was estimated by weight. Only ~100 mL of the methanol was circulated through the system to wash out the beads from the device (Figure 5.5); representing a great reduction in solvent requirements when compared to the 8 litres of ethyl acetate needed for two extractions of MELs using 1:1 solvent/broth volume ratio. The methanol was evaporated, and the beads weight was measured before and after drying at 60 °C for 2 days, to quantify their dry mass content. To estimate biomass content on the beads, those were then dissolved in ethyl acetate, the resulting solutions were centrifuged, and the obtained pellets dried, and the measured weight reported as biomass content. Finally, the solvent of the remaining ethyl acetate solution, containing MELs and lipids was evaporated and the

concentration of MELs and lipids, which include free-fatty acids, mono-, di-, and tri-acylglycerols were determined in the residue obtained. This data is presented in Figure 5.6.

Figure 5.5 The collected beads recovered from the bioreactor dissolved in methanol.

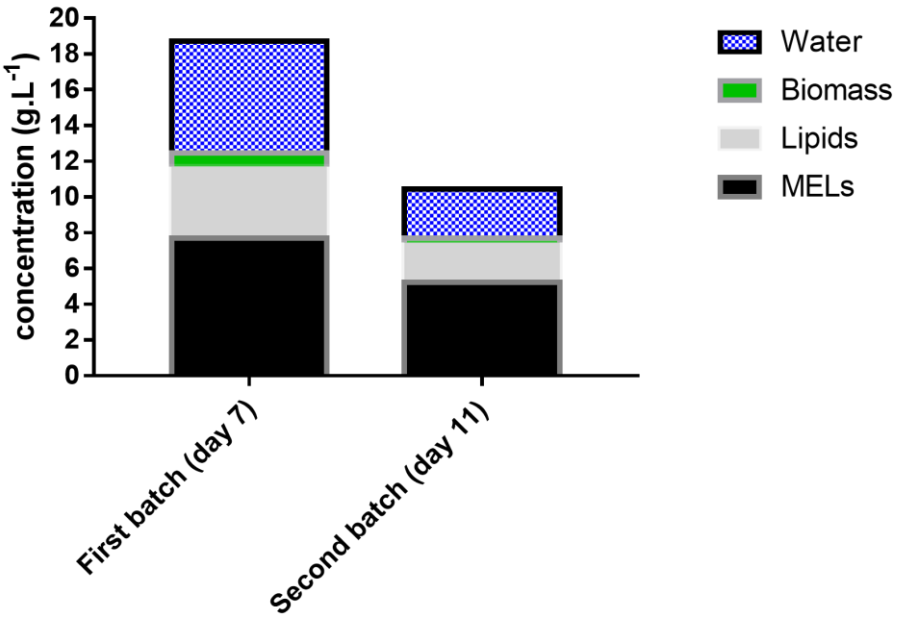

Figure 5.6 Composition of beads collected from the reactor. Concentrations given as grams per litre of fermentation broth volume.

The first harvest of MEL-rich beads, collected on day 7, yielded ~12 $g.L^{-1}$ of MELs with purity of 65%. This MEL was obtained from the two feeds of 20 $g.L^{-1}$ of WFO, on days 0 and 3. The second harvest of MEL-rich beads yielded 8 $g.L^{-1}$ of MELs with 70% purity and it was obtained from the following 20 $g.L^{-1}$ of WFO fed at day 7 after the first harvest. Both beads harvests had similar water content (33.4% and 26.33%, for the first and second harvest respectively).

After the second harvest, another 20 $g.L^{-1}$ of WFO was fed at day 11. However, formation of beads did not reach a sufficient high level, within the 16 days fermentation time, to justify the application of the device.

Considering the 11-day fermentation (i.e., disregarding the non-productive period of day 11 to 16), the two bead collections resulted in ~20 $g.L^{-1}$ of MEL, with 67% purity, in bead form using as carbon source feeds of 40 $g.L^{-1}$ of D-glucose and 60 $g.L^{-1}$ of WFO (over 3 feeds on days 0, 3 and 7). The resulting yield is 0.134 $g_{beadMELs}.g_{substrate}^{-1}$. Although these results might seem underwhelming, it should be highlighted that while the device only collected the large beads, the residual broth contains still significant amounts of MELs. The data presented in Figure 5.3 indicates that the remaining broths, after the removal of large beads, contains on day 7 and day 11 another 9.52 $g.L^{-1}$ and 12.24 $g.L^{-1}$ of MEL, respectively. The bead-free residual fermentation broth could be treated as an additional product, as it contains a significant amount of MELs as well as lipases and yeast cells.

5.5 Conclusions

Fermentations aiming at MELs using vegetable oils as substrates resulted in the formation of product-rich beads, due to high product titres. Shake flasks studies indicates that MEL fermentations can be performed in fed-batch culture, with multiple additions of the hydrophobic substrate. (Goossens et al. 2016) However, removing the MEL beads before the addition of

more lipid substrate promotes further extension of the fermentation and a route for product harvesting. This strategy allows to avoid several lag periods and growth stages for biomass build up and to decrease the time between the addition of the substrate and MEL collection from ~9-10 days to 4-5 days. MELs losses are reduced, as the dissolved MEL and small MEL beads are returned to the continuing fermentation feeding further formation of MEL-rich beads. Also, performing a single fermentation reduces the need for water used for the preparation of the growth medium, and reduces consumption of energy used for cleaning the bioreactor and sterilizing the medium. To enable the aforementioned strategy and in-situ MEL-rich beads harvesting, a beads collector microfiltration device was designed and applied on a 4 L bioreactor fermentation.

The feeding strategy and process parameters (agitation speed, reactor design) could be optimized, to secure production of larger and more rigid beads, which would simplify and increase the efficiency of the harvesting process. Additionally, larger feeds could be used to secure the production of beads over multiple times. Finally, additional nutrients could be fed, to avoid culture media components exhaustion. Collecting and treating the MEL-rich beads is beneficial compared to the conventional MELs collection method. Usually, MELs downstream comprises a liquid-liquid extraction, where the whole fermentation broth is extracted several times (at least two) with ethyl acetate (1:1 V). The use of the suggested method, employing the designed device, consumes only 5-10% of broth volume in methanol. This simplifies the downstream treatment and increases the overall sustainability of the MEL production process

Chapter 6

6 Effect of substrate selection on *Moesziomyces antarcticus* - impact on cell morphology and MELs production

6.1 Abstract

Mannosylerythritol lipids (MELs) are produced by a range of microorganisms. *Moesziomyces* genus yeasts seem to be the most promising producers in an industrial scale. The morphology of the cells in the broth was found to be related to their performance – when species such as *M. bullatus* are used (which grows as single-celled forms, suggesting reproduction by budding), a higher production of MEL-rich beads is achieved, despite the relatively lower total MELs concentrations. A relation between distinct cell morphologies and organization with cell hydrophobicity has been previous reported. (Min et al. 2020) On the other hand, it has also been reported that accumulation of intracellular glycerol, used to fight osmotic stress is accompanied by surface changes in the cells that affect their hydrophobicity. Thus, here it was hypothesized that the use of glycerol, instead of D-glucose, can have an impact on cell morphology and performance concerning MELs production. Glycerol was used as the main carbon source in various stages of the fermentation – inoculum preparation and main fermentation stage (including exponential growth and stationary phases). The use of glycerol in any stage resulted in a formation of a higher number of individual detached cells, and affected cell interaction, as indicated by sedimentation rate estimation. Although glycerol, when used during the main fermentation stage affected cell performance negatively, it was found to be beneficial when used for inoculum preparation. It had a lasting effect on the cells, promoting beneficial morphological changes, even when D-glucose was used as carbon source in the main fermentation, and resulted in a drastic increase of MELs beads production.

6.2 Introduction

MELs are a group of glycolipid biosurfactants, whose structure and properties have been discussed in detail in previous chapters. They are produced by microorganisms from the *Ustilaginaceae* family: yeasts of the *Moesziomyces* genus and the *Ustilago* smut fungi. This chapter is focused on production of MELs by *M. antarcticus* as one of the more promising working microorganisms, as it enables high product titres and efficient substrate to product conversion. (Kitamoto, Fuzishiro, et al. 1992; Morita et al. 2007a)

As mentioned before, MELs can be produced using a variety of carbon sources, both hydrophilic and hydrophobic. However, such substrates not only follow different metabolic pathway for MEL building blocks synthesis, but also affect the morphology of *M. antarcticus*. The goal of this chapter is to study how the use of hydrophilic substrates, D-glucose, and glycerol, in different stages of the fermentation affect the cell culture morphology – cell shape, size, flocculation, and proliferation. Finally, MEL production was quantified for the different conditions assessed.

6.2.1 Yeast cell organization

Usually, yeast-shaped cells perform better in large scale process than filamentous ones. Smaller, individual cells are more efficient in terms of mass transfer exchange. Filamentous cells form more agglomerates - biomass pellets, within which oxygen and substrate availability is lower and inhibitory products tend to accumulate. (Antecka, Bizukojć, and Ledakowicz 2016; Nielsen et al. 1995) Yeast shaped biomass also performs better in a large-scale bioreactor due to higher resistance to sheer stress caused by mixers, enabling more efficient parameter control. (Amanullah et al. 2002; Papagianni 2004)

In general, the ability to generally grow in a yeast-shaped morphology and avoid filamentous growth in submerse cultivation conditions enabled the use of Basidiomycetous yeasts, such as *M. antarcticus*, in biotechnological production of enzymes and MELs. (Kitamoto, Fuzishiro, et al. 1992)

The genetic similarities between *M. antarcticus* and *S. cerevisiae* were discussed in earlier chapters, as well as relevant stress-response mechanisms share by these species. Delicate underlying processes regulating cell morphology of *M. antarcticus* are less explored, so parallels are carefully made with literature data related to the thoroughly explored brewer's yeast.

Usually, yeasts switch to filamentous growth as a response to environmental signals and stress. (Mutlu et al. 2019) For *S. cerevisiae*, pseudohyphal differentiation is induced by nutrient stress – nitrogen limitation and short-chain alcohol presence. (Lorenz, Cutler, and Heitman 2000; Miled, Mann, and Faye 2001) This response is seen as a survival mechanism, related to foraging for nutrients and toxin avoidance. The underlying mechanisms for pseudohyphal differentiation are proven to involve a mitogen-activated protein (MAP) kinase cascade. (Madhani and Fink 1998; Pan and Heitman 1999) This mechanism was previously theorized in this thesis to be responsible for intracellular glycerol regulation in *M. antarcticus* as a response to osmotic stress. A family of glycosyl-phosphatidylinositol (GPI)-linked glycoproteins participates in adhesion processes of baker's yeast cells, whether for mating, intercellular adhesion or filamentation, or other purposes. (Brito et al. 2020; Guo et al. 2000) Cell adhesion plays a role in pseudohyphae formation, as newly created cells remain attached to the mother cells, forming a linear or branched string of cells.

6.2.2 *Moesziomyces* cell physiology and MELs relationship

In *M. bullatus*, Günther et al. (Günther et al. 2015) describes a relationship between glycolipid synthesis and propagation of morphological changes within the cell cultures. Usually, *M. bullatus* cultures are less filamentation, as can be seen in Figure 2.4. The findings in this paper related triacylglycerol presence in the medium with cell filamentous growth. *M. bullatus* oval cells become more elongated, and formed more lipid bodies, which were hypothesised to have a role in energy storage (Rau, Nguyen, Roeper, et al. 2005). The potential link was established to be a cluster of genes encoding membrane compounds, wall modifying enzymes, which were upregulated in the presence of soybean oil. As for *U. maydis*, it was also found that fatty acids triggered the fungi to grow filamentous. (Klose, De Sá, and Kronstad 2004) Furthermore, glucose seemed to suppress this morphological change in the microorganism. This phenomenon was theorized to be linked to plant pathogenesis - that the lipids acted as ligands to trigger the change into an invasive growth mode.

One of the rare literature reports examining *M. antarcticus'* pseudohyphal morphology concludes that MELs play a role in prompting the switch towards filamentous growth in the yeast. Morita et al. (Morita, Ito, et al. 2010) analysed the properties of a mutant strain in which the gene *ΔPaEMT1*, encoding an erythritol/mannose transferase, was knocked out, and thus MEL production was disabled. This strain tended to form small, yeast-shaped cells. However, when externally produced MELs is added into the broth, the culture started forming pseudohyphae. Similar phenomena was observed on solid-state growth, as was reported in a later paper. (Yoshida et al. 2014) The mechanism by which MELs affect cell culture morphology and organization is poorly understood. One of the possibilities is that MELs molecules adsorbed to the cell's surface affect their hydrophobicity, an important characteristic determining interactions between yeast cells. Other biosurfactants were proved to adsorb onto

the surface of cells and play a role in changes of cell hydrophobicity. The adsorption rate depended on the physiological status of the cells. This caused an interesting phenomenon – hydrophobic binding sites on the cells surface would attract the lipophilic moiety of the biosurfactant, exposing the rest of the molecule to the environment, making in turn the cell surface effectively more hydrophilic. (Zhong et al. 2007)

6.2.3 Yeast cell flocculation

Cell flocculation is another related phenomenon, also observed in *M. antarcticus*, characterized by cells clumping together, forming a pellet of varying density. Cell flocculation might be induced by external factors and employed to facilitate biomass removal from the fermentation broth by sedimentation. (Maekawa and Takegawa 2020) Thus cell flocculation is sometimes of interest for process intensification. However, it is mostly undesirable during fermentation, as cells within the dense floccule have less availability to oxygen and nutrients and are prone to toxic effects caused by accumulation of metabolic products. A variety of factors was proven to cause flocculation in different yeasts, including ions (especially calcium), pH, certain nutrients, and cell hydrophobicity. (Jin and Alex Speers 1998) Flocculation and filamentation are co-related, as part of the mechanisms for stress responses affecting the cell surface. (Chow, Starr, et al. 2019) Many of these topics were brought up in Chapter 3, where osmotic stress was found to induce a reduction in filamentation of *M. antarcticus* (Figure 3.10).

To prevent cell flocculation, as well as adsorption of lipids and MELs to the cell surface, tests were performed with alginate-encapsulated cells, as alginate is largely hydrophilic. These results are presented in Appendix 10.4. Several literature resources report the successful use of immobilized cells for the production of rhamnolipids. (Abouseoud et al. 2008; Bagheri

Lotfabad et al. 2017) The results for MELs production obtained with SBO and glucose for alginate encapsulated cells were worse than for free cells, suggesting that the alginate barrier, which may limit direct access to nutrients, is detrimental for MEL production. Other possible reasons for this poor performance of incapsulated cells could be oxygen limitation, or inhibition by calcium ions (which is used for alginate crosslinking).

6.2.4 Potential effect of substrates on yeast cell organization and research strategy

The effect of two hydrophilic substrates on all these properties was tested. The first substrate was glucose, the most commonly used carbon source. The second substrate was chosen to be glycerol. Besides being a cheap and sustainable substrate, as crude glycerol is an undesired by-product of biodiesel production (Yang, Hanna, and Sun 2012), it plays an important role in yeast's internal metabolism. Namely, glycerol is an osmolyte, present in proliferating yeast cells with the role of maintaining turgor and volume. (Tamás et al. 2003) It is generated rapidly by the yeast's metabolism to combat osmotic shock (Kayingo, Kilian, and Prior 2001), as discussed in Chapter 3. Besides secreting glycerol, same responsory pathways trigger changes in cell surface assembly. (Hohmann 2002a) As all these phenomena could possibly relate to cell hydrophobicity changes, leading to pseudohyphal growth, it was of interest to test glycerol impact on cell morphology.

In line with the research goals for this chapter, two groups of experiments are conducted. In the first, fermentations were carried out in which glucose and glycerol were used as carbon sources exclusively, and the impact of these two substrates on *M. antarcticus* cell physiology is estimated. A comparison is made between the cultures grown with the two substrates in terms of predominant cell structure. Then, the two substrates are used in different stages of the

fermentation, in an effort to determine which feed strategy gives optimal results in terms of cell physiology.

In the second part of the experimental work, the findings from the previous section were used to develop several feed strategies, including glucose, glycerol, and rapeseed oil (RSO), with the intention of exploring the impact of these feed strategies on the production of MELs. An analysis is also made of the impact of glycerol presence in the medium in various stages of the fermentation on the formation of MEL-rich beads.

6.3 **Materials and methods**

6.3.1 Microorganisms and maintenance

Moesziomyces yeast strain *M. antarcticus PYCC 5048^T* (CBS 5955) was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal, and maintained following a previously established protocol (Santos et al. 2019). Strains were plated on YMA (yeast extract (t.a., Oxoid LTD) 3 g L^{-1}, malt extract (t.a., Oxoid LTD) 3 g L^{-1}, peptone (t.a., BDH) 5 g L^{-1}, D-glucose (p.a., Fisher Chemicals) 10 g L^{-1} and agar (JMVP) 20 g L^{-1}) and incubated for 3 days at 30 °C. Cultures were kept at 4 °C and renewed every week and stored at −80 °C in 20% (w/v) glycerol (≥99.5%, JMGdS) to be recovered when necessary.

6.3.2 Media and cultivation conditions

Erlenmeyer flasks were used for preparation of inoculum and batch fermentation. The previously described media and conditions were used (Santos et al. 2019). Inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of medium containing 3 g L^{-1} $NaNO_3$ (p.a., PanReac AppliChem), 0.3 g L^{-1} $MgSO_4$ ((≥99.5%, Panreac AppliChem), 0.3 g L^{-1}

KH_2PO_4 (≥99.5%, Chem-Lab NV), 1 g L^{-1} yeast extract, 40 g L^{-1} D-glucose, and incubated at 27 °C, 250 rpm, for 48 h (orbital incubator, AraLab). Batch cultivations were performed in Erlenmeyer flasks containing 1/5 working volume of mineral medium (0.3 g L^{-1} $MgSO_4$, 0.3 g L^{-1} KH_2PO_4, 1 g L^{-1} yeast extract) supplemented with 40 g L^{-1} D-glucose or glycerol. The experiment started by transferring 10% (v/v) inoculum, corresponding to approx. 0.6 g L^{-1} of cell dry weight (CDW), followed by incubation at 27 °C, at 250 rpm.

In some cases, rapeseed oil (RSO) (refined, Salling Group, Poland), as well as additional feeds of glucose and glycerol, were also added in concentrations indicated in the experimental design.

6.3.3 Morphological structure determination and counting

Cell structures were categorized in four main morphology structures: yeast shaped single cells, pseudohyphae, clumps and pellets (as seen in Figure 6.1). The cells structures were counted at different time points of the culture and the respective profiles over time were reported. Samples were diluted appropriately, and a haemocytometer was used to count the number of occurrences of each structure. Counting was performed in quadruplicates.

6.3.4 Growth and biomass determination

Cell growth was quantified by cell dry weight (CDW), using a previously described protocol. (Santos et al. 2019) CDW was determined from 1 mL culture broth by centrifugation at 10 000 rpm for 10 min (Sartorius 1-15P centrifuge), washing with deionized water (twice) and drying at 60 °C for 48 h. Supernatant from the centrifuged samples was collected and used for various analyses.

In order to distinguish between the mass of small and big cell fractions, samples collected from the flasks were, at selected time points, filtered through 25 μm filter paper (Prat Dumas, France), under a vacuum. The filter paper was dried, and the ratio of small to large cell fractions was estimated based on the CDW in the whole sample, and the dry mass of cells on the filter paper.

To determine the viable cells content, samples were diluted and plated on YMA, and formed colonies were counted (Leica Microsystems microscope).

6.3.5 Analysis of fermentable sugars, nitrates, and glycerol concentrations

D-glucose and glycerol concentrations in the fermentation broths were quantified. Samples of the fermentation broth were collected and centrifuged (10000 rpm, 10 min) and supernatants collected, and when needed diluted. D-glucose and glycerol concentrations, were quantified by a high-performance liquid chromatography (HPLC) system (VWR Hitachi, Darmstadt, Germany) equipped with a RI detector (L-2490, VWR Hitachi, Darmstadt, Germany), UV-detector (L-2420, VWR Hitachi, Darmstadt, Germany) and a RezexTM RHM-Monosaccharide H+ (8%) column (300 mm × 7.8 mm, Phenomenex), at 65 °C. Milli-Q water was used as mobile phase at 0.5 $mL.min^{-1}$

6.3.6 MELs beads development tracking and retrieval

The appearance of MELs-rich beads was tracked daily. The color of the beads was noted, and their approximative size was measured using a ruler through the transparent bottom of the flasks. At the end of the fermentation, the broth was strained through a metal sieve (diameter opening ~0.5mm). The mass of the beads was measured before and after 48h of drying at 60°C,

to estimate their moisture content. Then, the beads were dissolved in ethyl acetate, centrifuged at 10000 rpm for 10 minutes and the liquid fraction was removed. The solid phase was dried for an additional 48h, to estimate biomass content in the beads. Finally, the liquid fraction was evaporated using a rotavapor, and the dry mass of the extract (a mixture of MELs and lipids) was submitted to methanolysis and GC-FID analysis of methyl esters.

6.3.7 Gas chromatography (GC) analysis of MELs and lipids

The fatty-acid composition of biological samples was determined by methanolysis and GC-FID analysis of methyl esters.16 Pure methanol (20 mL) was cooled down to 0 °C and 1 mL acetyl chloride was added to generate a water-free HCl/methanol solution. Culture broth samples (3 mL) were freeze-dried, weight and mixed with 2 mL HCl/methanol solution and incubated for 1 h at 80 °C for transesterification into methyl esters. Heptanoic acid was used as internal standard. The resulting product was extracted with hexane (1 mL) and 1 µL of the organic phase was injected in a GC system (Hewlett-Packard, HP5890), equipped with an FID detector and an Agilent HP Ultra2 capillary column (L 50 m × I.D. 0.32 mm, df 0.52 µm). The oven was programmed to an initial temperature of 140 °C and three temperature gradients were defined: 140 to 170 °C at 15 °C min^{-1}, 170 to 210 °C at 40 °C min^{-1} and 210 to 310 °C at 50 °C min^{-1}. A final time of 3 min at 310 °C was defined. Carrier gas was used with a split of 1/25. MEL were quantified through the amount of C8, C10 and C12 fatty acids 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.

6.4 Results and discussion

6.4.1 Moesziomyces antarcticus morphological structures characterization

M. antarcticus cells were found in various morphological structures. Based on classification used for the *Fungus Penicillium Chrysogenum* (Veiter and Herwig 2019), four main structures (Figure 6.1) were defined as:

1. Yeast-shaped single cells - small, individual slightly elongated cells
2. Pseudohyphae – longer structures composed of several chained cells, with branched structures
3. Clumps - loose groups of several pseudohyphae
4. Pellets - dense pseudohyphae agglomerates, with a non-transparent core

Yeast-shaped single cells were usually present in the most prominent numbers. When, upon cell division, the daughter cell remains attached to the mother cell, a string of two cells is formed. This cell string is the result of incomplete budding can then be further extended, and even branch, to form a pseudohyphae. Unlike hyphal growth occurring in fungi, in pseudohyphae structures there is not cytoplasmic connection between adjacent cells. (Min et al. 2020) As pseudohyphal growth is linked to increased cell hydrophobicity, pseudohyphae structures tend to agglomerate with each other's, forming loose clumps. Transition from this morphological structure to pellets can take place due to two possible mechanisms: (i) increase of the clumps density due to multiplication of the cells trapped withing the clump and/or (ii) change in cells surface hydrophobicity due stress caused to the cells within the clumps as this structure grows, leading to an "implosion" in which the cells collapse into this tightly packed structure that characterizes cell pellets.

Microscopic images of the inoculum prepared with glucose and glycerol are presented in Appendix 10.5.

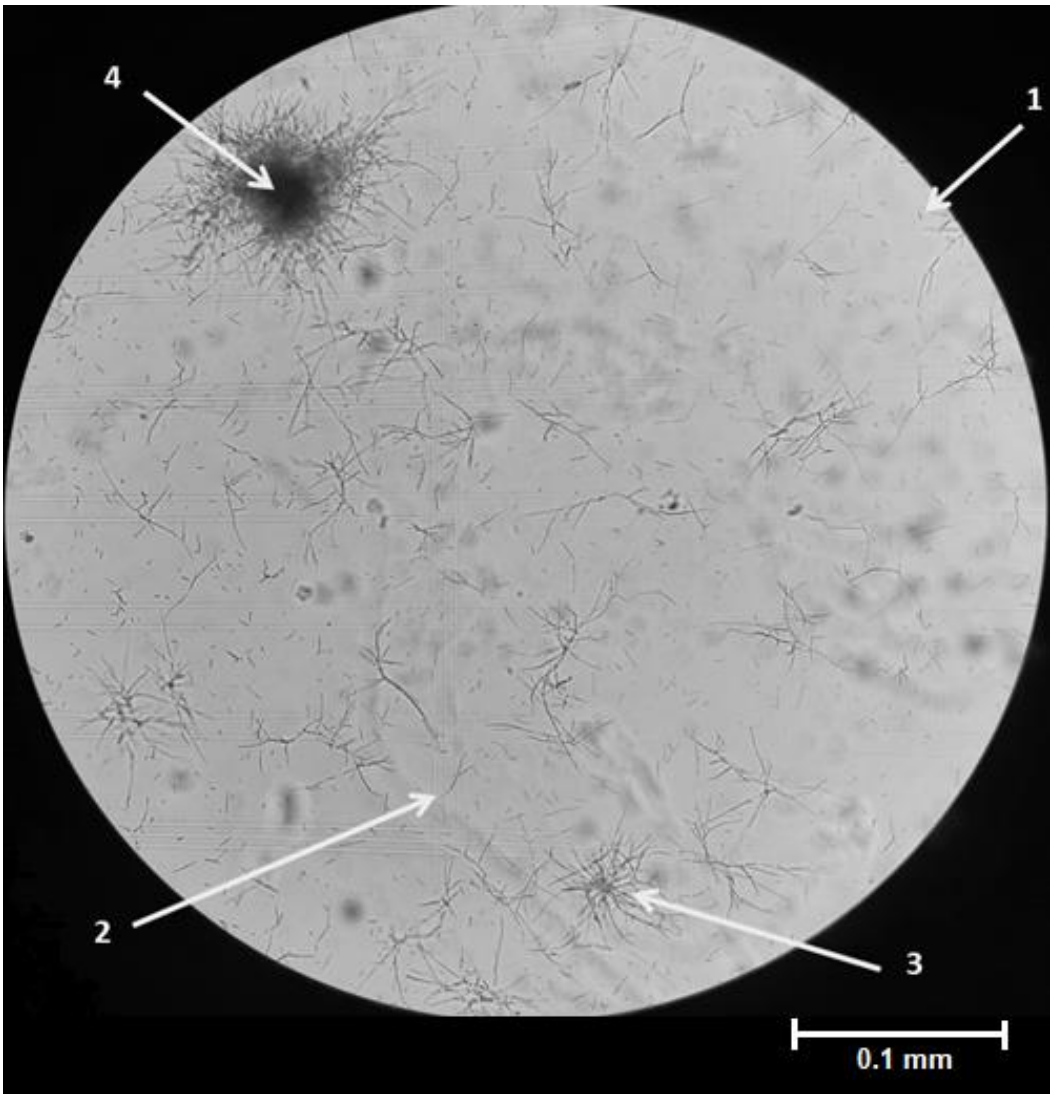

Figure 6.1 Four main morphology structures: 1 – Yeast cells; 2 – Pseudohyphae; 3 – Clumps; 4 – Pellets. Sample of fermentation broth collected at day 4, grown with 40 $g.L^{-1}$ D-glucose. 200x magnification.

6.4.2 Effect of the carbon sources, D-glucose, or glycerol, used on inoculum and main fermentation on cell morphology

MELs fermentations are relatively slow and typically include several feeds of various nutrients during the culture's progression. Several feed strategies of D-glucose and glycerol were designed to estimate how glycerol presence affects cultures in various stages of the fermentation. The biological impact of the substrate on cell morphology was assessed for the different feeding regimes studied. First, 2-day inoculums were prepared with either D-glucose or glycerol, which were used to inoculate the main fermentation flasks. In the main fermentations the carbon source substrate was added at 40 $g.L^{-1}$ day 0 and, after 4 days, more 40 $g.L^{-1}$ of same substrate was added. The aim of this set of experiments was to estimate whether inoculums prepared with different carbon source substrates impacted cell morphology in the main fermentation, or whether the cells were influenced only by the substrate used during the main fermentation.

These four sets of flasks had the feed strategies described in Table 6.1:

Table 6.1 Feed strategy for flasks with D-glucose and glycerol used in different stages of *M. antarcticus* fermentation – the main fermentation, and inoculum preparation stage

		Main fermentation substrate	
		Glucose	**Glycerol**
Inoculum substrate	**Glucose**	**1.** Inoculum: **Glucose** Day 0: 40 $g.L^{-1}$ of **Glucose** Day 4: 40 $g.L^{-1}$ of **Glucose** ***(glu glu)***	**2.** Inoculum: **Glucose** Day 0: 40 $g.L^{-1}$ of **Glycerol** Day 4: 40 $g.L^{-1}$ of **Glycerol** ***(glu gly)***
	Glycerol	**3.** Inoculum: **Glycerol** Day 0: 40 $g.L^{-1}$ of **Glucose** Day 4: 40 $g.L^{-1}$ of **Glucose** **(*gly glu*)**	**4.** Inoculum: **Glycerol** Day 0: 40 $g.L^{-1}$ of **Glycerol** Day 4: 40 $g.L^{-1}$ of **Glycerol** **(*gly gly*)**

Samples were periodically collected, and relevant analyses were performed. Dry biomass concentration (CDW) was measured to estimate the impact the carbon source on culture growth (Figure 6.2). It can be observed that the cultures grown with glucose had the highest biomass content throughout the whole fermentation. Still, some signs of lasting impact of the inoculum substrate could be seen, as the flasks inoculated with a glycerol feed had a somewhat lower CDW than their glucose counterparts, in particular for the cultures where glycerol was the carbon source on the main fermentation. While in the first four days all cultures maintained similar biomass profiles, after the addition of the second carbon source feed on day 4 differences between the cultures increased.

In order to distinguish between small and big cell fractions, samples collected from the flasks were filtered through 25 μm filter paper, under a vacuum. The filter paper was dried, and the ratio of small to large cell fractions was estimated (Figure 6.3).

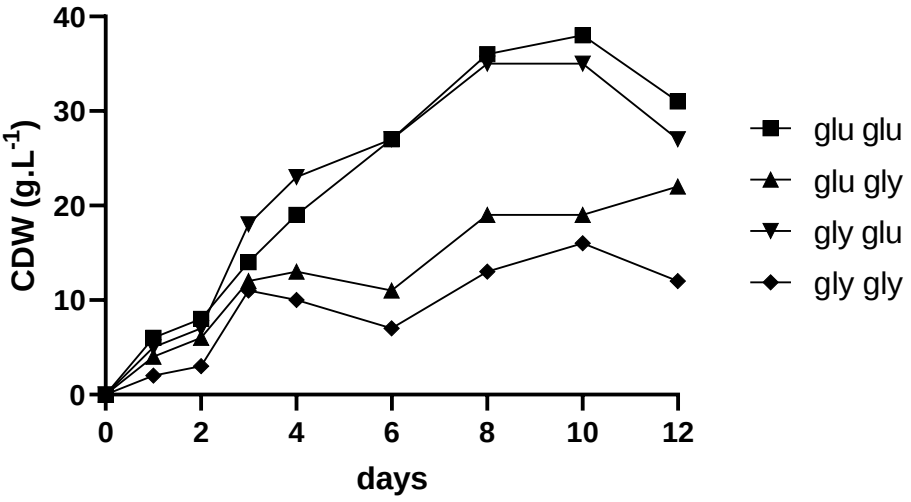

Figure 6.2 Cell dry weight profiles for 12 days fermentation of *M. antarcticus* with combinations of the substrate (D-glucose and glycerol) for inoculum/main fermentation. The second feed of main fermentation carbon source was performed on day 4.

In order to distinguish between relative amount of small and large cell's structures, samples collected from the flasks were filtered through 25 μm filter paper under a vacuum. The filter paper was dried, and the ratio of number of small to large cells was estimated (Figure 6.3).

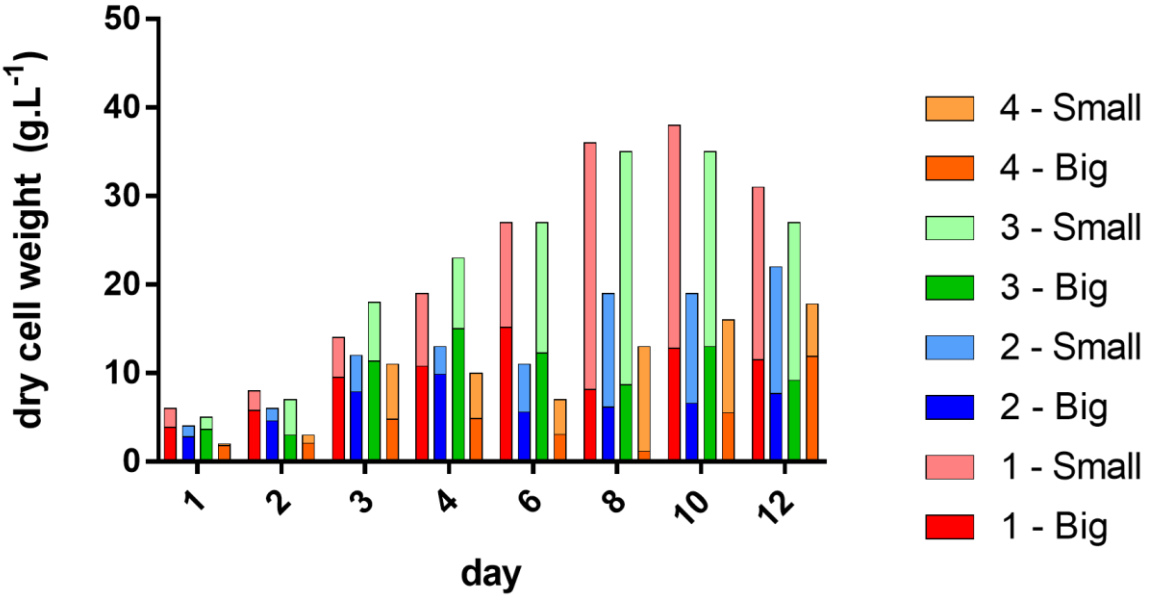

Figure 6.3 Small and large cell ratio in samples. Bottom (darker colour) – bigger fraction (on filter, > 25 μm); Top (lighter colour) – smaller fraction (passing through filter, < 25 μm)

Surprisingly, in the earlier stages of fermentation the participation in the total CDW of large structures was higher, with mass content of small structures only increasing in later stages of fermentation.

To fully understand the impact of feed strategy on the ratio of small to large morphological structures, the same data from Figure 6.3 is presented as ratios of small cells in the total cell dry weight, as % w/w (Figure 6.4).

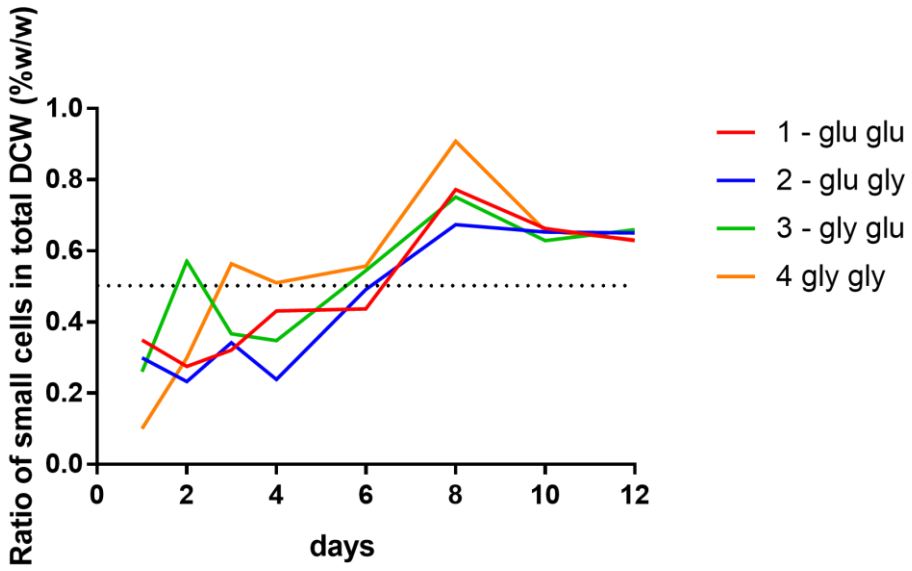

Figure 6.4 Ratio of small cell fraction dry weight to total CDW

Based on this figure, it is made more obvious that there is a shift towards a higher dry mass content of smaller cell fractions for all the feed strategies. A possible explanation for this would be that the cells which initially formed agglomerates remained stagnant, as their growth potential was limited by the poorer conditions for development within the agglomerates, while the small yeast-shaped cells proliferated after the addition of the second dose of substrate on day 4.

Measuring dry weight of the different size fractions was not sufficient in order to obtain a definite answer regarding changes in morphology depending on the substrate used during the inoculum phase and the main fermentation. Thus, different cell structures (as seen in Figure 6.1) were counted over time, and their profiles were formed. Samples were diluted appropriately, and a haemocytometer was used (Figure 6.5). Viable cell number determination was deemed inadequate in this case, as misleading results might be achieved due to cell agglomeration into big multicellular structures.

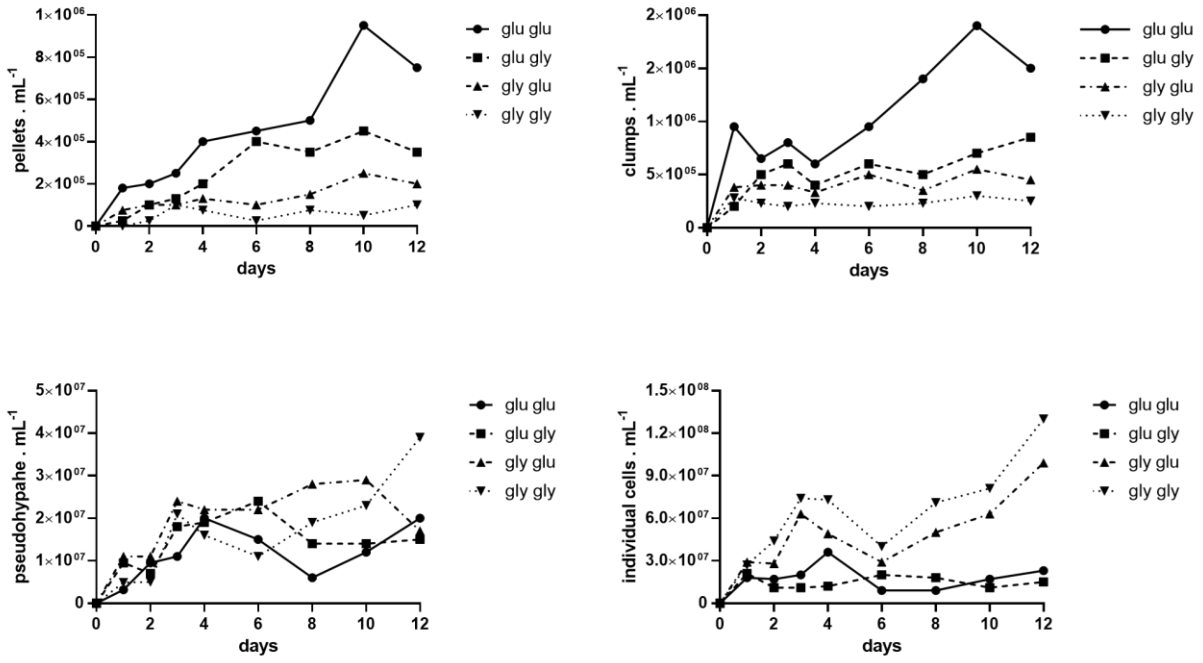

Figure 6.5 Profiles of various morphological structure profiles in 12-day fermentation of *M. antarcticus* using different combinations of D-glucose (glu) and glycerol (gly) in the inoculum and fermentation (e.g. *glu gly* corresponds to D-glucose used on the inoculum and glycerol in the fermentation).

Flasks prepared using D-glucose-based inoculum had more clumps and pellets, and less individual cells than ones with seeded with a glycerol-based inoculum, regardless of the substrate used in the main fermentation. Flasks prepared with glycerol-based inoculum had the highest content of yeast-shaped single cells throughout the whole fermentation.

Samples collected on day 6 of these were left to sediment, in order to roughly estimate the hydrophobic interactions between the cells. It was obvious throughout the duration of the fermentation that D-glucose presence negatively affected cell sedimentation rates. A set of Eppendorf tubes containing samples which were let to sediment for two hours is presented in Figure 6.6. The cells collected from the culture grown on glycerol through inoculum and main

fermentation stages sediment the most, and those in which glucose was used on both stages barely showed any signs of sedimentation. The results indicate that glycerol influenced sedimentation when it was the main carbon source during the main fermentation. Still, there was a difference between sedimentation rates between conditions 1 (*glu glu*) and 3 (*gly glu*), indicating that glycerol presence during the inoculum phase of growth had a lasting impact which affected cells during the main fermentation with glucose

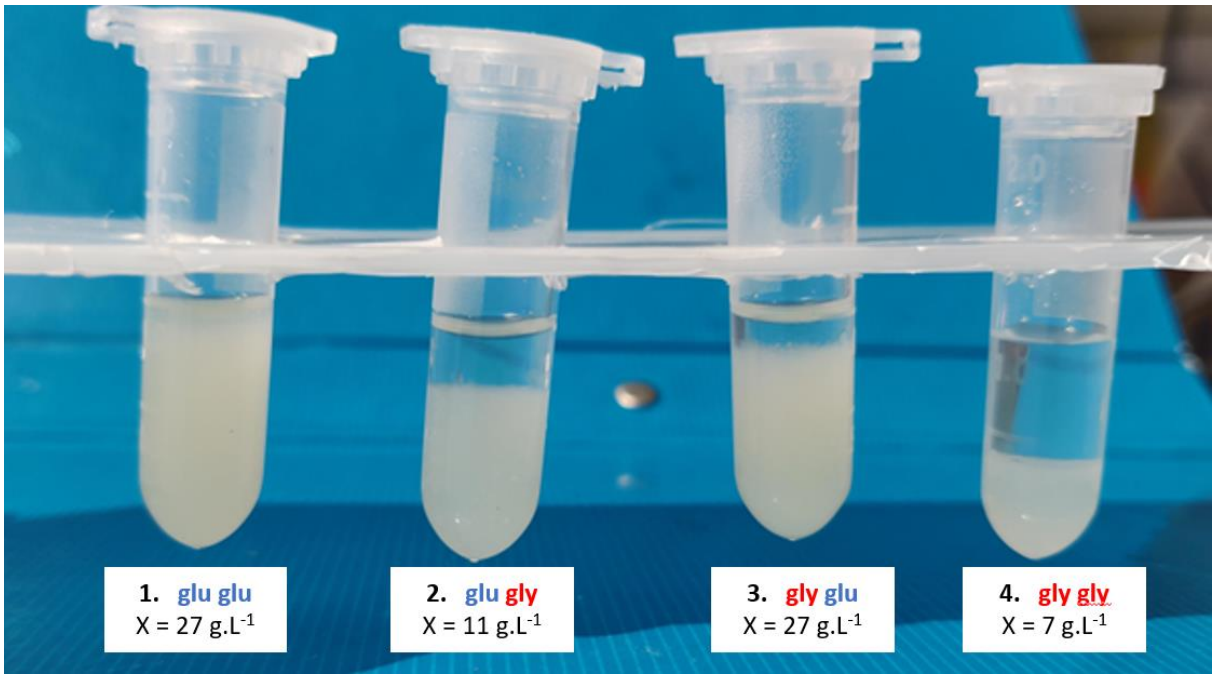

Figure 6.6 Cell sedimentation after 120 min of rest. Samples collected on day 6 of *M. antarcticus* fermentation. From left to right, samples with feed strategy 1, 2, 3 and 4.

Inoculum substrate did not influence CDW and size-to-weight ratio of cells. It did, however, play a role in determining cell interaction (sedimentation) and cell morphology; cells fed with glycerol had less intracellular interaction and more single yeast like better morphology (more small cells, less pellets); i.e., a morphology supposedly more prompt to MEL production and to support successful submerged bioreactor operations.

Substrate, glycerol or D-glucose, selected to be used on the main fermentation was influencing CDW, with cells fed with D-glucose presenting a faster and cell growth and leading to higher cell densities in comparison to cells fed with glycerol in the main fermentation, regardless of inoculum substrate used.

Results show that the substrate used in the inoculum had a lasting effect on the cultured cells morphology. The substrate, D-glucose vs glycerol, to which the cells were exposed during the inoculum propagation stage permanently influencing the shape that the cells take on the main fermentation. Cells grown, during the inoculum stage in glucose tended to be larger, filamentous and had a higher dry cell concentration, while cells grown on glycerol caused production of smaller cells that were more dominantly yeast shaped.

Finally, based on information from Figure 6.2, Figure 6.4, and Figure 6.6, it can be suggested that it is not the amount, but the shape of the biomass that affects cell sedimentation.

A side, but important, outcome of these results is that they show the unreliability of classic viable cell number determination. The fact that filamentous cells are interconnected would mean that the diluting and plating cells on Petri dishes with agar can give misleading results. Cells forming large agglomerates impact the credibility of these results, since their presence makes results from this method unrepresentative, and with a seemingly lower concentration of viable cells. However, one interesting observation from plating fermentation samples was the presence of two distinct colony groups. The first had a shiny and smooth surface, while the other were opaque and flaky. Microscopic investigation of needle scrapes obtained from these different colonies showed that the first group was mainly composed of detached yeast-shaped cells, while the other contained dense groups of pseudohyphal cells. This indicated that induction of pseudohyphal growth had some permanence, and cells, once they are cultured on the main fermentation, the cells would continue to grow in filamentous structures even during

plate growth. Similar observations are noted in literature for *S. cerevisiae* (Chow, Dionne, et al. 2019; Palecek, Parikh, and Kron 2002) in which cells retained their "invasive" growth mode when seeded on agar.

6.4.3 Feed strategy optimization

When glycerol was used as carbon source, it had a lasting effect by prompting cells culture organization into detached individual cells. Nonetheless, the results of the previous set of experiments were not clear to indicate whether glycerol had also a positive effect on production of MELs. Still, considering as valid the hypothesis that the use of submerged single cells cultures is beneficial for MEL production, glycerol should be used in inoculum and/or early fermentation stages, during biomass development. On the other hand, considering the metabolic pathway for the MEL production, previously described (Figure 2.3), D-glucose, rather than glycerol, is a more straight-forward route for MEL production, and this D-glucose in later stages of fermentation. Actually, the best MELs production results obtained, within this thesis, for MEL production combines an initial feed of glucose with a further feed of lipidic substrate (e.g., vegetable oils), would be added during the stationary phase. In this context a new set of experiments, assessing different feeding strategies, was designed (Table 6.2.) to investigate in which step of the fermentation to use glycerol instead of glucose will contribute to improve MEL production.

Table 6.2 Feed strategy assessed to study when the use of glycerol instead of glucose contributes to increase MEL production. Inoculum was prepared from cryopreserved stocks. Flasks of main fermentation were inoculated with 10% inoculum. All conditions provide roughly equimolar carbon content.

Culture		Inoculum	Day 0	Day 4	Total C added (g)
phase		Culture propagation	Exponential	Stationary	
Feed strategy	A	Glucose	Glucose (40 $g.L^{-1}$)	Glucose (30 $g.L^{-1}$) + RSO (40 $mL.L^{-1}$)	54.36
	B	Glycerol	Glucose (40 $g.L^{-1}$)	Glucose (30 $g.L^{-1}$) + RSO (40 $mL.L^{-1}$)	54.36
	C	Glycerol	Glycerol (40 $g.L^{-1}$)	Glucose (30 $g.L^{-1}$) + RSO (40 $mL.L^{-1}$)	54.01
	D	Glycerol	Glycerol (40 $g.L^{-1}$)	Glycerol (30 $g.L^{-1}$) + RSO (40 $mL.L^{-1}$)	53.75
	E	Glycerol	RSO (40 $mL.L^{-1}$)	RSO (40 $mL.L^{-1}$)	52.73

Flasks were prepared using the substrates, D-glycerol or glycerol, for inoculum, which was used to start 12-day of main fermentations. All fermentations were performed in duplicate. Samples were collected periodically to determine dry cell weight (DCW), MELs and lipid profiles.

Results for dry biomass content (Figure 6.7) show little difference in biomass development between the various feed strategies. The exception was feed strategy E (**gly rso rso**), which enabled the development of higher biomass concentrations in early stages of fermentation. Alternatively, this result could have been influenced by the accumulation of internally stored lipids within the cells.

Viable cell numbers (VCN) were estimated by counting colony forming units, to assess whether this increase in cell dry weight is related to an increased number of cells, or the cells became larger and heavier – namely due the presence of intracellular lipid bodies or adsorbed to the cell

surface of vegetable oil used as substrate. These results are presented in Figure 6.8. VCN estimations using microscopy can be tedious and reliability of the outcome can be affected by the presence of various cell morphological structures and oil droplets present in the samples.

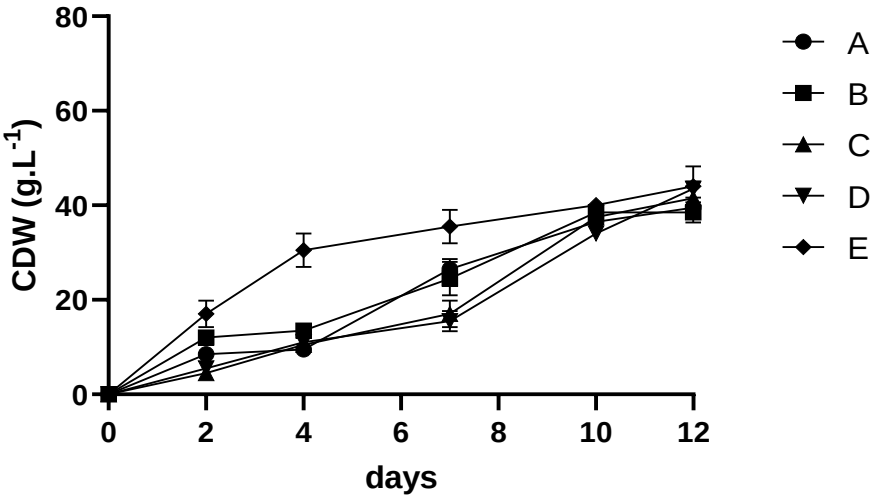

Figure 6.7 Dry cell weight profiles for conditions A through E

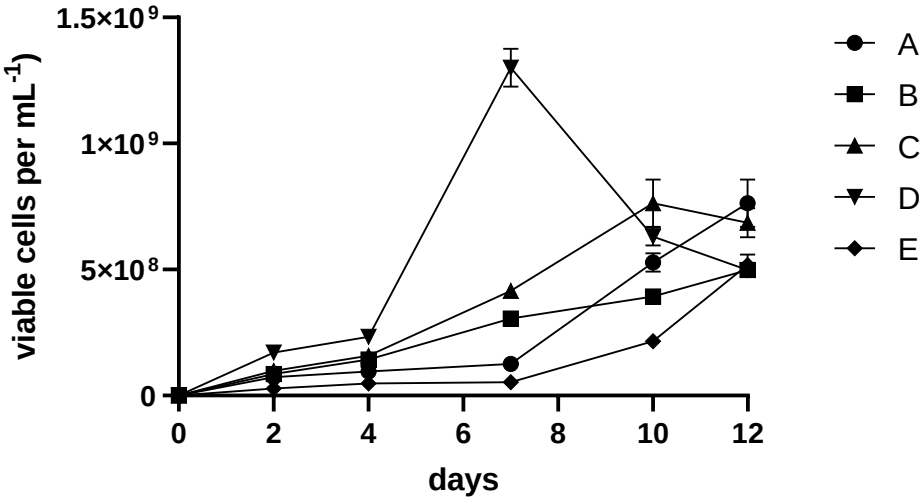

Figure 6.8 Colony forming unit number profiles for conditions A (**glu glu glu rso**), B (**gly glu glu rso**), C (**gly gly glu rso**), D (**gly gly gly rso**) E (**gly rso rso**)

Actually, the cells in condition E (**gly rso rso**), which had an initial feed of rapeseed oil and presented the higher CDW, had the lowest viable cell number throughout the duration of the fermentation. Therefore, this result that the early addition of oil caused the cells to swell and

accumulate lipids internally in the form of lipid bodies. Again, the cultures with inoculum prepared using glycerol, apparently, had a higher viable cell number compared to those whose inoculum was grown on glucose.

An important phenomenon which was impacted by the different feed strategies was the formation of MEL-rich beads. These formations would appear supposedly due to poor solubility of MELs and pose an opportunity for more efficient product removal. The beads would have varying consistency and size depending on the ratio of MELs to lipids. Their colour also depended on their purity, with beads richer in MELs having a darker orange colour. Beads rich in triacylglycerol would tend to float on the surface of the broth, and have a loose and viscous structure, with a greenish to yellow colour. In order to track the progression of the beads, observations regarding their size and colour were made, as well as rough measurements of their size through the bottom of the glass flasks. This data is presented in Table 6.3.

Table 6.3 Progression of bead development for conditions A through E. Size - Tiny: >1 mm; Small: 1-3 mm; Medium: 3-10 mm; Large<10 mm. Colour of the field in the table indicates the colour of the beads on that day.

Day	**0**	**…**	**7**	**8**	**9**	**10**	**11**	**12**
A	/	…	/	tiny	tiny	small	small	small
B	/	…	/	tiny	small	medium	large	large
C	/	…	/	/	/	/	/	tiny
D	/	…	/	/	/	/	/	/
E	/	…	/	/	small	medium	large	large

Flasks fed with glucose during the main phase of the fermentation proved to be the most proliferous in terms of bead development. Cultures exposed to glycerol, on the other hand, had little bead development, with the appearance of smaller beads appearing in the culture than in those with glucose used in the main fermentation. This made them impossible to be separated using a sieve due to their fragile structure. The cultures in which beads robust enough to be removed at the end of the fermentations were flasks with feed strategies A, B and E, and their appearance on the 12th day of fermentation is presented in Figure 6.9.

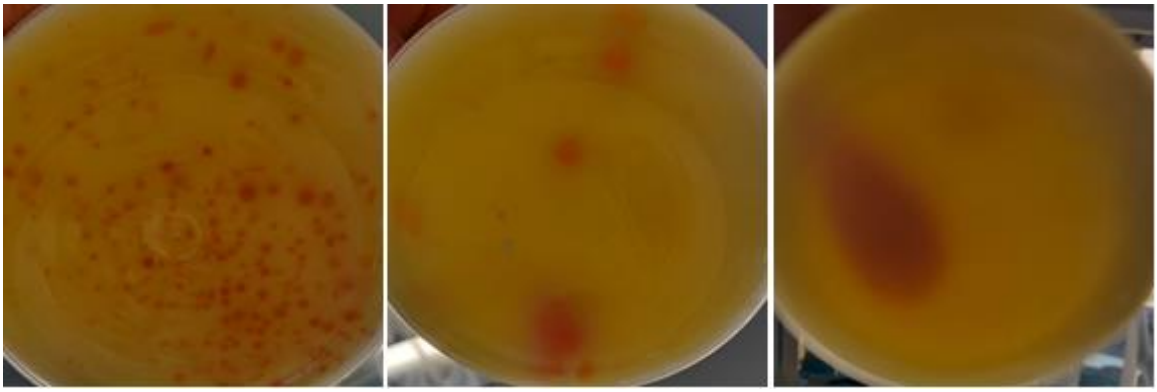

Figure 6.9 Beads in flasks of *M. antarcticus* fermentation, prior to harvesting. Left: condition A; Middle: condition B; Right: condition E. Condition E (with feeds of only RSO during the main phase of fermentation) formed large, gelatinous beads, which floated on the surface of the broth)

The beads were collected, dried and the extract was collected using ethyl acetate The solvent solution obtained was centrifuged and the pellet not removed with solvent supernatant is labelled as biomass. As in previous results, all free fatty acids and acyl acylglycerols were jointly marked as lipids. Concentrations were presented as normalized for the total broth volume i.e., 1 gram of MELs in beads in the 50 mL flask would correspond to 20 g.L^{-1} of MELs in bead form. The data on bead content is presented in Figure 6.10.

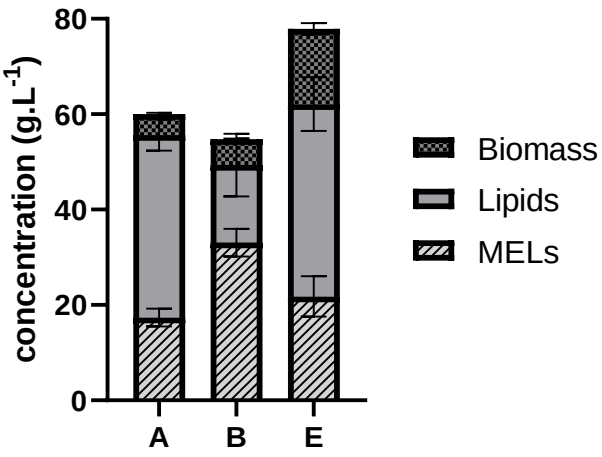

Figure 6.10 Component concentration of beads collected on day 12 of M. antarcticus fermentations, conditions A, B and E (see Table 6.2).

The results indicate strategy B (**gly glu glu rso**) to be the most promising for beads development. This strategy uses glycerol for inoculum preparation, glucose during the whole duration of the main fermentation including with rapeseed oil feed at day 4. These beads were richer in MELs and had lower levels residual lipids – beads from condition A (**glu glu glu rso**) had ~30% purity, while the beads with condition B (**gly glu glu rso**) had ~58% purity. The feed strategy A (**glu glu glu rso**) with glucose used in all phases of culture growth (combined with rapeseed oil at day 4) resulted in slightly more bead mass formed, however those beads contained a larger content of lipids. The feed strategy E (**gly rso rso**), where rapeseed oil was used as the sole source of carbon throughout the main fermentation, had a high concentration of biomass in the beads (~23%). The singular bead which was formed was glutinous and seemed to trap a higher fraction of cells and cell debris within it.

In addition to estimation of the MELs and lipids in the beads, the concentration of these compounds was also measured in the bead-free fermentation broth. Samples were carefully collected so they contain no beads, as they would interfere with the results. These profiles are

shown in Figure 6.11. For the last day of the fermentation, cumulative values for total concentration of MELs and lipids (both in the fermentation broth and the beads) is presented with red points. For some conditions, the apparent values for days 8 and 10 are lower than the real values, as bead presence and broth heterogeneity prevented collection of representative samples.

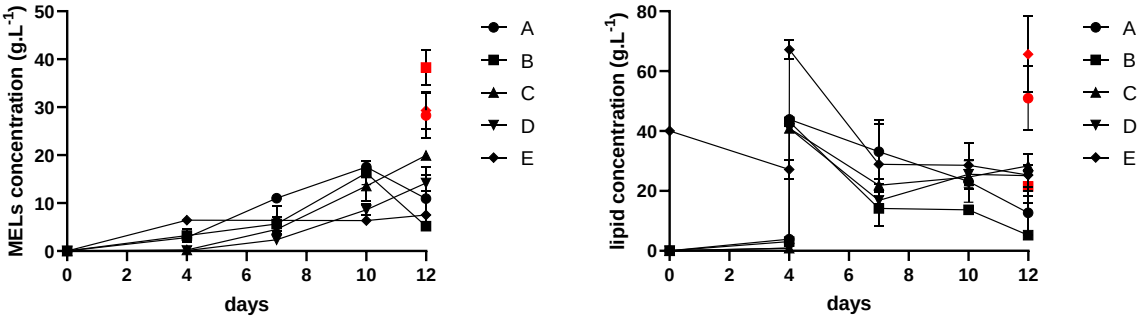

Figure 6.11 MELs and lipids concentration profiles for M. antarcticus with feed strategies A, B, C, D, and E (from Table 6.2). Red points represent cumulative value of component concentration in both broth and beads.

MELs and lipid profiles show interesting trends, which complement the understanding of bead development in this set of experiments. Values of MELs and lipids concentrations dropped between day 10 and day 12 for conditions in which glucose was the main hydrophilic nutrient during the main fermentation, indicating that bead formation was in progress. This drop indicates the transition of free MELs into bead form and their maturation into large beads proper to be harvested. This is especially prominent for the cultures in which glycerol was only used during inoculum preparation - condition B (**gly glu glu rso**), for which the average concentration of MELs in the bead-free broth dropped from 16.26 to 5.19 g. L^{-1} between days 10 and 12. Total MELs and lipid concentrations at the end of the main fermentation, and respective distribution in beads and bead free fermentation broth are present on Figure 6.12.

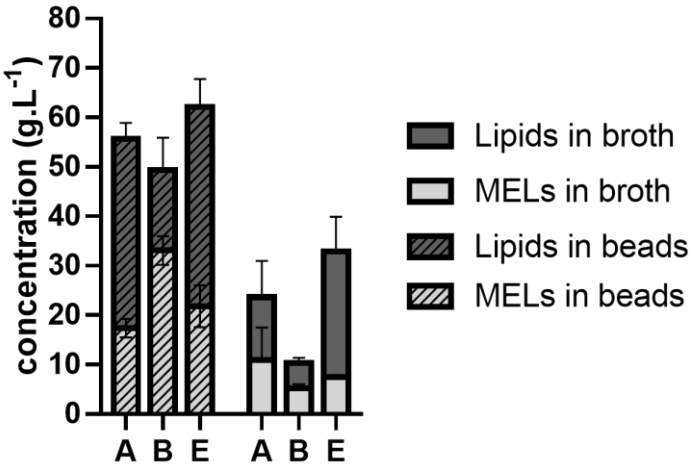

Figure 6.12 Total MELs and lipids concentration in flasks A (**glu glu glu rso**), B(**gly glu glu rso**), and E(**gly rso rso**), at the end of the fermentation. Values include MEL and lipids in bead form and in bead-free broth.

One can conclude that using glycerol for cell propagation during inoculum preparation proved to be beneficial for the culture. The culture whose inoculum was prepared with glycerol produced more MELs and had fewer residual lipids in the end of the fermentation, even for conditions with the same feed strategy during the main course of the fermentation, Condition B (gly glu glu rso) vs. Condition A (glu glu glu rso). Also, glycerol used for inoculum preparation resulted in higher production of MELs-rich beads, which is an interesting opportunity for retrieving the surfactant from the broth more efficiently at the end of the fermentation.

6.5 Conclusions

Glycerol was found to have a beneficial impact on *M. antarcticus* cell quality. During the initial stages of fermentation, yeast-shaped cell morphology is promoted. Furthermore, regardless of the carbon source used in further stages of the main fermentation, the initial substrate used

during inoculum preparation greatly determined cell quality throughout the fermentation. In other words, cells initially grown on glucose remained filamentous despite further feeds of glycerol. Similarly, cells initially grown on glycerol had higher numbers of yeast-shaped single cells throughout the fermentation, regardless of the other carbon source used.

Actually, cultures that were grown on glycerol during the inoculum phase, and later grown on glucose, showed higher capabilities of producing MELs, and generated more MEL beads of higher purity compared to those grown on glucose exclusively. They also consumed FFAs the fastest. Glycerol proved to be a good substrate to promote a long-lasting effect on the favourable cell morphology, benefiting the culture in terms of MEL production continuously, while glucose seems to be the substrate for the main fermentation which promoted MEL production. The morphology of *M. antarcticus* cells caused by glycerol seems to be the reason for better performance during the main fermentation. More small individual cells and lower numbers of large multicellular structures resulted in better metabolic activity of the culture as a whole. The extracellular glycerol probably affected the stress-response mechanisms of the cells, affecting their differentiation towards smaller yeast-shaped cells. The hydrophobicity of cells was probably affected, resulting in more hydrophilic cells with less agglomeration and adsorbed MELs. This resulted in formation of more MELs beads with a higher purity.

Chapter 7

7 Semi-continuous production of an aqueous MELs- (mannosylerythritol lipid) and lipase-rich solution by *M. antarcticus* resting cells

7.1 Abstract

The potential substitution of chemical surfactants by biosurfactants is being promoted by a wide range of industrial producers, due to their excellent performance at low concentrations, in a wide range of conditions, with a low environmental impact. Among the most promising biosurfactants are MELs, which are produced by yeasts of the *Moesziomyces* genus, which includes *M. antarcticus,* from which the genes used for the production of industrially applied lipase *Candida Antarctica* Lipase B (CAL-B) were retrieved.

The production of an aqueous-based product rich in biosurfactant and lipases has industrial interest for several applications in home care and industrial processes. For such a product, downstream processing should have low energy and solvent intensity (potentially avoiding any solvent use altogether). Thus, hydrophilic substrates should be preferably selected.

In an effort to develop a process able to produce large volumes of an aqueous solution containing both MELs and lipases, a semi-continuous production process is here proposed. Hydrophilic substrates were used as sole carbon sources, with frequent removal of the supernatant by centrifugation. The biomass would be transferred to a flask with fresh medium, containing more substrate, to promote the continuation of the fermentation with resting cells for multiple cycles. Such fermentations were performed with glucose and glycerol as main carbon sources, and a total of 8 cycles of supernatant were collected.

After analysis of the collected samples, a lipase productivity (units of activity per day) was 23.5% and 83% higher for glucose and glycerol, respectively, with a 52-day multi-step fermentation with 8 steps, than with a single-step 10-day fermentation. As for the biosurfactant, there was a significant drop in MELs concentration in the aqueous product as the fermentation progressed. Still, these low levels of biosurfactant concentration remain well above its CMC and are sufficiently high to enable the use of this product in a range of applications, allowing the product to be diluted before being applied. Finally, as there were no drastic differences in

performance between the two substrates, glycerol is proposed to be used in such process configurations, as currently it can be more sustainable than glucose.

7.2 Introduction

Mannosylerythritol lipids (MELs) are a group of non-ionic microbial biosurfactants, with a wide range of possible applications, due to their exceptionally low CMC value and high biodegradability. They are produced mainly by yeasts of the *Moesziomyces* genus, which includes *M. antarcticus,* from which the genes used for the production of industrially applied lipase *Candida Antarctica* Lipase B (CAL-B) were retrieved. (Lou et al. 2008)

MELs are usually produced using lipidic substrates and recovered from the fermentation broth by liquid-liquid extraction using organic solvents. After their removal, a crude mixture containing both MELs and lipids is obtained. As MELs are poorly soluble in water, applications which require an aqueous solution of MELs would require the use of additives such as dispersants. However, MELs are usually found in the broth in concentrations significantly higher than its CMC, which is possible due to the many different structures that MELs can form. (Imura et al. 2006)

Due to their non-ionic nature which eases sorption of soil and lipidic stains, biosurfactants have a great potential for detergency applications. (Ying 2006) As detergents used for cleaning hard surfaces and laundry are washed away into wastewater (in case of home care products into the municipal sewage system), the use of biodegradable biosurfactants can greatly mitigate this negative environmental impact. Finally, issues related to acute toxicity and skin/eye irritation in humans are lower for biosurfactants due to their biocompatibility.

Besides surfactants, enzymes have been introduced to detergents to increase their effectiveness. Proteases, lipases, cellulases and amylases play important roles in assisting the cleaning process, by removing soil and stains, and by facilitating fabric conditioning. (Olsen and Falholt 1998) As most lipases are stimulated by cations, as CAL-A produced by *Moesziomyces* species

is stimulated by calcium, their use is enabled even in water with a higher mineral content. (Domínguez de María et al. 2005; Niyonzima and More 2015)

As for other industries, lipases are used in food processing (for enhancement of dairy product maturation, for baked goods), in pulp processing and paper production, among others. (Guerrand 2017) Thus, an aqueous product rich in lipases would have a great market value, which could be possibly enhanced if a biosurfactant is present.

Batch fermentation is most often used in industrial production due to better process efficiency, as it enables achieving higher product titres. The fed-batch mode is usually used if substrate inhibition occurs. (Cardona and Sánchez 2007) Alternatively, continuous production is applied when lower product titres are not an issue, and it lowers equipment downtime and avoids culture development during lag-phase for secondary metabolism products.

This chapter aims to develop a semi-continuous process for producing a cell free aqueous solution rich in MELs and lipases. This will be accomplished by growing cultures on glucose and glycerol. After the initial 10-day fermentation, the culture will be centrifuged. The supernatant will be separated, while the biomass pellet will be transferred to a flask with fresh medium. After this, the process will be repeated, and in the stationary stage, the culture will be transferred to fresh medium every 6 days. The experimental plan is presented in Figure 7.1.

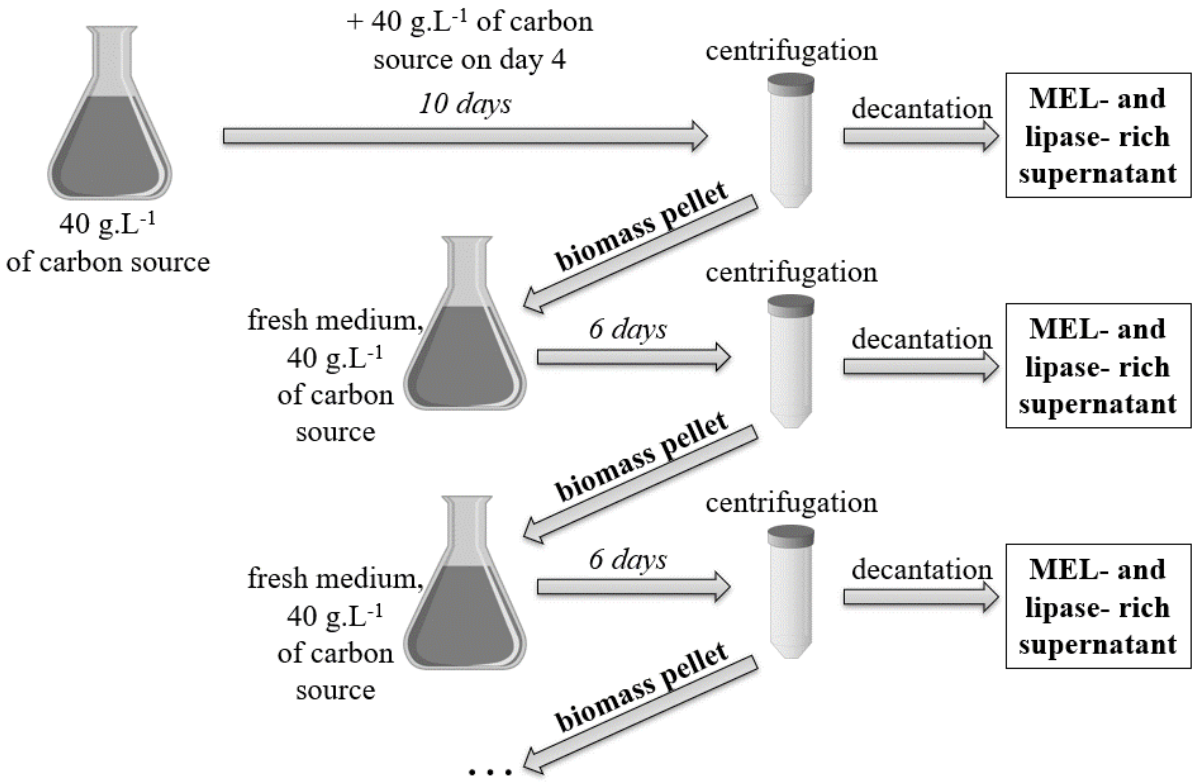

Figure 7.1 Illustration of the experimental setup for fed-batch production of lipase and MELs-rich supernatant using resting cells of *M. antarcticus,* with supernatant collection in multiple cycles.

Although high MELs titres are obtained using lipids as carbon source, the aim of this process is to achieve a ready-to-use aqueous product. Such a product should not have any residual lipids originating from the substrate used, as they would affect the product quality. Finally, due to MELs superior performance at exceptionally low concentrations, the fermentation broth when D-glucose or glycerol would be used would have sufficiently high concentrations of MELs needed for the envisioned applications, without need to add any lipidic substrate.

7.3 **Materials and methods**

7.3.1 Microorganisms and maintenance

Moesziomyces antarcticus PYCC 5048^{T} (CBS 5955) was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal. Strains were plated on YMA (yeast extract (t.a., Oxoid LTD) 3 g L^{-1}, malt extract (t.a., Oxoid LTD) 3 g L^{-1}, peptone (t.a., BDH) 5 g L^{-1}, D-glucose (p.a., Fisher Chemicals) 10 g L^{-1} and agar (JMVP) 20 g L^{-1}) and incubated for 3 days at 30 °C. Cultures were kept at 4 °C and renewed every week and stored at −80 °C in 20% (w/v) glycerol (≥99.5%, JMGdS) to be recovered when necessary.

7.3.2 Media and cultivation conditions

Inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of medium containing 3 g L^{-1} $NaNO_3$ (p.a., PanReac AppliChem), 0.3 g L^{-1} $MgSO_4$ ((≥99.5%, Panreac AppliChem), 0.3 g L^{-1} KH_2PO_4 ((≥99.5%, Chem-Lab NV), 1 g L^{-1} yeast extract, 40 g L^{-1} D-glucose or glycerol, and incubated at 27 °C, 250 rpm, for 48 h. The same substrate was always used for preparation of both the inoculum and the broth for the main fermentation. Batch cultivations were performed in Erlenmeyer flasks containing 1/5 working volume of mineral medium (0.3 g L^{-1} $MgSO_4$, 0.3 g L^{-1} KH_2PO_4, 1 g L^{-1} yeast extract) supplemented with 40 g L^{-1} D-glucose or glycerol. The experiment started by transferring 10% (v/v) inoculum, corresponding to approx. 0.6 g L^{-1} of cell dry weight (CDW), followed by incubation at 27 °C, 250 rpm, for 10 days.

After this period, the content of the flasks was transferred to sterile 50 mL Falcon tubes and centrifuged for 10 minutes at 10000 rpm (Centrifuge 5810 R, Eppendorf). The supernatant was removed by decantation (or pipetted in case of the appearance of lose biomass pellets), while

the pellet was transferred to a flask with fresh medium, starting the new cycle of the fermentation.

7.3.3 Growth and biomass determination

Cell growth was quantified by cell dry weight (CDW), using a previously described protocol. (Santos et al. 2019) CDW was determined from 1 mL culture broth by centrifugation at 10 000 rpm for 10 min (Sartorius 1-15P centrifuge), washing with deionized water (twice) and drying at 60 °C for 48 h. Supernatant from the centrifuged samples was collected and used for various analyses.

To determine the viable cells content, samples were diluted and plated on YMA, and formed colonies were counted.

7.3.4 Analysis of fermentable sugars, nitrates, and glycerol concentrations

In the collected sample supernatants, monosaccharides, nitrates, and glycerol were quantified in a high-performance liquid chromatography (HPLC) system (VWR Hitachi, Darmstadt, Germany) equipped with a RI detector (L-2490, VWR Hitachi, Darmstadt, Germany), UV-detector (L-2420, VWR Hitachi, Darmstadt, Germany) and a RezexTM RHM-Monosaccharide H+ (8%) column (300 mm × 7.8 mm, Phenomenex), at 65 °C. Milli-Q water was used as mobile phase at 0.5 mL.min-1.

7.3.5 Gas chromatography (GC) analysis

The fatty-acid composition of biological samples was determined by methanolysis and GC analysis of methyl esters. Pure methanol (20 mL) was cooled down to 0 °C and 1 mL acetyl chloride was added to generate a water-free HCl/methanol solution. Culture broth samples (3 mL) were freeze-dried, weight and mixed with 2 mL HCl/methanol solution and incubated for 1 h at 80 °C for transesterification into methyl esters. Heptanoic acid was used as internal standard. The resulting product was extracted with hexane (1 mL) and 1 µL of the organic phase was injected in a GC system (Hewlett-Packard, HP5890), equipped with an FID detector and an Agilent HP Ultra2 capillary column (L 50 m × I.D. 0.32 mm, df 0.52 µm). The oven was programmed to an initial temperature of 140 °C and three temperature gradients were defined: 140 to 170 °C at 15 °C min^{-1}, 170 to 210 °C at 40 °C min^{-1} and 210 to 310 °C at 50 °C min^{-1}. A final time of 3 min at 310 °C was defined. Carrier gas was used with a split of 1/25. MELs were quantified through the amount of C8, C10 and C12 fatty acids 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. (Faria, M. V. Santos, et al. 2014)

7.3.6 Lipase activity analysis

The enzymatic assays were performed as described in Gomes *et al* (Gomes et al. 2011). The substrate used for the enzymatic assays was p-nitrophenyl butyrate. All enzymatic activities were carried out in a 96 well microplate, and the reaction mixture was composed by: 2.63 mM of p-nitrophenol butyrate was dissolved in 50 mM acetate buffer (pH 5.2) and 4% of triton-X-100.To initiate the enzymatic assay, 10 µL of the supernatants was added to 90µL of p-nitrophenol butyrate 2.63 mM solution. The reaction mixture was incubated at 37ºC for 15 minutes, and after that, the reaction was stopped by adding 200 µL of acetone. The absorbance was measured at 405 nm in a microplate spectrophotometer (MultiskanTM GO, ThermoFisher

Scientific), and the enzymatic activity was determined. One unit (U) of lipase activity is defined as the amount of enzyme releasing 1 μmol p-nitrophenol per minute.

7.4 Results and discussion

7.4.1 Production of supernatant using resting cells in "cycles"

A total of 8 cycles of fermentation were performed in triplicates. After each cycle, the supernatant was harvested after removing the biomass by centrifugation. Fermentations were performed with D-glucose and glycerol as sole carbon sources. Each time the collected biomass was transferred to a new flask, fresh medium was added. The concentration of biomass for both substrates obtained at the end of each cycle is presented in Figure 7.2.

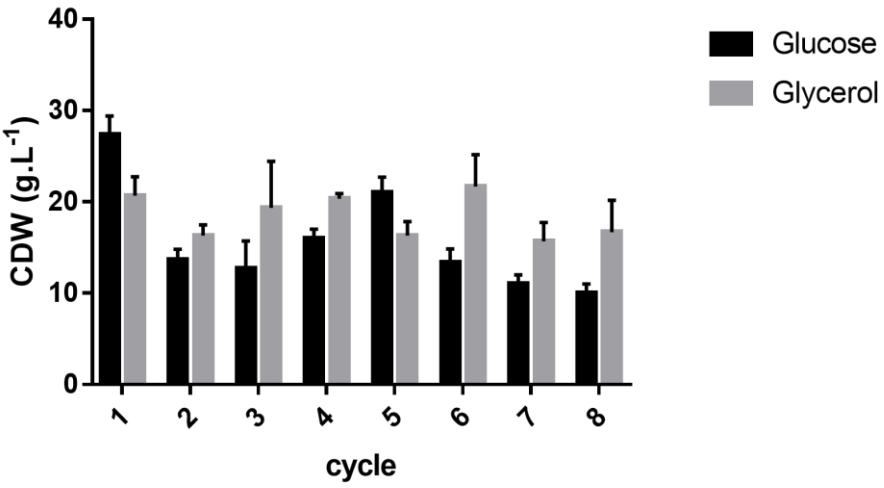

Figure 7.2 Biomass concentration at the end of each cycle for *M. antarcticus* cultures grown on D-glucose and glycerol, in total 52 days of fermentation. Cycles ended, and biomass was transferred to new flask with fresh medium (at the end of a cycle), on days 10 (end of cycle 1), 16 (2), 22 (3), 28 (4), 34 (5), 40 (6), 46 (7) and 52 (8).

Excluding the first cycle, the average biomass concentration obtained for glucose cultures was 13.95 ± 3.64, while for glycerol it was 18.04 ± 3.23. A two-way ANOVA analysis of the data showed a *p* value of 0.0014, signifying statistical difference between the two substrates' impact on biomass growth. This was unexpected as glucose proved to be the better substrate for biomass growth in terms of CDW, as seen in full biomass profiles from Figure 7.3 (and as discussed in Chapter 6). However, glycerol might be better for facilitating the development of cells in a resting-cell culture. This is supported by the fact that fermentations with resting cells (the second cycle and subsequent ones) maintained a higher concentration of biomass. Also, cell agglomerates (more common when glucose is used) might perform poorly compared to single cells due to nutrient and metabolite diffusion limitation.

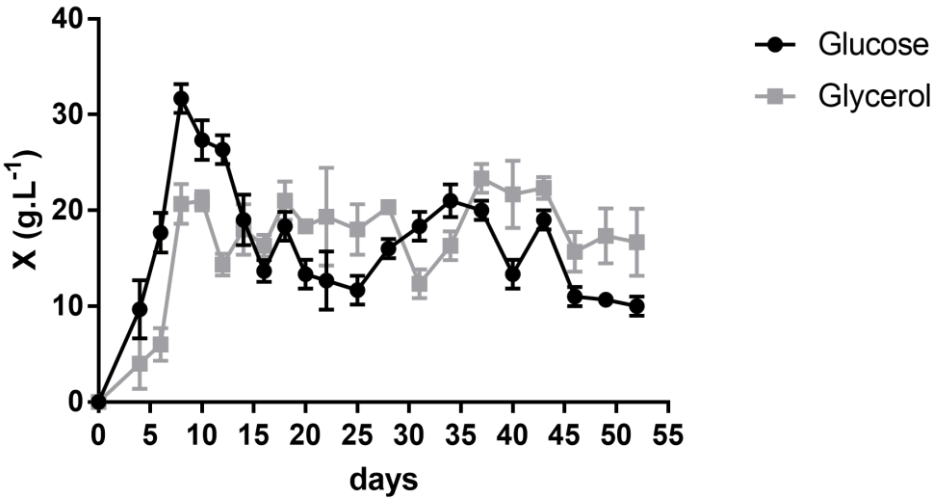

Figure 7.3 Biomass concentration profiles for *M. antarcticus* cultures grown on glucose and glycerol. Cycles ended, and biomass was transferred to new flask with fresh medium, on days 10 (end of cycle 1), 16 (2), 22 (3), 28 (4), 34 (5), 40 (6), 46 (7) and 52 (8).

The complete consumption of substrate in the case of producing a ready-to-use product is important, as any residual carbon source would be undesirable in such a product. Also, it would secure that the feed strategy is optimized, and the metabolic potential of the microorganism is

fully utilized. The concentrations of residual glucose and glycerol in samples of supernatant collected at the end of each cycle are presented in Figure 7.4

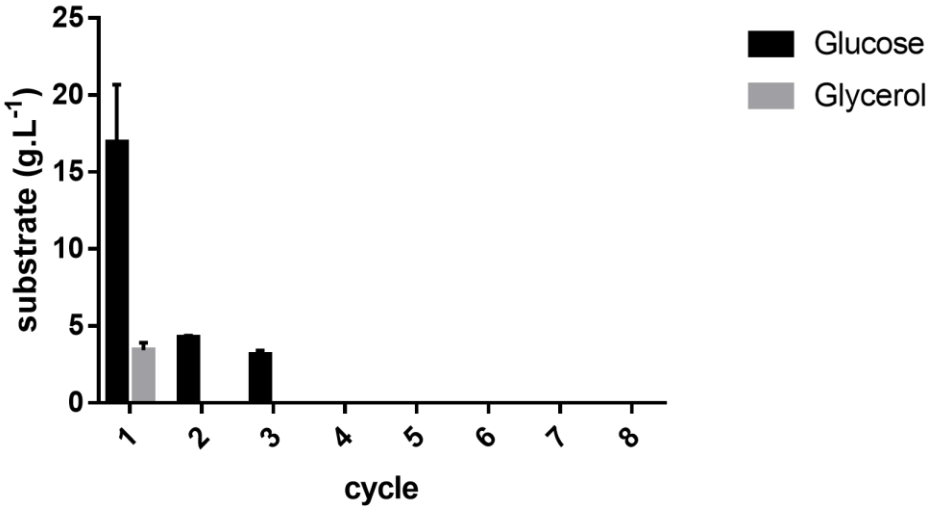

Figure 7.4 Residual glucose and glycerol concentration at the end of each cycle for *M. antarcticus* cultures grown on 40 $g.L^{-1}$ D-glucose and glycerol. In fermentations 4 to 8, carbon source concentration is below the detection limit (0.14 $g.L^{-1}$).

The results indicate that the culture fed with glycerol showed higher substrate consumption rare. After 2 cycles the glycerol was depleted in the media. D-Glucose consumption rate was lower the glycerol, being depleted only after 4 cycles.

Samples were collected at the end of each cycle for establishing the MELs concentration profile in the full fermentation time (Table 7.1).

Table 7.1 Concentration of MELs in fermentation broth samples collected at the end of each cycle for M. antarcticus cultures grown on D-glucose and glycerol.

Substrate		**Glucose**		**Glycerol**	
Day	End of cycle	MELs ($g.L^{-1}$)		MELs ($g.L^{-1}$)	
10	1	5.82	± 0.14	1.95	± 0.30
16	2	7.25	± 1.51	5.48	± 0.91
22	3	5.88	± 0.34	4.88	± 1.36
28	4	4.91	± 1.04	4.49	± 3.03
34	5	5.96	± 0.99	4.20	± 1.32
40	6	4.46	± 0.87	3.91	± 0.61
46	7	3.69	± 0.43	3.10	± 1.19
52	8	6.95	± 1.68	4.57	± 2.10

In the conditions tested, no beads were formed in any of the flasks at any point throughout the experiment, which is expected for fermentations where no lipidic substrates are used, as the threshold concentration of MELs needed for beads to form was not achieved.

The full profiles of MELs and lipids in the broth, during the extended 52-day fermentation, are presented in Figure 7.5. The results for glucose and glycerol overlap, although it is visible that glycerol performed poorly in terms of MELs production during the first two cycles, until resting cell mode was reached.

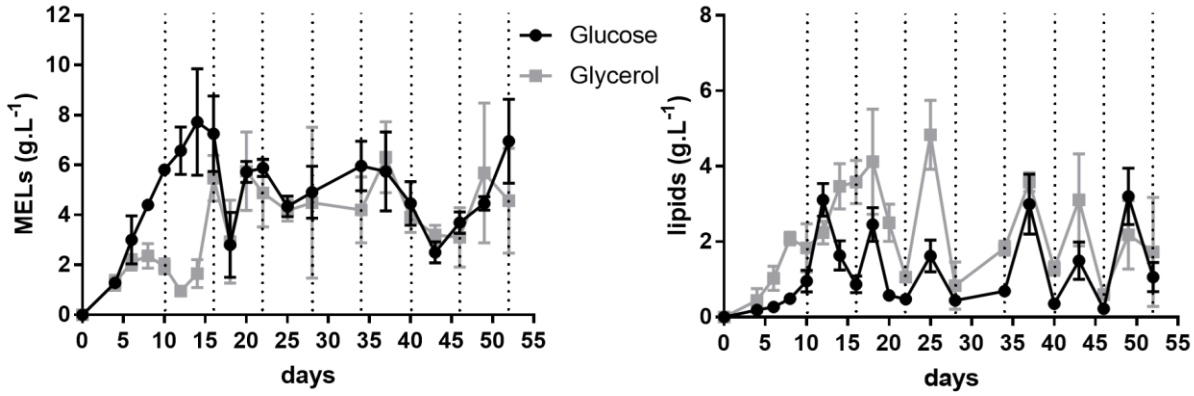

Figure 7.5 GC results for concentration profiles of MELs and lipids in fermentation broth samples collected from cultures grown on glucose and glycerol. Cycles ended, and biomass was transferred to new flask with fresh medium, on days 10, 16, 22, 28, 34, 40, 46 and 52 (indicated by vertical dotted lines).

7.4.2 Characterization of the produced supernatant of *M. antarcticus* cultures grown on D-glucose and glycerol

The volume of the collected supernatant was determined, and these results are given in Figure 7.6. The reason for a lower volume obtained from the first cycle with D-glucose was the inability to form a compact biomass pellet (as was observed in Figure 6.6 of Chapter 6). As a consequence, a higher average volume of supernatant was obtained in the second cycle (when using D-glucose), as the initial volume of the second cycle was slightly higher. Supernatant volume obtained from *M. antarcticus* cultures grown on glycerol was similar and constant over the cycles. This difference between biomass obtained from glucose and glycerol can be explained by findings from Chapter 6, related to substrate effects on cell morphology and sedimentation. After some time, the values for average volume for all flasks stabilized at ~40 mL, which is ~80% of working volume.

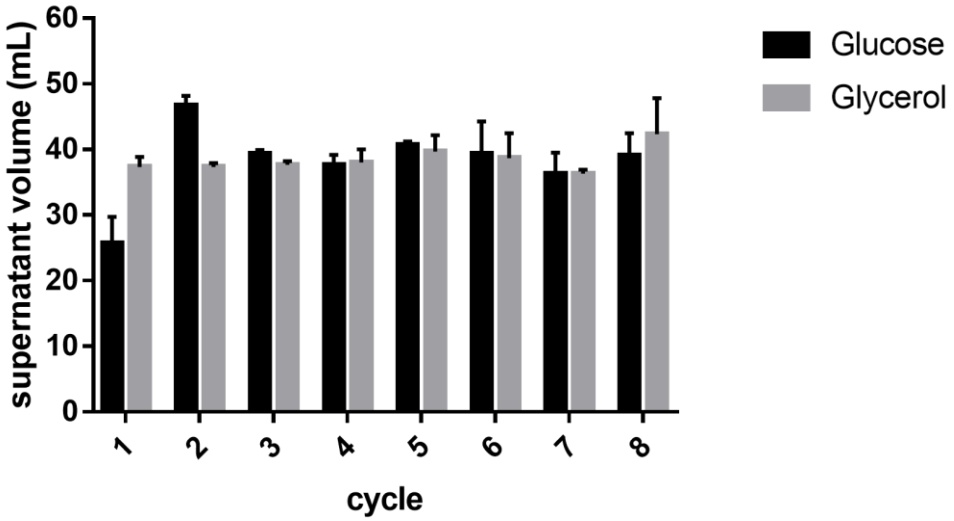

Figure 7.6 Volume of supernatant collected at the end of each cycle for *M. antarcticus* cultures grown on D-glucose and glycerol.

Extracellular lipase activity was assessed in those (cell-free) crude extract (Figure 7.7) which peaked in cycles 3 and 4 for both substrates. There was a reduction in lipase activity in subsequent cycles, where it stabilized at a value of ~5 $U.mL^{-1}$.

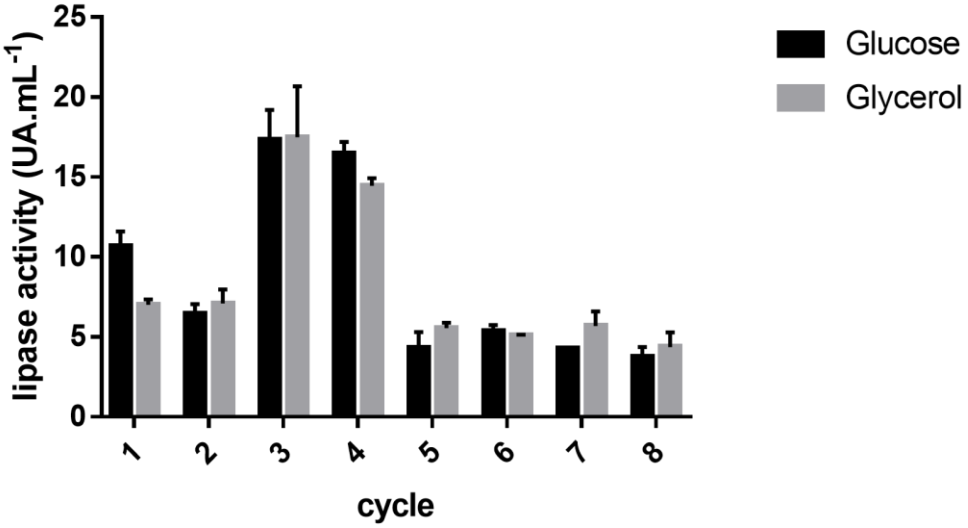

Figure 7.7 Lipase activity in samples collected from supernatant produced from cultures of resting cells with glucose and glycerol as carbon substrates

There was no difference in the value for average lipase activity among all samples results obtained with glucose (8.57 ± 5.26 $U.mL^{-1}$) and glycerol (8.34 ± 4.64 $U.mL^{-1}$).

Although cells retained their performance in terms of lipase production throughout the experiment, a wide variation appeared in terms of MELs concentration in the collected supernatant (Figure 7.8). The cultures peaked early on (cycle 1 for glucose and cycle 2 for glycerol), after which the concentration of dissolved MELs dropped to a fraction of a $g.L^{-1}$, much lower than what would be expected when considering significantly higher concentrations of MELs in the whole fermentation broth (before supernatant separation) at the end of each cycle (as seen in Table 7.1).

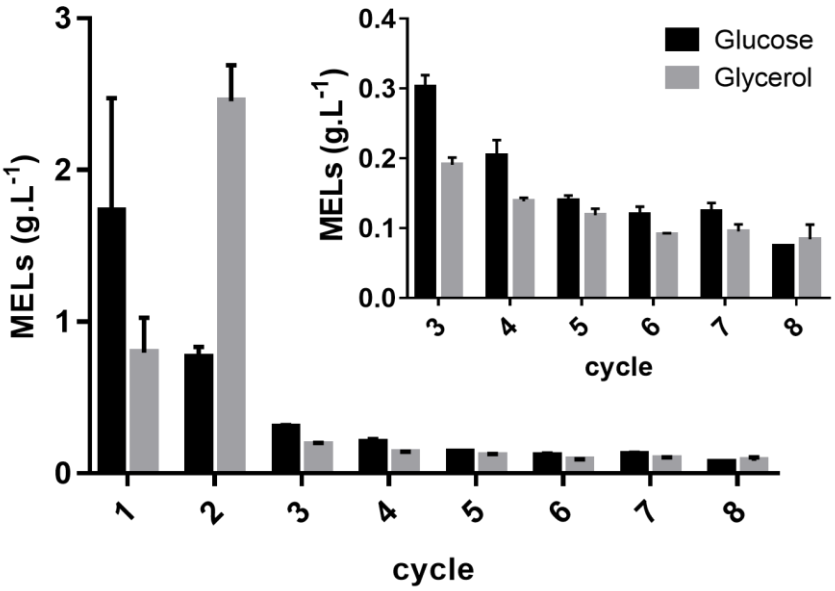

Figure 7.8 Concentration of MELs in samples collected from supernatant produced from cultures of resting cells with glucose and glycerol as carbon substrates

The reason behind this drop could be related to the aging cells and consequent adsorption of most of the produced MELs, as discussed previously in Chapter 3. This hypothesis is further

supported by the fact that MELs were found in much higher concentrations in the complete fermentation broth, which contained the biomass as well. (Table 7.1, Figure 7.5) Additionally, the resting cells could have started to accumulate the MELs intracellularly, in internal lipid bodies, which are known to appear in presence of excess hydrophilic feeds (Morita et al. 2007b).

Yet, when the profiles of MELs concentrations are presented in relation to the CMC Figure 7.9, it is made obvious that even these low titres are sufficiently high to be considered successful for the intended product. The seemingly poorly performing cycle 3 to 8 had a concentration of MELs which was 50 to 150 times higher than the CMC of MELs (2.4 $mg.L^{-1}$) (Morita et al. 2009b).

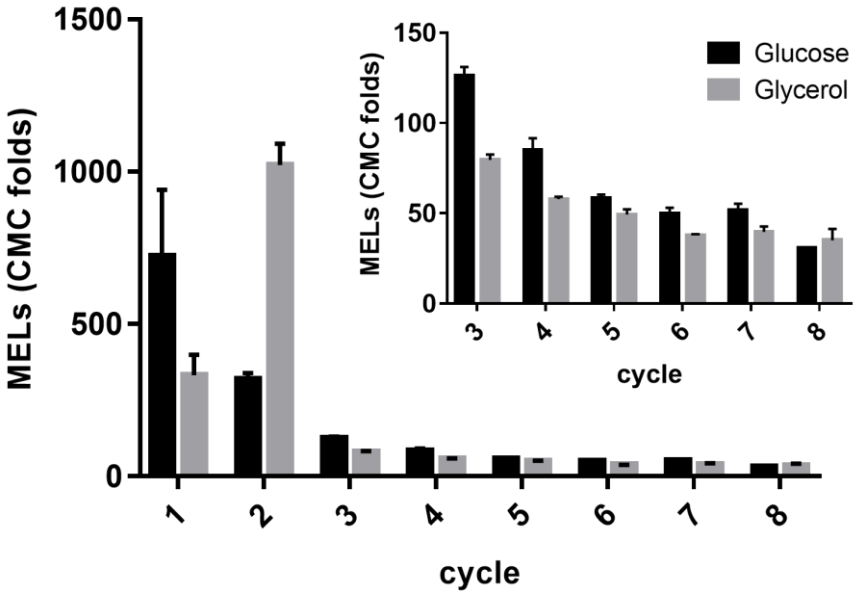

Figure 7.9 Concentration of MELs in samples collected from supernatant, with concentration presented in terms of CMC folds

Lipase and MELs productivity was calculated for the entire process at the end of each cycle. This was done for lipases and MELs, and these results are presented in Figure 7.10.

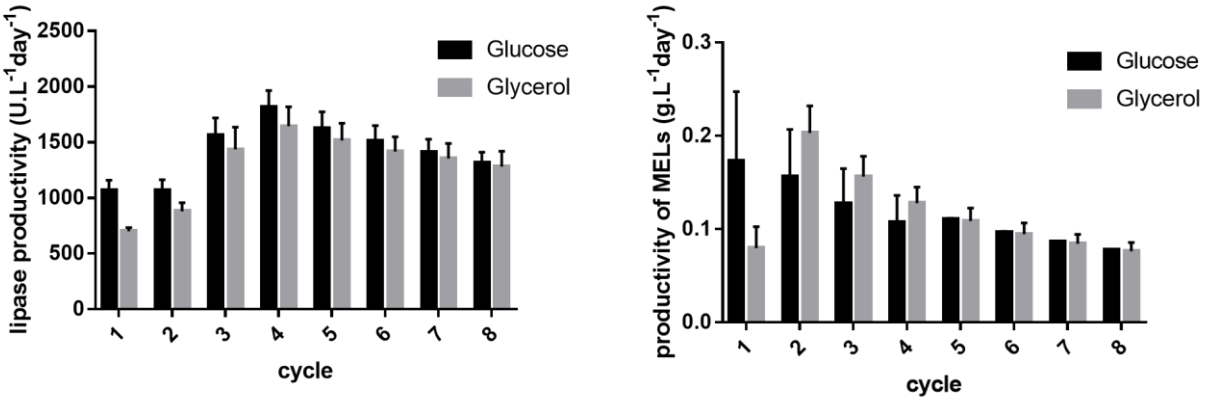

Figure 7.10 Productivity of the entire process at the end of each cycle for lipases (left) and MELs (right) (cumulative values)

Productivity for lipases increased with time, and peaked mid-way through the experiment, after which it started to gradually decrease. Still, even after eight cycle and 52 days of fermentation, the productivity of lipases was higher than for a single cycle fermentation. Productivity of lipase (units of activity per day) was 23.5% and 83% higher for glucose and glycerol, respectively, with a 52-day multi-step fermentation with 8 steps, than with a single-step 10-day fermentation (comparing data obtained after one cycle and eight cycles).

7.5 Conclusions

The process was successfully able to generate significant amounts of lipase using resting cells. The lipase activity and in the solution remained high after several rounds of fermentation, reaching a peak value after several rounds. The productivity for lipases is higher for the multi-step process, compared to the conventional single-step fermentation, especially for glycerol. MELs presence in the cultures reached a certain threshold early on and remained stable. The MELs, however, were not recovered by centrifugation. Instead, they remained in the biomass

pellet (intra- or pericellularly), and the aqueous phase was becoming increasingly depleted of MELs with the progression of the experiment.

Due to MELs extraordinary tensioactive potency, their concentration needed for most applications in which an aqueous solution is used would not need to be remarkably high. The MELs effectiveness would not be significantly increased in concentrations above its CMC. Other biosurfactants tested as laundry detergents were successfully used in concentrations of less than 100 ppm (Helmy, Gustiani, and Mustikawati 2020; Kasturi, Baeck, and Wolff 1998). Literature sources show high cleaning efficiency with lipases in concentration of less than a hundred $U.L^{-1}$.

Detergents for hard surfaces and laundry are usually diluted 50-100 times for use. Supernatant obtained from cycles 3 to 8 could be diluted ~50 times, with the concentration of MELs remaining well above the CMC value (as seen in Table 2.2), and a lipase concentration of 100-200 $U.L^{-1}$. This would make it able to be successfully used in detergency applications.

Both substrates behaved similarly, and selection of substrate can be made based on other relevant factors: sustainability issues or desired application for the product. For most applications, glycerol would be the preferred substrate, as residual glycerol found in the product would not affect its quality. Besides the fact that it can be obtained in a more sustainable manner than glucose, as it is not a food condiment and is generated as waste by other industries, glycerol is already being added to cleaning formulations of commercial products as a builder. This would make a promising, more sustainable alternative to producing aqueous solutions with lipases for various applications.

Chapter 8

8 Novel downstream processing setup for MELs produced from lipid-based substrates

Author contribution:

This chapter was developed in co-operation with PhD Student Miguel Nascimento, and the part of the experimental work related to nanofiltration was performed by him.

8.1 Abstract

In an effort to become competitive in the market, the cost of MELs production has to decrease drastically. Downstream processing contributes greatly to both production costs and lower production efficiency due to product losses. To overcome this issue a new downstream treatment process is proposed, including simple operational steps which avoid the use of mixtures of solvents. The use of a single solvent facilitates to recycle it by simple distillation. First, several solvents were assessed concerning their affinity to MELs and components usually found in the crude MELs collected at the end of the fermentation. Methanol was found to dissolve MELs and smaller lipidic contaminant, as triacylglycerols of larger molecular weight remained undissolved, allowing separation of the fractions by decantation. This feature was harnessed to remove any residual triacylglycerols from the broth by washing the extract. Then, the removed MELs and contaminants were treated by nanofiltration, which allows separations based on molecular weight, with retention of the larger MELs and permeation of the smaller free fatty acids and monoacylglycerols, resulting in a product of increased purity. The decantation and nanofiltration fractions enriched in lipidic contaminants could be reused as substrate in the following fermentations. Finally, MELs were purified with activated carbon, which was used to remove any pigmented compounds created during fermentation, increasing product value. The use of this efficient downstream setup results in a higher process efficiency, enabling to feed larger vegetable oil as carbon source, and potentially reducing the relevance of any unmetabolized residual lipids at the end of the fermentation.

8.2 Introduction

Biosurfactants of microbial origin are produced extracellularly or as part of the cell membrane, by various bacteria, yeasts, and fungi. (Mulligan, Environmental applications for biosurfactants, 2005) These microorganisms produce these compounds in order to form biofilms, attach to water-insoluble substrates or hydrophobic surfaces of plants, or to enhance the availability of lipidic substrates to the cells. (Ratledge, 1997) Compared to synthetic surfactants, mostly produced by fossil fuel derivatives, biosurfactants are more biodegradable, less toxic to humans and the environment, more potent, produce less harmful waste during production and can be produced from renewable resources. (Makkar and Rockne 2003) Glycolipid biosurfactants are the most prominent group of microbially produced biosurfactants, due to their potency, versatility, and high productivity. They consist of a hydrophilic moiety, usually carbohydrates, and a hydrophobic fraction consisting of fatty acids. (Mnif, Chaabouni Ellouz, and Ghribi 2018) Some of the most interesting biosurfactants, concerning industrial production, include biosurfactants produced by yeasts and fungi: Sophorolipids, produced by the yeast *Starmerella bombicola* and Mannosylerythritol lipids (MELs), produced by *Moesziomyces* spp. yeasts (formerly *Pseudozyma*) and *Ustilago maydis* fungus; as well as Rhamnolipids, the most explored bacterial biosurfactant, produced by *Pseudomonas* species. (Jezierska, Claus, and Van Bogaert 2018; Lang and Wullbrandt 1999; Mulligan 2005)

Microbial surfactants, in particular MELs, are able to drastically reduce surface tension of solutions at exceptionally low concentrations. Moreover, these glycolipids biosurfactants form complex supramolecular structures, which translates into promising applications in various fields. These include large volume applications in the production of cosmetics, detergents, wetting agents, enhanced oil recovery agents and bioremediation products. These molecules can also be applied on some high-end pharmaceutical and biomedical applications, as they are able to form microcapsules, show to have anti-tumor effects, and interact with stem cells and

affect their differentiation. (Kitamoto, Isoda, and Nakahara 2002; Mnif et al. 2018; Rodrigues et al. 2006)

However, several factors affect the market competitiveness of biosurfactants compared to chemical surfactants. The main issue, currently hindering the cost-effectiveness of large-scale biosurfactant production, is their production cost price due to low productivity, high raw-material costs, and costly and inefficient downstream processing. (Henkel et al. 2012) The need to develop efficient and cost-effective product extraction technologies has been reported as one of the key steps crucial to enable biosurfactant large-scale production and foster biosurfactant uptake of by many industries. (Campos et al. 2013) In the case of rhamnolipids, a recent review claims that up to 80% of total production cost is attributed to downstream processing, as an economically convincing method was still not developed. (Sekhon Randhawa and Rahman 2014)

Biosurfactants can be produced from a variety of substrates, including lipids, but also hydrocarbons and glycerol. (Marchant and Banat 2012) However, developing production with cheap renewable substrates is key towards a successful transition to large-scale sustainable production of biosurfactants. (Kosaric 1992) These include waste and low-value substrates. The highest productivity of most MELs obtained in this thesis is achieved by using carbohydrate-based substrates in combination with lipids, such as vegetable oils, which can drastically increase productivity by aiding the production of the hydrophobic moiety of the molecule.

However, separating residual triacylglycerols or lipid derivatives from the produced biosurfactant is challenging, due to the formation of stable emulsions and other supramolecular structures - biosurfactant/water/oil systems. (Worakitkanchanakul et al. 2008, 2009) The presence of these contaminants in the final product lowers its quality, applicability, and

potency, which creates the need for an efficient and cheap downstream process for treatment of these products.

Downstream processes currently used are focused on the separation of the crude biosurfactant fraction from the culture broth, either by extraction (with ethyl-acetate, chloroform, alkanes, ether, etc.) or with sedimentation/decantation, often coupled with heating/boiling, precipitation (with acid or ammonium sulfate) or foam fractionation. (Makkar, Cameotra, and Banat 2011) These heavily contaminated products require further treatment for removal of residual lipids by multiple organic solvent extraction steps with hexane, pentane or t-butyl methyl ether, and dehydration by distillation with polyhydric alcohols. To further purify the product, adsorption or column separation could be used, requiring large amounts of solvents (chloroform, methanol, acetone, etc.) in various ratios, which prevents solvent reuse. (Smyth et al. 2010) These steps, besides being costly and unsustainable due to the use of large amounts of non-renewable solvents, decrease the overall efficiency of the overall process due to losses of unrecovered biosurfactant and thermal degradation of the molecule. Furthermore, these processes can generate byproducts which make the final product undesirable in terms of sensory characteristics (smell, odor), which is particularly important for detergent, food, and cosmetic applications. Finally, the use of toxic solvents, such as chloroform, during downstream treatment can prevent product application in the food, cosmetics or pharmaceutical industry due to safety regulation. (Jordan, Stoy, and Sneddon 2021) The environmental impact of various solvents, their toxicity to humans, as well as the possibility of their reuse are all characterized through the GSK's (GlaxoSmithKline) solvent sustainability index (Table 8.1), an award-winning guide for sustainable process design. (Alder et al. 2016; Henderson et al. 2011)

Table 8.1 GSK's Solvent sustainability guide – sustainability assessment for selected solvents for selected relevant parameters. Higher scores indicate favourable performance in the category

Solvent	Recycling	Biotreatment	Aquatic impact	Air Impact	Health Hazard	LCA
Isopropanol	5	3	8	7	10	4
Chloroform	9	5	7	5	4	6
MTBE	8	4	7	5	7	8
MeOH	7	3	10	7	4	9
Ethyl Acetate	6	5	9	5	10	6
DCM	10	4	8	6	7	7
Hexane	8	4	3	5	7	7
Water	2	4	10	8	10	10

Although nanofiltration is extensively used in biotechnology, there is no literature information regarding the use of nanofiltration in downstream processing of biosurfactants. Only relevant report, by de Andrade et al. (Andrade et al. 2017a) refers to the use of ultrafiltration for the separation of protein contaminants from the foam fraction collected from the bioreactor, which contains MELs.

Membrane filtration was reported to be used for small-scale separation of surfactin, where the biosurfactant was retained in the form of micelles by ultrafiltration, with only 70% product purity achieved. (Sen and Swaminathan 2005)

Activated carbon (AC) is an amorphous solid material with an exceptionally large specific surface area. Due to its extraordinary absorptive properties, it has a history of use in purification of drinking water, in industrial processing and medical and pharmaceutical applications. (Baker et al. 2000; Van Wagenen et al. 1975) It has a low water affinity, enabling its use for absorption

for a range of inorganic and organic compounds from aqueous solutions. There are only a few studies that use of AC for the purification of biosurfactants. Dubey et al. (Dubey, Juwarkar, and Singh 2005) produced rhamnolipids with a medium based on a distillery wastewater and these biosurfactants were recovered with AC, as conventional downstream route would yield rhamnolipids with an undesired coloration. Several other sources (dos Santos Mendes de Oliveira et al. 2013; Saranya, Swarnalatha, and Sekaran 2014) discuss the recovery of various biosurfactants by absorption to AC.

The downstream process proposed in this chapter was developed with the aim to provide an efficient solution for the purification of biosurfactants , and MELs in particular, when those are produced from hydrocarbon and lipid-based substrates. Special focus was put on avoiding the use of unsustainable solvents, or solvents mixing, to enable solvent recovery and reuse. The proposed process includes a nanofiltration step, with the goal of removing lipid derivatives (free fatty acids, mono- and diacylglycerols) of molecular size smaller than MELs. Prior to this, a step including removal of triacylglycerols (oil) with methanol is used, as the nanofiltration step cannot efficiently separate these two groups of compounds due to similarity in molar mass of the molecules (vegetable oils: 870-930 g/mol; MELs ~ 676 g/mol). This separation is based on the poor solubility of triacylglycerols in methanol and extraction of MEL in the liquid fraction. This downstream process is presented schematically in the figure below
(Figure 8.1).

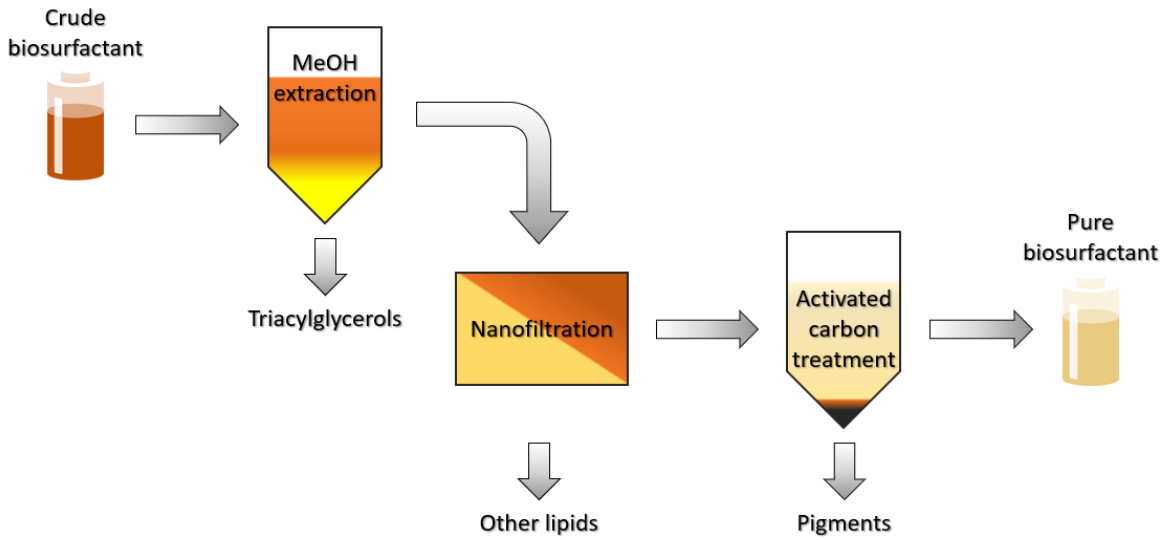

Figure 8.1 Schematic representation of proposed downstream process for glycolipid biosurfactants produced from hydrocarbon and lipid-based substrates

In all steps, single solvents are used, and they can be easily recovered and reused. The recovered lipid fractions are also pure and can be reused as substrates in new fermentations.

8.3 Materials and Methods

8.3.1 Materials and solvents used

Ethyl-acetate, methanol, isopropanol, chloroform, methyl tert-butyl ether (MTBE), dichloromethane (DCM), hexane, and acetone, all were produced by Fischer®, with analytical purity. α-Naphthol with 99% purity produced by Sigma-Aldrich. Sulphuric acid (98% purity) produced by JMGS. Acetyl Chloride produced by Fluka.

Activated carbon produced by Merck, with analytical purity.

Commercial CAL-B produced by Novozym® 435, Novozymes, Denmark, was used.

8.3.2 Mannosylerythritol lipids (MELs)

MELs were produced by Moesziomyces yeast strain *M. antarcticus* PYCC 5048T (CBS 5955) that was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal, by an already established fermentation medium and conditions (Rau et al, 2005). After 12 days, the broth was extracted with ethyl-acetate (with 2:1 EtAc : broth ratio) and the solvent was evaporated using a rotavapor.

8.3.3 Simulation of realistic, impure biosurfactant extract

Commercial immobilized CAL-B enzymes were used by diluting with the appropriate amount of sterile Milli-Q water, enough to enable hydrolysis of vegetable oil. This mixture was kept at 50 °C, mixed with a magnetic stirrer at 400 rpm, and after 24h the organic phase was collected. This mixture of lipids and lipid derivatives was characterised, and mixed with MELs in various ratios, to simulate the extracts collected from fermentations with residual lipid impurities. This protocol is explained in more detail in Chapter 4, for the preparation of the FFA partially hydrolysed vegetable oil.

8.3.4 Removal of triacylglycerols with Methanol washing

Crude extract samples were dissolved in methanol, in the ratio of 10:1 (sample:MeOH, w/w). The solution was mixed vigorously on a vortex mixer for 1 minute, transferred to a separation funnel and the fractions were left to separate for 15 minutes. Then, the bottom, triacylglycerol-rich phase was removed, and the top phase containing the biosurfactant was recovered. Both phases were analysed separately.

8.3.5 Thin layer chromatography (TLCs)

Samples of MELs, lipids and other solutions were eluted using various solvents (specified by the experimental plan: isopropanol, chloroform, MTBE, methanol, ethyl acetate, DCM, hexane, water, EtOH, acetone) in a closed TLC development chamber. The standard solvent mixture used for separation of different MEL homologues includes the use of a solvent system of chloroform/methanol/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}). To reveal the compounds, a 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 at high temperatures.

8.3.6 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 DMAc, PBI Performance Products Inc., USA) was diluted with N,N-dimethylacetamide (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, which was then left still for 24 hours 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), then immersed in a distilled water precipitation bath (1 hour, three times), and then in an isopropanol (Carlo Erba, Spain) bath (1 hour, three times) for water removal and kept on isopropanol until use. All the processes were performed at room temperature. The OSN membrane was not crosslinked and used directly for nanofiltrations.

8.3.7 Nanofiltration of MEL extract

A dead-end Sterlitech HP 4750 Stirred Cell fitted with a circular piece of the home-made PBI OSN membrane with an area of 14.6 cm^2 was used to carry out the filtrations. Replicate were performed using different membrane pieces. A pressure was applied using pressurized nitrogen, providing the driving force of 15 bar applied pressure for the filtrations. All experiments were performed under magnetic stirring of 300 rpm and assays only performed after membrane preconditioned by filtering pure solvent, until a constant solvent flux was obtained, at room temperature.

The membrane rejection values (R) for solutes can be estimated based on Equation 8.1 according with solute concentration in feed (Cf) and permeate (Cp).

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

Equation 8.1

A diafiltration strategy was then performed to purify MEL, retaining this molecule while pushing the smaller lipidic molecules through the OSN membrane. Again, the diafiltration was started by adding 50 ml of contaminated MEL in MeOH solution and, using an HPLC pump Series I, Scientific Systems Inc., fresh MeOH was add as required to keep the retentate nanofiltration cell volume constant, compensating for the volume leaving the system through the permeate. Samples were collected after addition of 2, 4 and 6 diavolumes (DV), with diavolume corresponding to the volume of fresh solvent added by OSN feed volume, i.e., 1, 2, 3, 4 diavolume correspond to 50, 100, 150, 200 mL of MeOH. Under such conditions the decrease on solute on the OSN nanofiltration (i.e., MEL losses or contaminants removal) can

be calculated by equation 8.2, which depends only on membrane rejection to the solute and diavolume used.

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

Equation 8.2

8.3.8 Analysis of fatty acids, mono-, di- and triacylglycerol concentrations

The content of fatty acids, mono-, di- and triacylglycerols was analyzed by a HPLC method (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 centrifugated at 10000 rpm for 2 minutes. The organic phase was recovered and used for analysis by high-performance liquid chromatography (HPLC), with 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 and using a Chromolith Performance RP-18 endcapped (100 mm x 4.6 mm x 2 μm) column. 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).

8.3.9 Analysis of MELs concentrations

MELs concentrations in the samples were calculated based on the results acquired by gas chromatography (GC) of methyl-esters. Solvent was evaporated from the samples, and a transesterification reaction of the lipid chains was performed. Pure methanol (20 mL) was cooled down to 0 °C and 1 mL acetyl chloride was added to generate a water-free HCl/methanol solution. Culture broth samples (1 mL) were freeze-dried, weight and mixed with 2 mL HCl/methanol solution and incubated for 1 h at 80 °C for transesterification into methyl esters.

Heptanoic acid was used as internal standard. The resulting product was extracted with hexane (1 mL) and 1 μL of the organic phase was injected in a GC system (Hewlett-Packard, HP5890), equipped with an FID detector and an Agilent HP Ultra2 capillary column (L 50 m × I.D. 0.32 mm, df 0.52 μm). The oven was programmed to an initial temperature of 140°C and three temperature gradients were defined: 140 to 170 °C at 15 °C.min-1, 170 to 210°C at 40 °C.min-1 and 210 to 310 °C at 50 ºC.min-1. A final time of 3 min at 310 °C was defined. Nitrogen was used as carrier gas with a split of 1/25. MELs were quantified through the amount of C8, C10 and C12 fatty acids considering a molecular weight between 574 and 676 g.mol-1 depending on the length of the two-acyl chain (C8–C12). The quantification of glycolipids based on a specific moiety was previously described. (Faria, M. V. Santos, et al. 2014)

8.3.10 Coloration assays and activated carbon purification

Absorbance in a 450-650 wavelength spectres were determined in freshly made samples, autoclaved samples, and autoclaved samples treated with 1% activated carbon. The specific samples assessed are described in the section of Results and discussion. Autoclavation was carried out by placing solutions in flasks to be sterilized in an autoclave (AJC, Uniclave 88) for 20 min at 121 ºC and 1 bar, following manufacturer instructions. For Activated carbon (AC) treatment, the autoclaved samples were added to falcon tubes with 1% (w/w) Activated Carbon. The contents of the tubes were vigorously mixed using a vortex mixer for 30 seconds. The tubes were then centrifuged for 8 min at 10000 rpm, to precipitate the AC. The aqueous phase was separated, and absorbance was measured.

For solutions absorbance measurements, 200μL samples were collected and placed in plates to be measured in a spectrophotometer for absorbance between 450 and 650nm. To the absorbance

value measured for each sample it was subtracted the value obtained by a blank measurement, containing the solvent (water, ethyl acetate or methanol) to subtract the contributions of plate and solvent to the measurement.

8.4 Results and Discussion

The cost of downstream significantly contributes to the economic cost-efficiency of glycolipid biosurfactants production process. MELs are usually recovered from the fermentation broth at the end of the fermentation using a liquid-liquid extraction with organic solvents, usually with ethyl acetate, hexane, or MTBE. Due to the similar polarity of the biosurfactant and lipidic contaminants, they are extracted together, resulting in a crude product with low purity. Since the lipids and biosurfactants have an antagonistic interaction, impurities of this kind lower the performance and quality of the final product. As the highest MELs titers and yields are obtained with lipidic substrates (vegetable oils), this creates a need for additional purification steps in order to obtain a more refined product. The conventional methods for separation of MELs from lipidic contaminants at laboratory scale include column purification or solvent extractions using multiple solvents, often toxic and/or unsustainable ones, in various ratios. This prevents their separation and reuse, affecting dramatically the sustainability of the process.

An effort was made to develop a simple and efficient downstream processing strategy for MELs, using a single solvent and alternative unit operations. The resulting strategy can be further explored for other biosurfactants, to create a solution which could be applied to other glycolipids produced from lipid-based substrates. With the development of an efficient downstream method, higher substrate concentrations of substrate during fermentation could be used, to achieve higher MELs titres, without compromising the quality of the product and the cost efficiency of the process.

8.4.1 Selection of solvent for separation of MELs from lipids

First, the affinity of various solvents for MELs and lipids was assessed. TLC separation was used as a simple representation of solvent interactions with MELs and vegetable oil, in this case soybean oil (SBO).

A group of solvents with different properties was selected, based on their known use in industrial downstream treatment and their sustainability performance following GSK's Solvent sustainability guide information. The selected solvents were non-halogenated compounds, which are produced from renewable resources in a sustainable manner.

The goal was to find a sustainable solvent that can separate MELs and vegetable oil by dissolving one completely, while not interacting with the other one. This would be applied in a "washing" step, in which the crude MELs would be separated from triacylglycerols.

The use of TLC allows to identify the compounds that moved along with the eluent (indicating interaction with the eluent) from the solutes that present a low interaction with the solvent and remain on the position of the TLC sheets were they were applied. This study was performed with MELs with ~80% purity (without triacylglycerols present), and soybean oil. The results for the eluents assessed are presented in Figure 8.2.

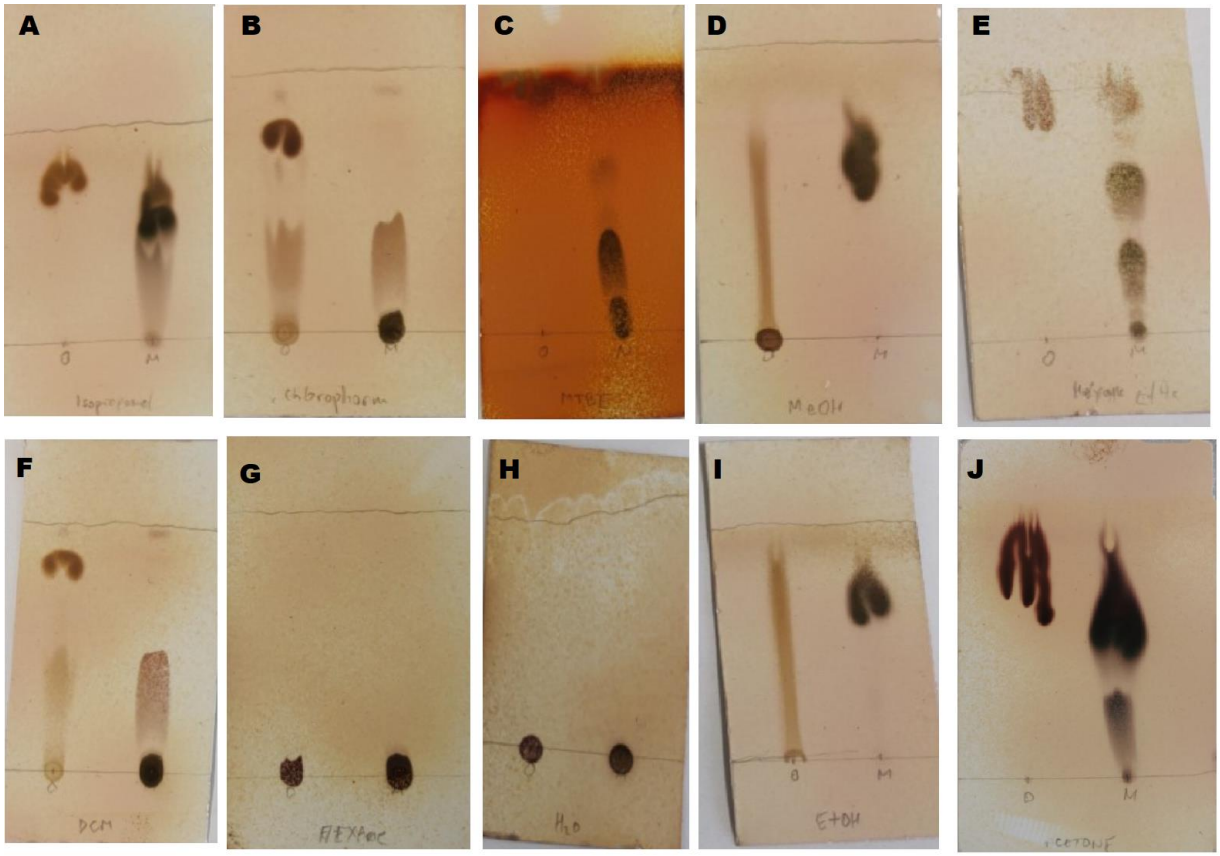

Figure 8.2 TLCs with different solvents. Left dot - Soybean oil. Right dot - MELs (with some residual fatty acids). A - Isopropanol; B - Chloroform; C - MTBE; D - Methanol; E - Ethyl Acetate; F - DCM; G - Hexane; H - Water; I - EtOH; J – Acetone

The Rf values for the two tested samples were calculated based on the migration along the silica gel and are presented in Table 8.2.

Table 8.2 Rf values for MELs and SBO for various solvents

Eluent	Rf Oil	Rf MELs	ΔRf
Isopropanol	0.79	0.58	**0.21**
Chloroform	0.80	0.00	**0.80**
MTBE	1.00	0.39	**0.61**
Methanol	1.00	0.60	**0.60**
Ethyl Acetate	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**
Ethanol	0.00	0.73	**0.73**
Acetone	0.85	0.56	**0.29**

The 5 eluents with the largest difference between Rf values MELs and oil were: DCM, Chloroform, Ethanol, and MTBE, and Methanol. To test whether they could be used to separate MELs from triacylglycerols by a "washing out" one component from the mixture, a preliminary experiment was designed.

5 mL of solvent was mixed with 0.1 g of SBO and 0.1 g of MELs in 15 mL Falcon tubes. The contents of the flasks were mixed and left to separate into phases. DCM, chloroform, and MTBE dissolved both components. Ethanol formed an opaque solution, indicating a formation of an emulsion. However, this emulsion was stable, and these solvents showed no sedimented fraction after being vigorously mixed and left 5 minutes to rest and sediment. After centrifugation for 3 minutes at 4000 rpm, only the Falcon tube with ethanol had sedimented oil.

Methanol proved to be effective at separating the compounds, and phase separation was visible, with oil forming a bottom layer. With methanol 88% of oil was removed, while ethanol managed to separate only 40% of the oil.

The use of other solvents which were found to have beneficial interactions with MELs and triacylglycerols was explored in column separations. Preliminary results for these experiments are presented in Appendix 10.6.

8.4.2 Removal of triacylglycerols with methanol

After testing interactions with triacylglycerols and MELs, the selected candidate that was selected was methanol. This alcohol dilutes MELs and free fatty acids completely (Figure 8.3), with virtually no triacylglycerols dissolved, which enables separation of residual oils from the extract. The solubility of vegetable oil in methanol is negligible (<1g.L-1), however separation efficiency drops with higher concentrations of MELs, since the biosurfactant and the oil form macromolecular structures, stabilizing the oil in the methanol solution.

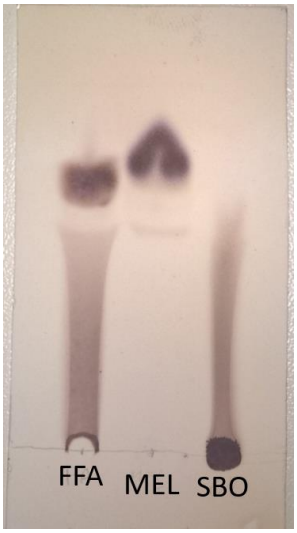

Figure 8.3 TLC with methanol as eluent. FFA - partially hydrolysed oil with free fatty acids (Used for experiments in Chapter 4)

To test the separation efficiency with methanol, a test was performed using a sample of MELs that was mixed with SBO, and SBO partially hydrolysed using the protocol from Chapter 4. This was done to simulate MELs extracted prior to the complete consumption of the lipid substrate.

The mixture of MELs and lipids was dissolved in 20 ml of methanol, and a mild centrifugation (2 minutes at 4000 rpm) was used to speed up the phase separation. Then, the top phase, rich in MELs, was separated, while the bottom phase, a precipitate containing oil, was used for the next step in the separation process. This was repeated two more times. The results of the analysis of the phases obtained in this three-step separation process are presented in Figure 8.4. The percentages of components in each fraction are shown, while the totals indicate the total dry mass of the sample.

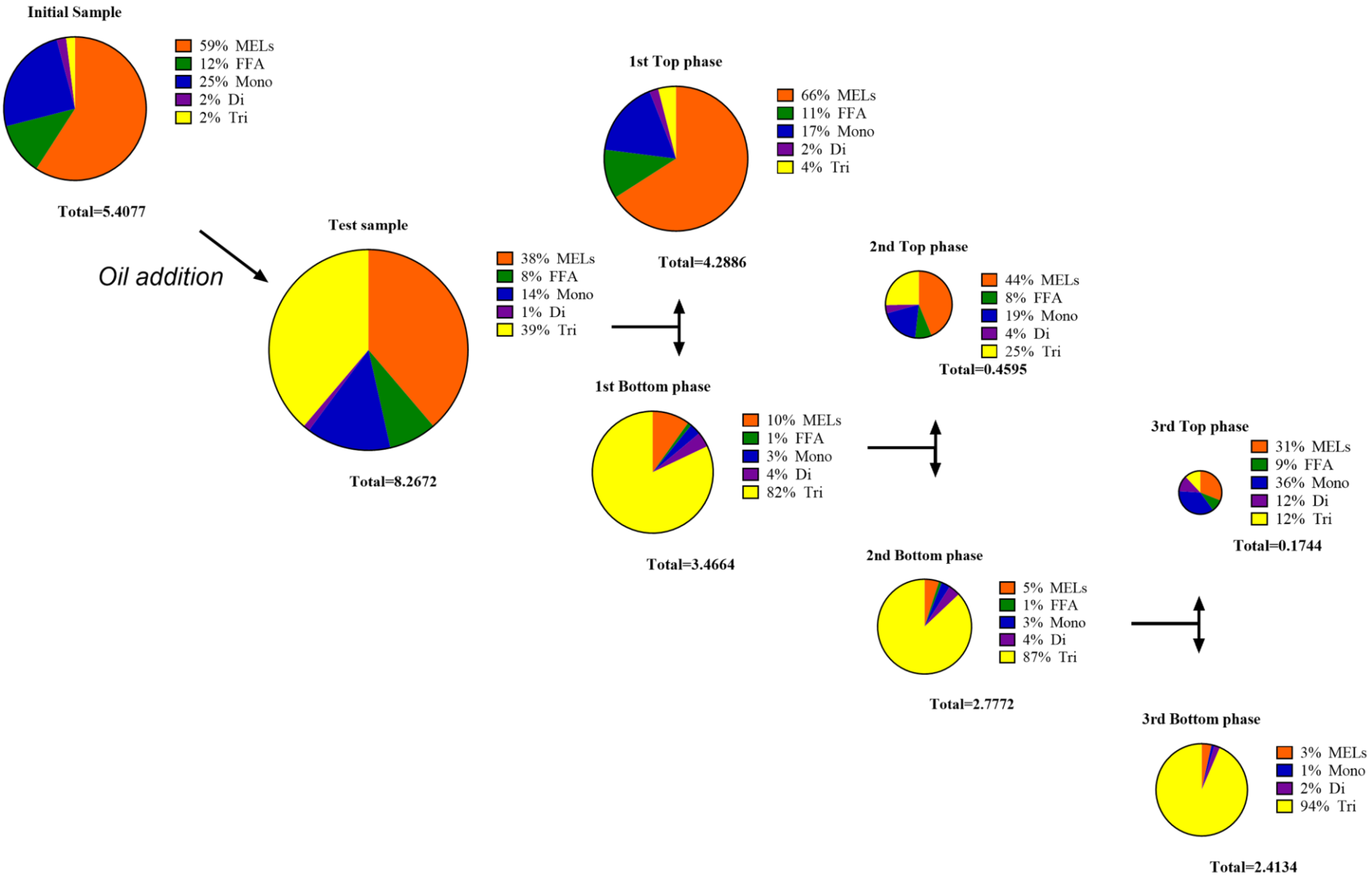

Figure 8.4 Results for three-step separation of MELs and SBO with methanol

After the first step, 89% of the oil was removed, with 11.6% losses of MELs. After the additional methanol "washings" of the bottom phase, the total losses of MELs were reduced to 3.6%. The top phases were added together, while the bottom phase could be reused as a feed to the next fermentation, as the traces of methanol present in it will not affect the culture negatively. Data obtained by analyses of separation efficiency is given in Table 8.3.

Table 8.3 Analysis of efficiency after each step of purification with methanol

	Initial sample	**After 1st step**	**After 2nd step**	**After 3rd step**
Conc. of triacylglycerols (%)	38.73	4.01	6.07	6.28
Conc. of MELs (%)	38.71	65.99	63.84	62.67
Loss of MELs (%)	0	11.56	5.28	3.59

This downstream method can be applied to other biosurfactants produced from lipid-based substrates, as two of the most commonly used biosurfactants, sophorolipids, and rhamnolipids, both have similar interactions with methanol in terms of solubility.

8.4.3 Nanofiltration

Triacylglycerol presence can have a drastic impact on the final product quality, still the fermentation can be optimized to avoid residual triacylglycerols and to facilitate their complete hydrolysis. In such cases, the typically remaining lipidic contaminants are free fatty acids and monoacylglycerols, which are seldom used completely and often remain in the final product in varying concentrations. However, for separation of such residual contaminants, one can explore

the difference in molecules molecular weight (MELs ~ 676 g/mol; Oleic acid ~ 282 $g.mol^{-1}$; Glyceryl monooleate ~ 356 $g.mol^{-1}$). For this case a nanofiltration can be applied using a OSN with a molecular weight cut-off (MWCO) of 300-500 Da. (Wu et al. 2017), where MWCO is defined as molecular size of the solute which membrane rejection is 90%. Although filtration operations are extensively used in biotechnology, there is no literature information regarding the use of OSN in downstream processing of biosurfactants. The only relevant report, by de Andrade et al. (Andrade et al. 2017a), refers to the use of ultrafiltration for the separation of protein contaminants from the foam fraction collected from the bioreactor, which contains MELs.

When considering the whole fermentation broth liquid-liquid extraction, ethyl acetate is used as extracting solvent and OSN could be used to purify MEL from the ethyl-acetate solution, pushing free fatty acids and monoacylglycerols to the permeate, while MEL is concentrated and recover on the OSN retentate. However, methanol is the solvent used when considering case studies with collection of crude MEL from the device (chapter 5) and/or triacylglycerols removal by extraction/decantation. Thus, methanol as solvent was selected to nanofiltration studies.

In-house OSN membranes, made as previous described from different PBI concentrations, were used in a dead-end filtration setup pressurized at 15 bar to filtrate 50 $g.L^{-1}$ solutions of crude MELs in methanol. Membranes prepared from different PBI concentrations will have different MWCO and therefore different rejections for MELs and lipids. Diafiltrations with three different PBI membranes and the MEL purity and MELs losses were estimated for 2, 4 and 6 diavolumes (DV). These results are presented in Figure 8.5.

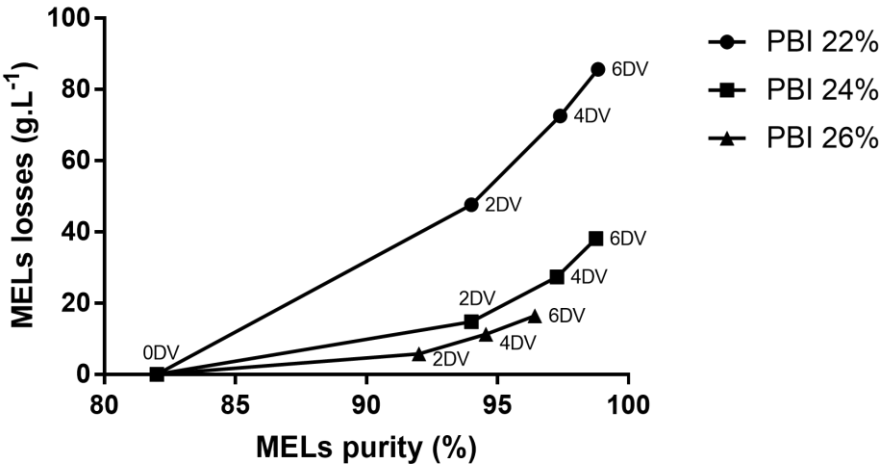

Figure 8.5 Nanofiltration performance after 2, 4 and 6 diavolumes using membranes with 22, 24 and 26% PBI. Initial MELs purity 82%.

The use of PBI 22% membrane resulted in significant losses of MELs. The membrane could be eventually used in a multi-step cascade filtration, in order to reduce losses of the product, however, it does not satisfy the solvent intensity requirements for the intended process.

The PBI 26% membrane obtained a satisfactory purity of ~92% with minimal losses (~5%), with the use of just two diavolumes. For the same conditions, the PBI 24% membrane generated MELs with slightly better purity (94%), however with three times higher MELs losses (15%). As the goal of this work is to develop a sustainable solvent-efficient downstream processing system, the performance of the PBI 26% membrane is satisfactory, and will be used in the remaining experimental work.

8.4.4 Activated Carbon purification

Regardless the purity obtained by extensive downstream processing, MELs obtained through fermentation have a distinct coloration ranging from yellow to brown. Although for some applications this is acceptable, in other cases the refinement of MELs would increase the

biosurfactants relative value. Still, there is little or no information concerning which substances contribute to MELs pigmentation or if such colour is due to the biosurfactant itself. Therefore, this section aims to briefly study MELs crude adsorption on AC to gain insights on the source of coloration of MELs. Three hypotheses can be briefly assessed:

(i) the colour is driven from a medium component and /or formed during the medium sterilization process, which takes place in an autoclave at heightened temperature and pressure,

(ii) the colour is driven from a component generated during the fermentation or the interaction between the cell's metabolic products and a medium components; and

(iii) the colour is due to MEL itself, in such case MELs decolouration would necessarily imply large product loss during AC, due to adsorption of the biosurfactant to the activated carbon.

8.4.4.1 Medium compounds as a source of coloration

Table 8.4 provides a list of medium components, carbon sources, and final products, along with a brief explanation of their significance and possible source of pigmentation. Pigmentation could occur due to the natural coloration of the compound, or it could be generated during media sterilization.

The absorbance of these components in solution was measured before and after sterilization in autoclave, on order to determine if some pigmentation is generated during sterilization. The autoclaved solutions were treated with AC (10 g L^{-1} of solution) and reduction of absorbance was determined.

Table 8.4 Components tested for pigmentation removal by the use of AC

Components	Significance / Possible pigmentation origin
Medium (w/o Glucose)	Used in shake flasks, since C and N are autoclaved separately
Glucose (stock)	Caramelization could generate brown coloration
Medium + Glucose	Used for reactors, C and N autoclaved together
Glucose + Nitrate	Possible pigmented products
Yeast Extract	Pigments present
Yeast Extract (stock)	Concentrated, used as stock solution for medium preparation
WFO	Pigments and oil oxidation products
MELs in EtAc	Solvent interaction effecting MELs purification with AC
MELs in MeOH	
MELs in MeOH after NF	Presence of FFA

Medium stock compounds solutions are usually prepared at higher concentrations (50 times more concentrated than in final media) and then mixed and diluted with water to achieve the desired concentration. For bioreactors, the complete medium is prepared prior to sterilization, and added to the bioreactor before autoclave treatment. A mild increase of coloration is usually observed in the sterilized medium in bioreactors, possibly due to the interaction of glucose, nitrates, and minerals. Therefore, the following samples were prepared: (i) the complete medium without glucose, (ii) the medium with glucose, (iii) the stock solution of glucose, (iv) stock solutions of yeast extract as well as, at the concentrations at which they are present on the medium solutions of (v) the yeast extract alone, (vi) glucose and nitrate and (vii) WFO, which was tested in its pure form, as it is usually added in this form to the bioreactor and flasks after medium sterilization.

For those samples, absorbance in a 450-650 wavelength spectres were determined in freshly made samples, autoclaved samples, and autoclaved samples treated with 1% activated carbon. The reduction in absorbance due to AC treatment was quantified. Fresh, unsterilized components were not treated with AC, as they are never added to the fermentation broth without autoclaving. The changes in total absorbance within the spectrum after sterilization, as well as after activated carbon treatment are presented in Table 8.5. From all the values the value of the blank (the absorbance value for the plate and solvent) was deducted.

Concerning assessing coloration of the product, the same sample of MEL with 80% purity was dissolved on ethyl-acetate at a concentration of 50 g L^{-1} and then submitted either to AC or to OSN, generating the three analysed samples: (xix) MELs in ethyl-acetate after AC and (xix) MELs in methanol after AC, (xx) MELs in methanol after nanofiltration. Nanofiltration was operated with a PBI 26% and 2 diavolumes and MELs purity in methanol after nanofiltration was 92%.

Table 8.5 Values for absorbance (integrated value for visible light spectrum 450-650 nm, with the value for blank deducted) change after sterilization and activated carbon treatment for samples from Table 8.4. Stock solutions are 50 times more concentrated than normal concentrations present in medium.

Component	Operation	Change (%)	Comment
Medium (w/o Glucose)	after autoclave	45	
	after AC	-7	
Glucose (stock)	after autoclave	471	Caramelization
	after AC	-16	
Medium + Glucose	after autoclave	100	
	after AC	-50	
Glucose + Nitrate	after autoclave	-6	
	after AC	-58	
Yeast Extract	after autoclave	143	
	after AC	-24	
Yeast Extract (stock)	after autoclave	231	Highest intensity of colouration
	after AC	-33	
WFO	after autoclave	4699	Increased A due to oil clouding, not real pigmentation
	after AC	-27	
MELs in EtAc	after AC	-90	
MELs in MeOH	after AC	-947	
MELs in MeOH after NF	after NF	-82	

The results presented in Table 8.5 show a drastic reduction in absorbance when the methanol solution of MELs was treated with AC. This is a reduction is 10-fold greater than the one obtained by diafiltration, indicated that adsorption was more efficient than molecular weight based OSN to remove pigments responsible for the product colour. Compared to the use of methanol, its seems ethyl acetate is inhibiting pigment AC removal from MELs crude sample; suggesting either that pigments have a higher affinity to ethyl-acetate than to methanol or potential competition between ethyl acetate and pigments to active adsorption sites. Concerning

medium components contribution to pigmentation, most probably the major responsible is yeast extract (YE), as the stock solution of this component seems to have the highest pigmentation after autoclave and to be more resilient to activated carbon purification. The glucose stock solution also significantly gained coloration, which could contribute to the colour of MELs produced in shake flasks, where glucose is used in stock form. However, solutions of glucose did not gain pigmentation in its diluted form, regardless of the other components present. As MELs have equal pigmentation when produced in bioreactors (where diluted glucose is sterilized) and shake flasks, glucose is probably not contributing to the pigmentation of the final product.

Most peaks contributing to the integrated value for absorbance appear in the range of 500 and 600 nm. Still, after dilution, YE contribution for overall media colour is at the same range of other components such as glucose + nitrate, thus further investigation on the YE involvement on product contamination is needed.

8.4.4.2 Decolouration of YE and MELs by Activated Carbon

To gain further insight whether the source of coloration of the biosurfactant is the yeast extract (YE) used for medium preparation, MELs were produced conventionally – using YE without AC treatment, as well as with separately sterilized YE treated by AC prior to fermentation. If the MELs produced from AC-treated YE would have drastically lower pigmentation (indicated by a reduction in absorbance) compared to MELs produced using untreated YE, it would show that the pigments originates from the YE.

Firstly, it was important to prove that the treatment of YE with AC does not compromise MELs production. After 8 days of fermentation, MELs were extracted from the flasks and analysed.

The data regarding the resulting MELs produced by YE with and without AC treatment are presented in Table 8.6.

Table 8.6 MELs and lipids obtained from fermentation broth (standard medium, components sterilized separately in stock concentrations, with MELs collected by liquid-liquid extraction using ethyl acetate) after 8 days using media prepared with yeast extract without any treatment, or YE treated with AC prior to fermentation.

Component	**No AC treatment of YE**	**AC-treated YE**	**Reduction (%)**
MELs ($g.L^{-1}$)	5.25 ± 0.38	4.99 ± 0.21	4.74 ± 2.89
Lipids ($g.L^{-1}$)	0.13 ± 0.07	0.06 ± 0.03	52.50 ± 2.50

In spite the fact that AC can absorb bioactive compounds from the YE, this impact on MELs production is negligible. Although the concentration of lipids in the extracts was reduced by half, such a reduction is also negligible and not statistically significant, when the low concentration of the lipids obtained at the end of this fermentation is considered. Furthermore, as the range of error for both samples overlap, one can concluded that AC treatment of YE did not affect this fermentation outputs.

MELs produced with pre-treated YE were treated by AC, in order to determine whether post-fermentation pigmentation occurred. Absorbance was measured, as previously reported, in this AC-treated sample of MELs, as well as in samples discussed in Table 8.6. A photograph of the samples measured in the spectrophotometer and spectra obtained are shown in Figure 8.6.

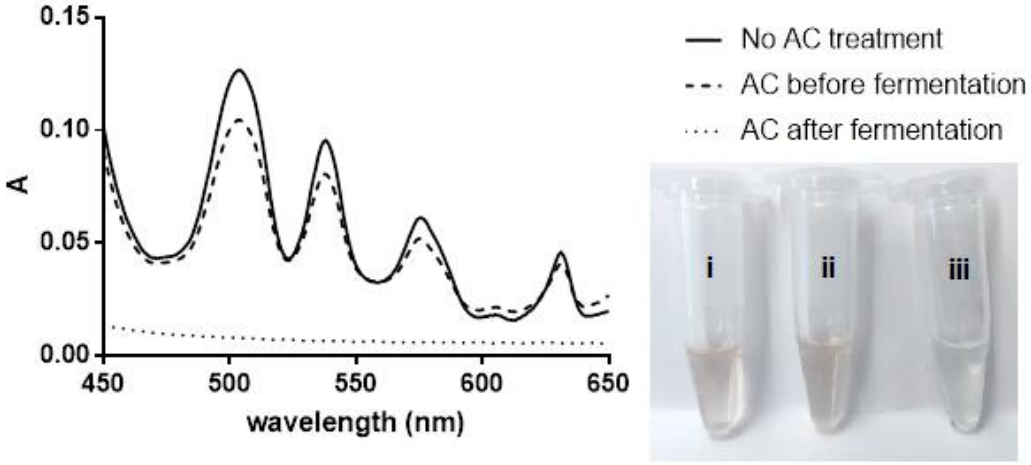

Figure 8.6 Solutions and Absorbance profiles of MELs solutions prepared with yeast extract with no treatment, yeast extract treated with AC prior to fermentation, and MELs solution treated with AC after fermentation.

Interestingly, four distinct peaks existed in the absorbance profiles of MELs withing the visible light spectrum. They could probably be attributed to specific compounds which in combination contribute to the coloration of MELs. In line with this, changes in absorption caused by treatment of extracted MELs with AC were determined not only for the total integrated absorbance area for the visible light spectrum, but for the individual peaks, in order to determine if some of them are affected by the AC treatment more than others.

Table 8.7 Results for Absorbance of MELs solutions prepared with yeast extract with no treatment, yeast extract treated with AC prior to fermentation, and MELs solution treated with AC after fermentation. Values of absorbance at peaks presented. The reduction of A at peak wave lengths given, as well as the reduction of the total integrated absorbance area for the visible light spectrum.

Peak λ (nm)	**Intensity**		**AC treatment before fermentation Reduction (%)**	**Intensity**	**AC treatment after fermentation Reduction (%)**	**Colour**
	No treatment	AC treated YE		AC treated MELs		
504	0.1268	0.1046	**17.51**	0.0078	**92.54**	Green
538	0.0956	0.0806	**15.69**	0.0065	**91.94**	Green
575	0.0611	0.0519	**15.06**	0.0060	**88.44**	Green/yellow
631	0.0458	0.0407	**11.14**	0.0056	**86.24**	Blue/cyan
Average reduction of peaks			**14.85**		**89.79**	
Total absorption change			**8.68**		**84.88**	

Based on the visible coloration of these solutions and respective absorbance spectrum (Figure 8.6 and Table 8.7), two conclusions can be made. First, the treatment of yeast extract by activated carbon prior to fermentation did not significantly reduce the coloration of the resulting MELs. Indeed, when the YE was treated prior to the fermentation, there was only a slight reduction in the coloration and absorbances of the final product. Therefore, the pigmentation cannot be allocated to YE solely. Second, the MELs produced from AC-treated YE, when treated by AC themselves, were seemingly completely decolorized.

Indeed, a significant reduction in absorbance profiles were attained after the AC was used to treat the MELs itself, visible both on the spectrum data and calculated absorbances reduction

values. This indicates that most of the coloration is generated during fermentation, or it is driven from other substrate than not YE. After solvent evaporation, the obtained dry product had a bright yellowish colour, in contrast to the initially brown coloured crude MELs.

To test whether there was loss of product during the AC treatment of MELs, which could affect the coloration of the solution, the samples were analysed by GC, with results reported in Table 8.8.

Table 8.8 Analysis of crude MELs composition before and after treatment with AC

Component	**MELs before AC treatment**	**MELs after AC treatment**
MELs ($g.L^{-1}$)	49.9 ± 2.13	49.12 ± 2.04
Lipids ($g.L^{-1}$)	0.61 ± 0.28	0.70 ± 0.45

MELs were quantified before and after AC procedures with results reported in Table 8.8. Although literature sources state that AC can interact with other glycolipid biosurfactants, and can be used for their recovery (dos Santos Mendes de Oliveira et al. 2013), in this case there were no losses of MELs due to adsorption to the AC.

The purification step with AC was tested for other biosurfactants as well and managed to reduce the coloration measured by absorbance within the visible light spectrum by 88.27% for rhamnolipids, and for 12.88% for sophorolipids. The poorer performance obtained with sophorolipids could be related to the substrates used and the working microorganism, which did not generate pigmented compounds during fermentation. Nevertheless, more research is needed for support this hypothesis.

8.5 Conclusion

Downstream processing significantly adds to the cost of biosurfactant production, reducing market competitiveness of these valuable products. Conventional downstream processing setups require the use of large volumes of mixtures of non-sustainable solvents, which cannot be reutilized without energy-consuming distillation processes, which are often cumbersome due to the formation of heterogeneous mixtures and azeotropes.

A simpler and more sustainable downstream processing strategy is proposed, using single solvents at a time, including three steps: methanol washing to remove residual triacylglycerols, followed by nanofiltration to remove smaller lipidic contaminants. Finally, activated carbon was used to remove pigmentation from the final product. Note that methanol was also the same solvent used as carrier to remove MEL crude from the harvesting device.

In an effort to find a solvent which would easily separate triacylglycerols from MELs, a screening was performed using TLCs with multiple solvents as eluents. Methanol was selected, as it fully dissolves MELs and smaller lipidic contaminants (free fatty acids, monoacylglycerols and diacylglycerols), while triacylglycerols dissolve very poorly. With this single solvent system, a sample containing roughly 1:1 ratio of MELs to SBO was purified in a three-step process with only 4% loss of product.

After the first step, 89% of the vegetable oil was removed. The development of this efficient and simple method of separating triacylglycerols from the final product would not only increase the value of the product, but would also increase the production efficiency, as larger feeds of vegetable oil could be used without raising concerns of residual unmetabolized triacylglycerols at the end of the fermentation.

Nanofiltration was used to separate the MELs from smaller lipidic contaminants, including FFA and monoacylglycerols. A screening was performed using several *in-house* manufactured membranes, containing varying levels of PBI. The crude MELs were dissolved MeOH for these tests at a concentration of 50 $g.L^{-1}$. The best results, in terms of avoiding of MELs losses, was achieved using PBI 26% membrane. With this system, a 92% purity of product was achieved with a moderate amount of solvent (2DV), and acceptable product losses of just 5%.

In a final effort to purify the glycolipids from pigmented compounds, they were treated with activated carbon. This method was successfully applied to MELs, for which the pigmentation was reduced significantly, without any losses of product. The yeast extract used in the medium formulation was treated with AC in an effort to assess whether it is the source of the coloration of the final product. However, this step did not reduce the coloration of the MELs, and it can be concluded that the pigmentation is driven from other component fed to the fermentation or generated during fermentation as a product of the microorganism's metabolism. The AC treatment was successfully applied to rhamnolipids, reducing their coloration by 88%, as measured by spectrophotometric absorbance in a segment of the visible light spectrum.

The use of methanol as the solvent in all the steps of the process avoids the use of ethyl-acetate or other more complex mixtures and enables the direct processing of beads harvested using the bead retrieval system presented in Chapter 5.

Chapter 9

9 Conclusions

9.1 Conclusions

The aim of this thesis was to explore pathways toward the improvement of sustainable production of Mannosylerythritol lipids. This aim was tackled from several perspectives and specific research questions are addressed from Chapter 3 to 8. Those include strategies both to increase productivity, titres and MEL-rich beads production and develop novel efficient downstream process, as well as studies to gain insights on the effect of specific stresses on *Moesziomyces* yeasts physiology that affect MELs production performance.

9.1.1 Work summary

A holistic approach was taken to improve the MELs production process as a whole and considering all the key process stages and envisaged application (Figure 1.2). Several aspects of the production process were addressed, in order to improve performance by obtaining better yields in a shortened period or in more sustainable manner.

- The possibility of using sea water was explored, in order to increase the sustainability of the process, as well as to make moves towards the development of a marine-based process for a product used in marine bioremediation. Although the metabolism of the yeast was affected, it promoted formation of MELs in bead form, offering a chance for simpler downstream processing.

- Strategies to improve process upstream stages performance include the use of yeasts and enzymes in combination, namely using the native lipases produced by the MELs-

producing microorganisms to partially degrade the lipids used as a carbon source in the fermentation; a strategy that was effective in shortening fermentation time.

The heart of the production process – the bioconversion of substrates to product, needed to be improved in order to obtain better titres and improve productivity.

- The effect of various feeding strategies on the production of MELs and lipases, as well on the cells' physiologies, was studied. Glycerol was found to be beneficial when used in the inoculum preparation, as it promoted the formation of smaller yeast morphological structures, which in turn are better MELs producers. Deeper information on how the process is affected by different cell physiology was obtained, as well as the underlying effects that determine cell differentiation.

- The formation of MEL-rich beads was investigated, which were the focus point of several chapters of the thesis. Although this phenomenon did not get much attention in scientific literature, it shows great potential to improve the efficiency of the production of MELs in an industrial scale. Information regarding the factors determining their appearance, disappearance, and features was obtained, opening the possibility towards the development of methods to stimulate their materialization. A relation between bead formation and cell properties was noted. Changes in surface hydrophobicity caused by stress, also causing differentiation into various cell morphologies, was hypothesised to be connected to the concentration of "free" MELs in the fermentation broth, which tends to agglomerate into beads.

For a production process of a biosurfactant or any similar microbial product to be effectively produced in a larger scale, its downstream processing should be effective, cheap, and simple.

Several downstream strategies were explored, which would prevent the use of non-renewable and toxic chemicals, while still enabling the recovery of a product with relative purity

- A new device was developed, specifically designed for harvesting *in-situ* the MEL rich beads, several times over a single fermentation without its interruption; while there are several possible options to collect the MELs beads from such device, in the current study a small amount of methanol was used.

- Methanol was used to remove residual triacylglycerols from the extract of the broth at the end of the fermentation, as triacylglycerols stay on a bottom rich phase and MEL and free fatty acids are carried out dissolved on the methanol top phase; this finding allows to move the focus from complete utilization of substrate towards higher product titres.

- Nanofiltration was used do remove smaller residual lipidic molecules (e.g., free fatty acids and monoacylglycerols); the nanofiltration was performed in methanol and a smaller number of 2 diavolumes is recommended to yield a MEL of higher purity, with minimal MEL losses.

- Finally, the product was purified with activated carbon to remove pigmented compounds giving MELs their distinctive colour, which were found to be created during fermentation. The final product had a mild yellowish coloration, in contrast to the intense brown colour of crude MELs.

The decision-making during the development of other stages of the bioprocess was done with the intention of opening the possibility of product use in various applications. Namely:

- Ecotoxicity of MELs was tested to enable the use of the biosurfactant in marine and terrestrial bioremediation efforts, showing their better performance compared to other glycolipid biosurfactants.

- A fed-batch production process with and integrated manufactured device for bead retrieval resulted in a potentially completely solvent-free product, giving it a greater value in certain branches of industry.

- Finally, the possibility to produce an aqueous product based on MELs and lipids using resting cells was explored, with potential applications in home care product formulations

The overview of the connections between the chapters – how findings from one inspired the work performed in the other, and how the whole work comes together, is all illustrated in Figure 9.1.

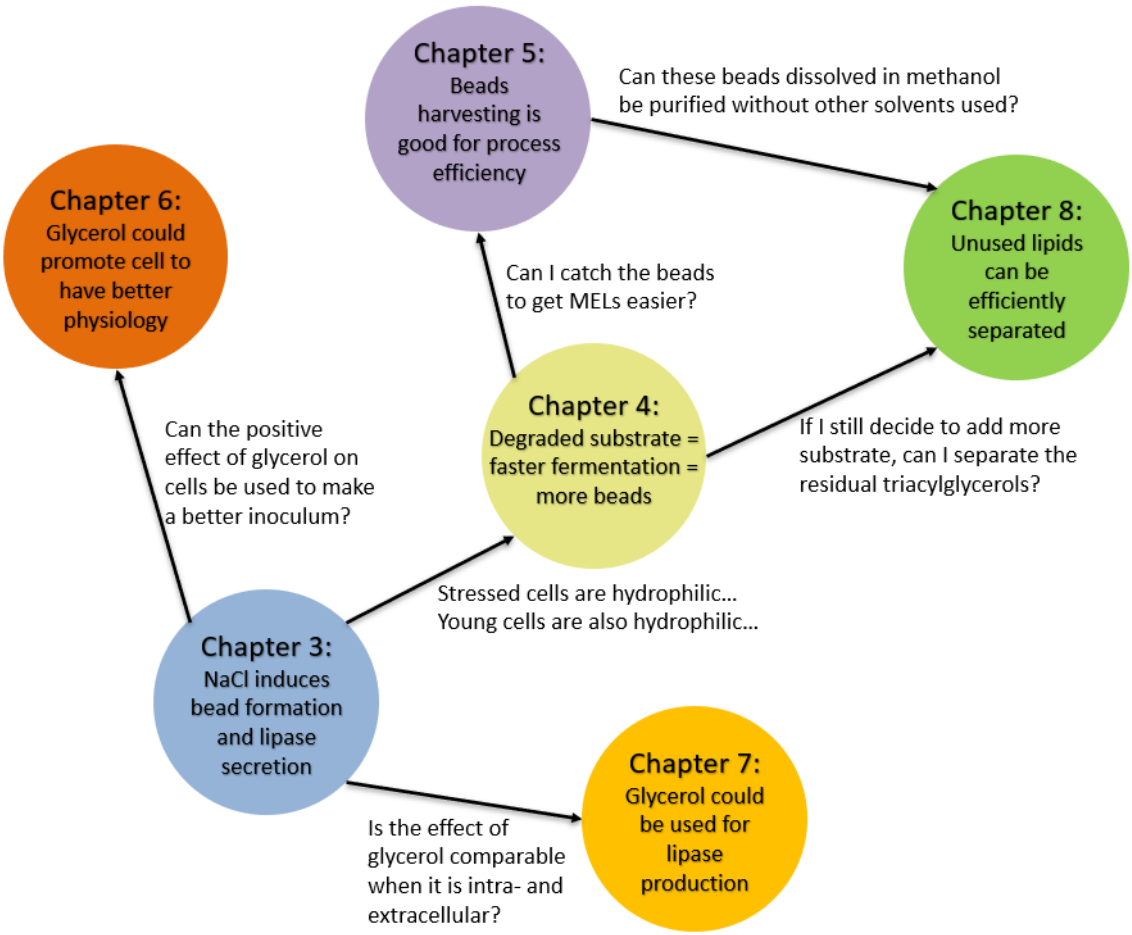

Figure 9.1 The interconnections of the experimental work performed in different chapters

9.2 Process integration

In conclusion, a MELs production process could be hypothesises which integrates improvements developed in this thesis.

- Glycerol is used for inoculum preparation, allowing the formation of favourable yeast physiological structure in the main fermentation
- The substrate is pretreated with native lipases, produced in a separate process, possibly using an aqueous lipase-rich product, produced in a continuous process including supernatant collection in cycles.

- This results in higher yields of MELs in bead form, which are collected using a integrated device for *in-situ* bead collection.
- The collected beads are dissolved in methanol, and any residual triacylglycerols are precipitated and removed, obtaining a solution containing a mixture of MELs and smaller lipid derivatives.
- These remaining lipids, including free fatty acids, mono-, and diacylglycerols, are removed by nanofiltration.
- The separated lipids are reintroduced as substrates to subsequent fermentations after solvent evaporation
- Finally, the obtained lipid-free MELs are treated with activated carbon, which removes the pigmentation generated during fermentation, resulting in pure, sustainably produced MELs.

9.3 Future perspectives

Use of renewable substrates

The integration of the MELs production process within a biorefinery is facilitated when an array of waste material and renewable complex substrates can be used as substrates. Therefore, the substitution of the hydrophilic and hydrophobic carbon sources assessed within the current thesis, by different agricultural and food processing wastes should be assessed. Ideally, media composition should be optimized to avoid the need of addition of mineral nutrients. Furthermore, waste waters and streams with a high moisture content should be favoured, as their use would reduce the need for addition of fresh water in the medium. Although no sustainable hydrophilic substrates were tested within this thesis, some preliminary results for sugar beet processing waste are presented in Appendix 10.7.

Novel bioreactors for challenging a fermentation

The MELs production process in technologically complex: a combination of hydrophobic and hydrophilic substrates is used, solids form throughout the fermentation, the product is tensioactive which causes foaming, etc. Note that in the conventional bioreactor it is challenging to maintain a high oxygen supply to the cells while avoiding formation of foam. To address this challenge alterative aeration and agitation techniques should be explored, and novel fermentation-support systems should be developed for better control of key parameters.

Reaching higher MELs titres

Improvements made to the MEL production process are still not sufficient to make it effective in an industrial scale, especially when compared to other biosurfactants. The aim should be to reach titres of MELs in the order of hundreds of $g.L^{-1}$, in an effectively sustainable manner. This can be achieved by the development of novel feed strategies, media formulations, and improvements to the working microorganism's genome. The production cost should be lowered enough to make the product competitive in the surfactant market, as only then its full potential for diverse applications could be harnessed.

Developing understanding of the bead formation phenomenon

Although some knowledge was gained regarding the mechanism that determine the appearance of MEL-rich beads, there is still the need for future research to understand the formation of such structures. The observation that such MEL rich beads do dissipate in later times of the fermentation, despite the fact that there is no drop in MELs concentration, should be further explored. On the other hand, the exact causes for beads to form in various points throughout

the fermentation, when different microorganisms and feed strategies are used, it is not yet clear. The possibility of forecasting - and possibly triggering bead formation - should be studied, either by introducing hydrophobic seeding materials to induce bead nucleation, or by some other induction method. Additionally, the study of how MELs interacts with the surface of the cell, and what can trigger detachment of MEL from the cells should be explored when envisaging the developing of MEL rich aqueous products.

Exploration of MELs' supramolecular structures

Although the structure of the MELs molecules is well known, a deeper understanding is lacking in the scientific literature concerning the supramolecular structures formed by these biosurfactants. State-of-the-art microscopy and analytical techniques should be harnessed to gain knowledge of all the complex structures the different homologues form. Along with the exploration of MELs interactions with other lipids, this would contribute to modelling the formation of beads during fermentation. Furthermore, innovative applications for the biosurfactant, mainly in the fields of pharmacology and medicine, could be developed based on the exploitation of these unique properties.

Exploring innovative applications

The effectiveness of emerging and innovative products is often measured by their ability to perform better than the ones they are aimed to replace. Although this might not be challenging for biosurfactants, bioproducts in general should be envisioned as not only alternatives to conventional fossil fuel-based chemicals which they will outperform, but as products which will be used in novel, previously impossible manners. The full potential of MELs, supported by its biocompatibility, biodegradability, effectiveness, and structural diversity features should

be considered when new applications are developed. At first, while the production costs are still high, focus should be placed on high-end product applications, especially in the field of medicine, pharmaceuticals, and cosmetics. This would pave the way towards other utilizations in which MELs will be used as bulk chemicals, and large amounts of low-grade biosurfactant could be produced with economic feasibility.

Lifecycle analysis (LCA) and study on economic feasibility

To fully comprehend the impact changes in process configuration proposed in this thesis would make on the overall sustainability of the MELs production process, both a Lifecycle Analysis and an economic feasibility study should be made. These analyses would enable to quantify the specific improved made by the novel aspects in the process, and to compare them with alternatives.

9.4 Final remarks

The COVID-19 pandemic prevented the development of several planned real-life applications for MELs which would require activities outside the laboratory. Some promising preliminary results were obtained for certain applications, however they were not presented in this thesis in order to not compromise any future intellectual protection, such as in the form of patents.

The general decision-making was dictated by improvements to the overall sustainability of a future industrial-scale MELs production process. The author hopes that the findings in this thesis could be applied to other similar bioprocesses, as they might face similar challenges in their transition to a commercially viable production process. Finally, steps taken in the

development of this bioprocess were made with keeping in mind its possible integration into a larger bioconversion system within a biorefinery.

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10 Appendix

10.1 Supporting Information for Chapter 3: Profiles of relevant parameters for flasks exposed to NaCl

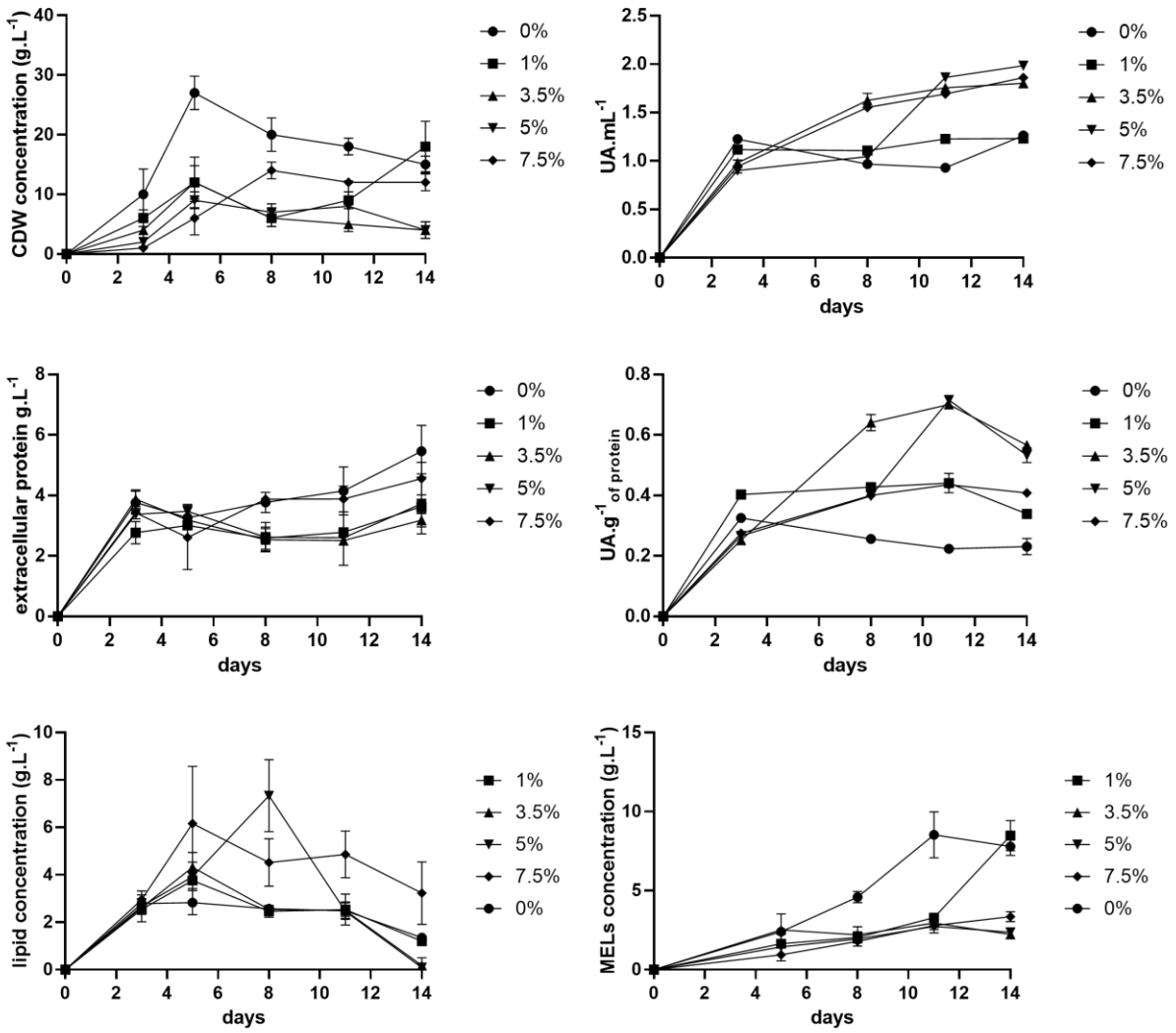

Figure 10.1 Profiles of relevant parameters in flasks containing various concentrations of NaCl: Biomass; Extracellular Protein; Lipase activity; Lipase specific activity; Free Fatty Acids; MELs (concentration of MELs calculated based on samples obtained

10.2 Supporting Information for Chapter 3: Phytotoxicity of MELs

For evaluating phytotoxicity of MELs, lettuce seeds (Lactuca sativa) (Flora Lusitana, Portugal) were used, following a reported protocol. (Mañas and De las Heras 2018) For these experiments, fresh water was used (0% NaCl).

Seeds were placed in Petri plates between two layers of filter paper, covered with 5 mL of MEL solution and incubated at 25 °C for 5 days, exposed to indirect sunlight. After that period, percentage of germinated seeds was calculated, and root and hypocotyl were measured to determine impact of MELs on their elongation. Results for root and hypocotyl elongation are presented in Figure 10.2.

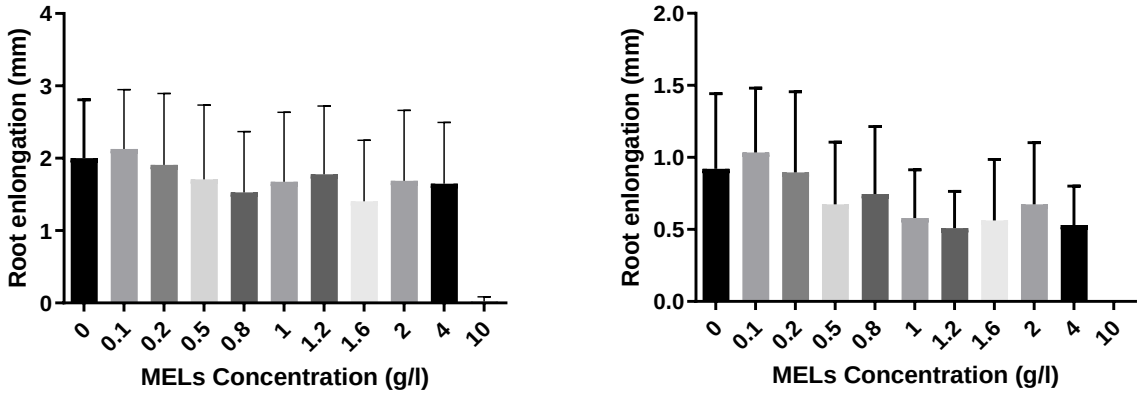

Figure 10.2: Root (left) and hypocotyl (right) elongation for lettuce seeds germinating in presence of various concentrations of MEL.

Results clearly indicate MELs low phytotoxicity, with no toxic impact observed in relevant concentrations. Although not presented here, germination was within the same values for all tests, except for the plate with 10 $g.L^{-1}$ of MELs, where 10% of seeds germinated. Liduino et al. (Liduino, Servulo, and Oliveira 2018) report rhamnolipid toxicity for sunflower seeds, where at 10 mg/kg of soil lethality for seeds was 100%, which is drastically lower than the values in

this paper. In this study, sophorolipids showed no toxicity, and other reports explored stimulative effects of biosurfactants on seeds in their germination phase. (De Andrade 2020) As no phytotoxic effects were observed at relevant concentrations (an IC50 below 1 $g.L^{-1}$), MELs can be considered non-toxic for plants. MELs' CMC is ~3 $mg.L^{-1}$ (Kim et al. 2002), much lower than 10 $g.L^{-1}$, the only value where a drastic effect on the seeds was observed. High seed mortality at this concentration could be attributed to seed respiration inhibition due to the layer of the biosurfactant on the seed.

10.3 Supporting Information for Chapter 5: Study on effects of calcium presence on bead formation

Moesziomyces yeasts are potent producers of CAL-B, a versatile lipase enzyme, which is widely industrially produced. However, another major lipase produced by these yeasts is CAL-A, which is dependent on Ca ions (Anderson et al. 1998; Kirk and Christensen 2002), which act as a cofactor for the activity of this enzyme. In order to test of Ca presence in the medium is affecting lipid degradation, and thus MELs production, some experiments were performed with a medium supplemented with calcium, in the form of 1.5 $g.L^{-1}$ of calcium nitrate. In order to keep the same C/N ratio for both media, the calcium nitrate substituted half of the sodium nitrate, which is normally added, and the remaining sodium is added in the form of sodium carbonate. Detail on both media is presented in Table 10.1.

The beneficial effect of calcium in the media was initially theorized based on the results obtained with cheese whey as a substrate obtained within our team, since higher titers and yields of MELs were observed, with fast production of a large amount of MEL-rich beads.

Table 10.1 Composition of the conventional medium and newly proposed Ca-supplemented medium, for initial addition of Ca, as well as Ca addition on day 4

Media Component	**Old medium**	**Ca medium day 0**	**Ca medium day 4**	
			Day 0	Day 4
Glucose	40 $g.L^{-1}$	40 $g.L^{-1}$	40 $g.L^{-1}$	
Yeast Extract	1 $g.L^{-1}$	1 $g.L^{-1}$	1 $g.L^{-1}$	
KH_2PO_4	0.3 $g.L^{-1}$	0.3 $g.L^{-1}$	0.3 $g.L^{-1}$	
$MgSO_4$	0.3 $g.L^{-1}$	0.3 $g.L^{-1}$	0.3 $g.L^{-1}$	
$NaNO_3$	3 $g.L^{-1}$	1.5 $g.L^{-1}$	1.5 $g.L^{-1}$	
$Ca(NO_3)_2$	/	0.94 $g.L^{-1}$	/	0.94 $g.L^{-1}$
Na_2CO_3	/	1.45 $g.L^{-1}$	1.45 $g.L^{-1}$	

In order to estimate the effects of Ca on fermentation performance, a group of fermentations with these proposed feed strategies were performed. Compared to the standard medium used in the rest of this thesis, an additional medium, supplemented with calcium was tested. The calcium was added initially, at day 0, or at day 4, according to Table 10.1. The results for biomass concentration in the flasks are presented in Figure 10.3

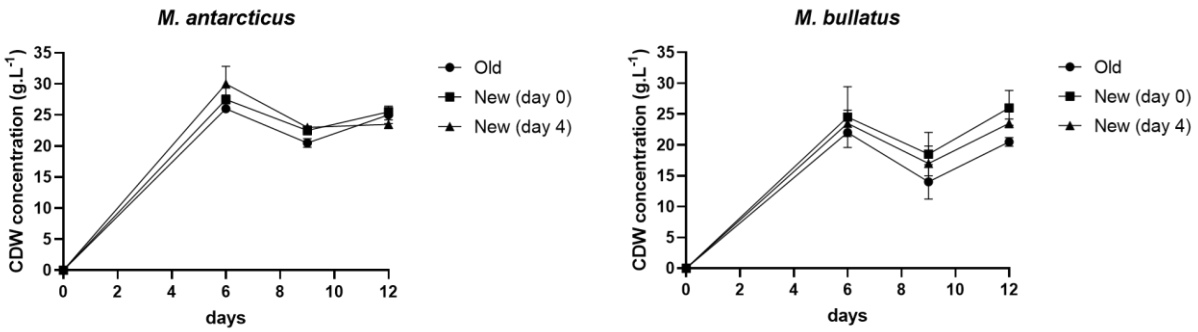

Figure 10.3 Profiles of CDW concentration for cultures with feed strategies without (old medium composition) and with Ca addition (new medium composition, Ca added on day 0 and day 4).

The results showed minor difference between the various cultures in terms of biomass profiles. The results of MELs profiles are presented in Figure 10.4.

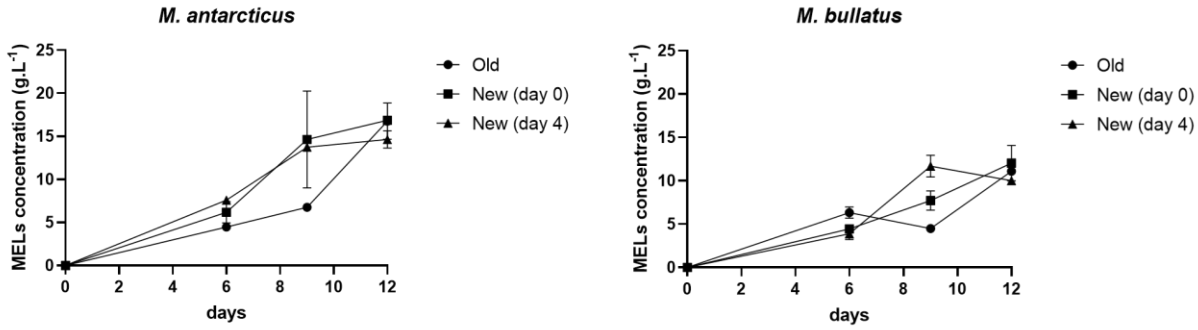

Figure 10.4 Profiles of MELs concentration for cultures with feed strategies without and with Ca addition (on day 0 and day 4)

The addition of calcium to the media was beneficial for bead formation, causing larger, more orange beads to appear earlier on (Table 10.2). This can be caused either by activation of CAL-A, which helps with faster oil degradation, or by causing other effects which affect the cell walls (i.e., formation of Ca-bridges, etc.). Nonetheless, further research should be carried out to explore this phenomenon.

Table 10.2 Bead presence and appearance for different experimental setups: working microorganisms, substrates (SBO - soybean oil, FFA - free fatty acid, FAME - fatty acid methyl esters, as used in Chapter 4), feed strategy (day of lipid substrate addition) and medium (blank - conventional medium, * - Ca added - medium supplemented with calcium nitrate at day 0). Color of field indicates the color of beads on certain day, and sizes expressed as: T - tiny - <1 mm; S - small - 2-5mm; M - medium - 5-10 mm; L - large - >10 mm.

Microorganism	**Substrate**	**Day of addition**	**day**											
			1	2	3	4	5	6	7	8	9	10	11	12
M. antarcticus	SBO	4							T	T	S	S	M	
M. antarcticus	SBO	4*							S	M	L	L	M	
M. bullatus	SBO	4							S	M	M	L		
M. bullatus	SBO	4*						M	L	L	L	S		
M. bullatus	FAME	0			S	M	L	L	M					
M. bullatus	FAME	0*			M	L	L	L	S					

Initial experimental results show that the addition of inert, highly hydrophobic particles of silane-coated cellulose, promoted bead formation as these particles acted as seeds on which the MELs was able to adsorb. More research should be performed in this line of work, in order to alleviate complex MELs separation strategies and simplify MELs removal from reactors.

10.4 Supporting Information for Chapter 6: Immobilized cells for MELs production

High yields of MELs can be achieved with oil feeds during fermentation. However, multiple smaller feeds of vegetable oil are usually performed, as high concentrations of lipids in the fermentation broth have potential negative interactions with the fermentation, affecting cells and broth properties, as well as reducing lipase activity through interactions with enzymes. In order to prevent this, an estimation of MELs production was made with immobilized *M. antarcticus* yeast in hydrophilic hydrogel beads.

10.4.1 Materials and methods

10.4.1.1 Media and cultivation conditions

Erlenmeyer flasks were used for preparation of inoculum and main fermentation. They had 1/5 working volume and contained medium composed of 3 $g.L^{-1}$ $NaNO_3$, 0.3 $g.L^{-1}$ $MgSO_4$, 0.3 $g.L^{-1}$ KH_2PO_4, 1 $g.L^{-1}$ yeast extract, 40 $g.L^{-1}$ D-glucose, and were incubated at 27 °C, 250 rpm. Inoculum flasks were incubated for 48h, after which they were used as a 10% (v/v) inoculum for main cultivation flasks, corresponding to approx. 1 $g.L^{-1}$ of CDW. These flasks were incubated for 3 days. After this period, the contents of the flasks were transferred to Falcon tubes (50 mL) and centrifuged at 10000 rpm for 8 minutes to separate the biomass. The biomass pellet was then transferred for fermentation with free cells to new flasks, while for others the yeast biomass was encapsulated in Ca-alginate and used for fermentation with immobilized cells.

In the main fermentation, 40 $g.L^{-1}$ D-glucose was used for some flasks, while 20 $g.L^{-1}$ of SBO was used for others as carbon sources.

10.4.1.2 Cell encapsulation

The encapsulation protocol reported by Fraser and Bickerstaff was used. (Fraser and Bickerstaff 2008) Sterile sodium alginate solutions (2% w/w) (*BDH* Chemicals Ltd, Poole, UK) were prepared, and the yeast biomass pellet was dissolved in 50mL of solution. Uniform spheres were created with a diameter of Ø=2.5-3.0 mm using a syringe needle (1.2 x 40 mm) and a peristaltic pump (Watson Marlow, UK), by dripping into a 0.15M $CaCl_2$ solution (Figure 10.5). The beads were mixed in the calcium solution for 20 minutes, to enable sufficient level of polymer crosslinking.

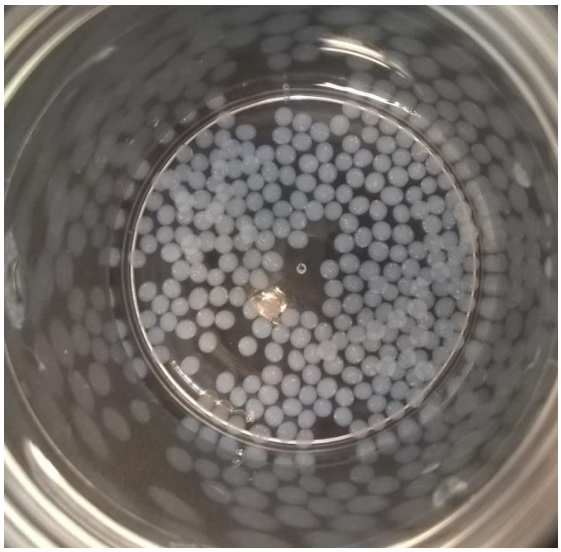

Figure 10.5 Encapsulated yeast beads

10.4.2 Results and discussion

Fermentations were performed using the yeast collected after three days of fermentation using glucose as the main carbon source. The yeast biomass was separated from the supernatant by centrifugation, and the pellet was transferred to a flask with fresh medium, either as free cells or encapsulated in alginate (Figure 10.6). Samples were taken periodically to establish concentration of free cells in the broth, as well as concentration of MELs.

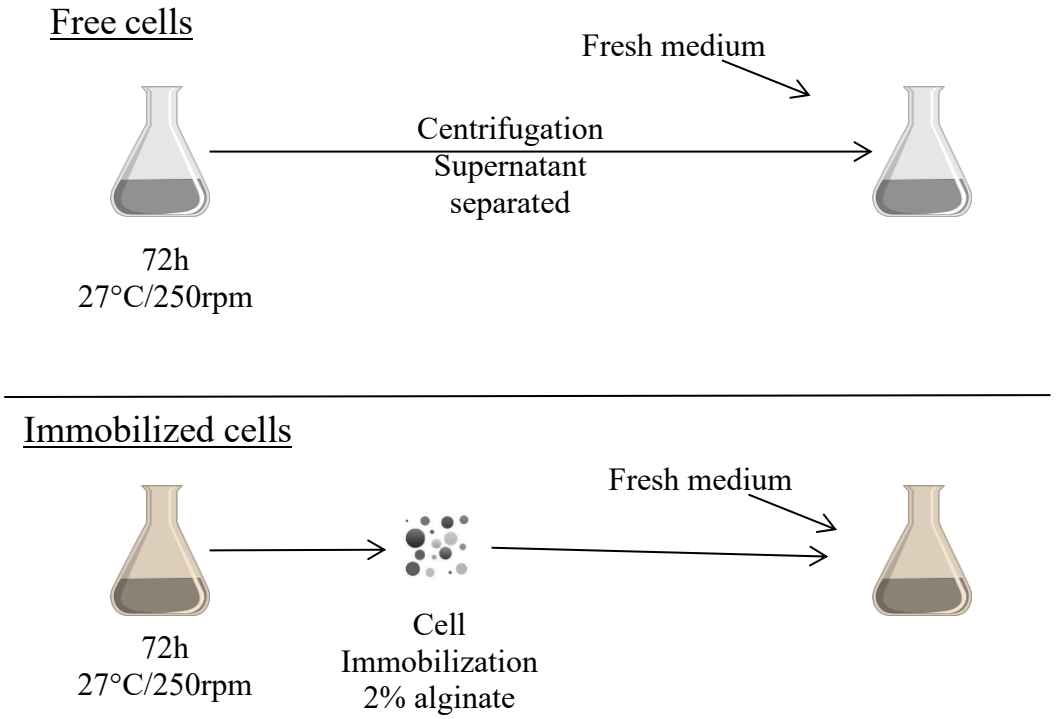

Figure 10.6 Experimental protocol for fermentations with free cells and immobilized cells

Results for biomass profiles (Figure 10.7) shows that in the fermentations with free cells biomass profile remained relatively stable throughout the fermentation, with a slight drop near the end of the fermentation. For the flasks with immobilized cells, there seems to be an initial leakage of biomass, which continued to proliferate in the broth.

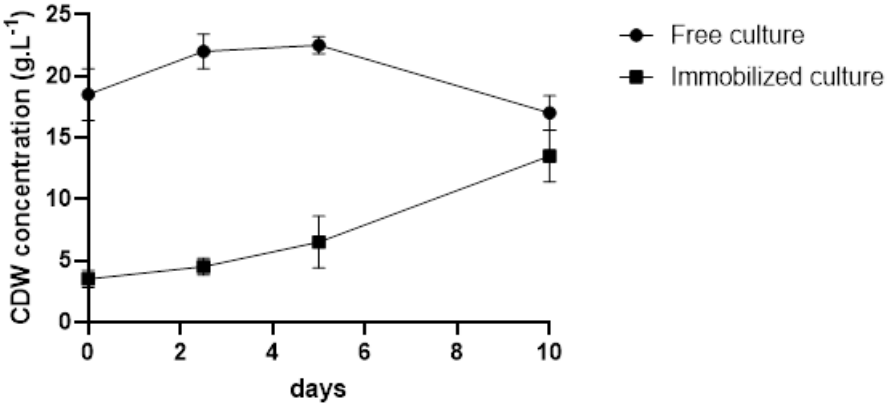

Figure 10.7 Profile of free cells in broth (represented as CDW) for fermentations with free cells and immobilized cells

The profiles for MELs (Figure 10.8) reveal that cultures with free cells outperformed immobilized yeast cultures for both glucose and SBO. Even despite significant levels of free cells in cultures with immobilized biomass, concentration of MELs was drastically lower than those containing free cells exclusively.

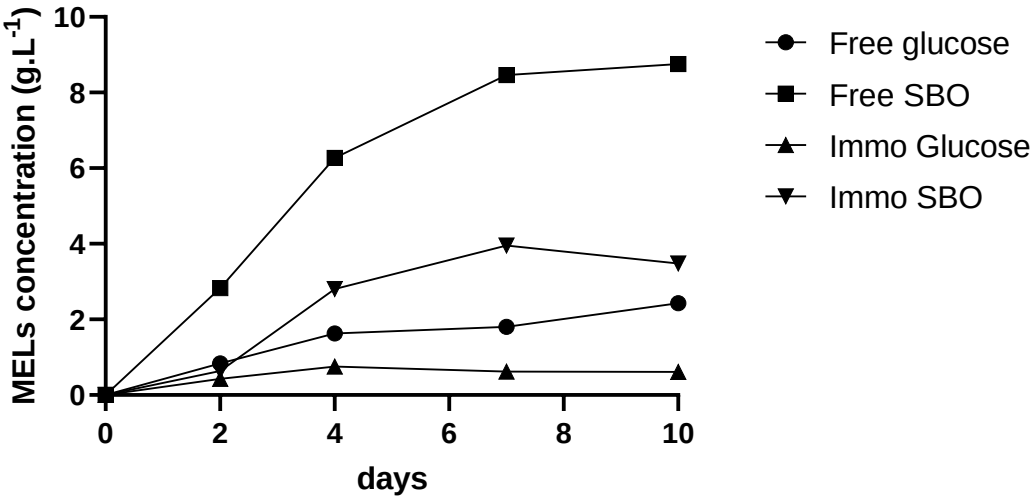

Figure 10.8 Profile of MELs for fermentations with free cells and immobilized cells.

As expected, production of MELs was better with the hydrophobic substrate compared to glucose. However, MELs titres were lower than in normal fermentations which are obtained after the same period of time (~12-15 $g.L^{-1}$). Interestingly, despite prominent levels of free cells growing in the medium besides the immobilized culture, levels of MELs remained low.

The possible reason for the inability to produce MELs with immobilized yest cells, is the natural hydrophilicity of alginate (Maiti and Kumari 2016), which possibly prevents contact between the yeast and the lipid substrate. However, as the production of MELs is also lower for glucose, indicating that the issue lies in the lower activity of immobilized cells.

10.5 Supporting Information for Chapter 6: Cell morphology in glucose and glycerol inoculum

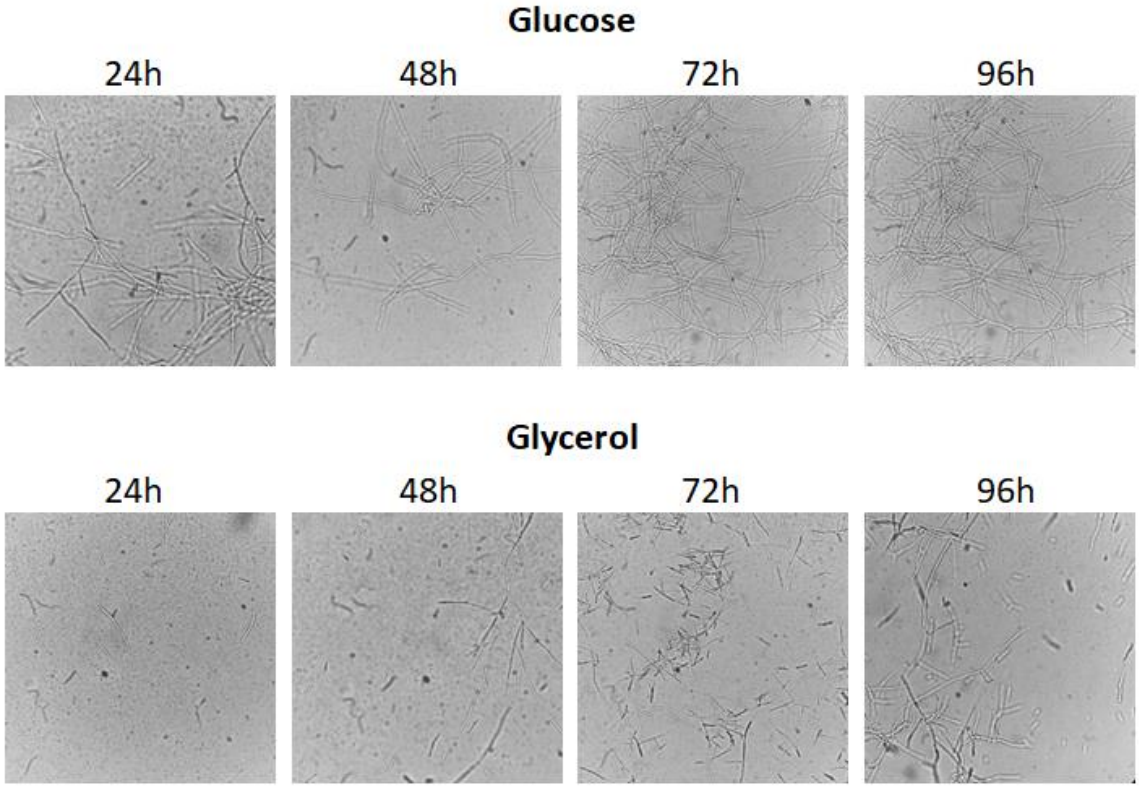

Figure 10.9 Microscopic images of fermentation broth during first four days of inoculum development. (500x magnification)

10.6 Supporting Information for Chapter 8: Proposed column separation of MELs from Fatty Acids and Oil

The existing column separation setup includes the use of several solvents in various ratios. This prevents recovery of solvent by simple evaporation. The process included the use of 1l of different solvents for separation of 1g of MEL.

TLCs were performed to estimate solvent performance on oil and fatty acid separation, and in order to simulate behaviour in the column. Solvents that were tested are presented in Figure 8.2, and their sustainability score is presented in Table 8.1

Isopropanol, Hexane, Water, and Acetone failed to separate the components efficiently. Chloroform, MTBE, Methanol, Ethyl-Acetate, DCM, and Ethanol separated the components with various efficiency.

For some of them, more detailed analysis was performed. Samples applied to TLCs were

- full MELs samples (with residue oil and FA)
- Soybean Oil
- FA- Hydrolysed oil (FA/Oil mix)

10.6.1 TLC test with DCM

Dichloromethane (DCM) was tested as an alternative to chloroform. Dichloromethane (DCM) very successfully separated oil from the rest of the mixture (Figure 10.10). Fatty acids moved slowly, while MELs remained unaffected. This means DCM can be used for a quick oil separation in a column. However due to the presence of chlorine in the molecule, the eluent was not used in the main body of the thesis for sustainability concerns.

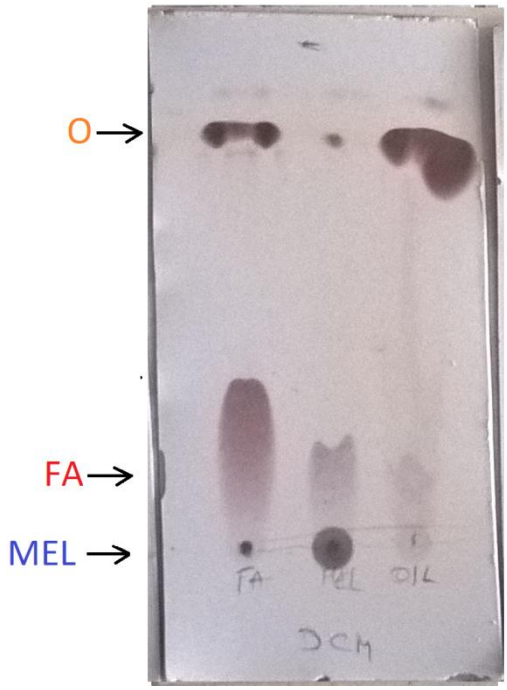

Figure 10.10 TLC performed with DCM as eluent. Left - Partially hydrolysed SBO rich in FFA; Middle - MELs; Right – SBO. Components (vertically separated): MEL – MELs, FA – free fatty acids, O – triacylglycerols (oil)

10.6.2 TLC test with Ethyl-Acetate

Ethyl-Acetate also successfully separated oil from the mix, as well as FFA, however MELs were less affected and the individual MELs homologues moved in separate groups. Namely, MEL A was moving faster along the solvent flow, and separated from the other groups.

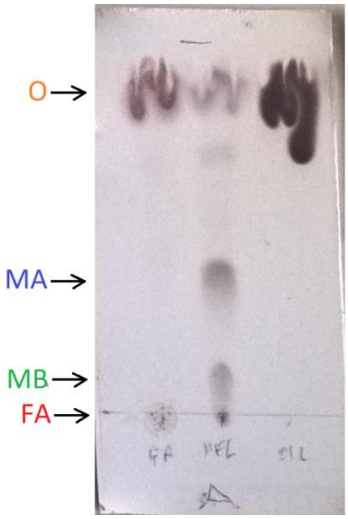

Figure 10.11 TLC performed with ethyl acetate as eluent. Left - Partially hydrolysed SBO rich in FFA; Middle - MELs; Right – SBO. Components: FA – free fatty acids, MB – MEL-B; MA – MEL-A; O – triacylglycerols (oil)

10.6.3 “Microcolumn” separation with DCM and Ethyl-acetate

Before testing the new column separation method in full scale, a small test was performed in order to save reagents and time. A glass Pasteur pipette was used as a "microcolumn" for a flash separation, in order to get qualitative results of solvent performance (Figure 10.12). The MELs used contained both free fatty acids and triacylglycerols as impurities.

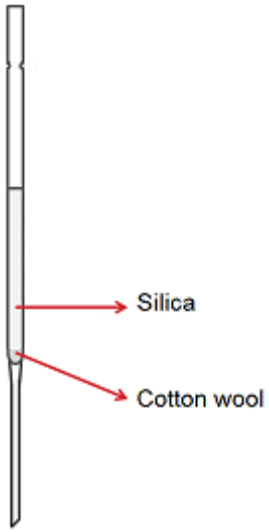

Figure 10.12 Pasteur pipette “microcolumn”: Silica gel mass – 0.5g; Sample mass 0.03 g

The experiment was performed in two steps: fist, DCM was added to separate the oil fraction, and after EA was added to separate the fatty acids from the MELs.

The results of the column separation are presented in Figure 10.13.

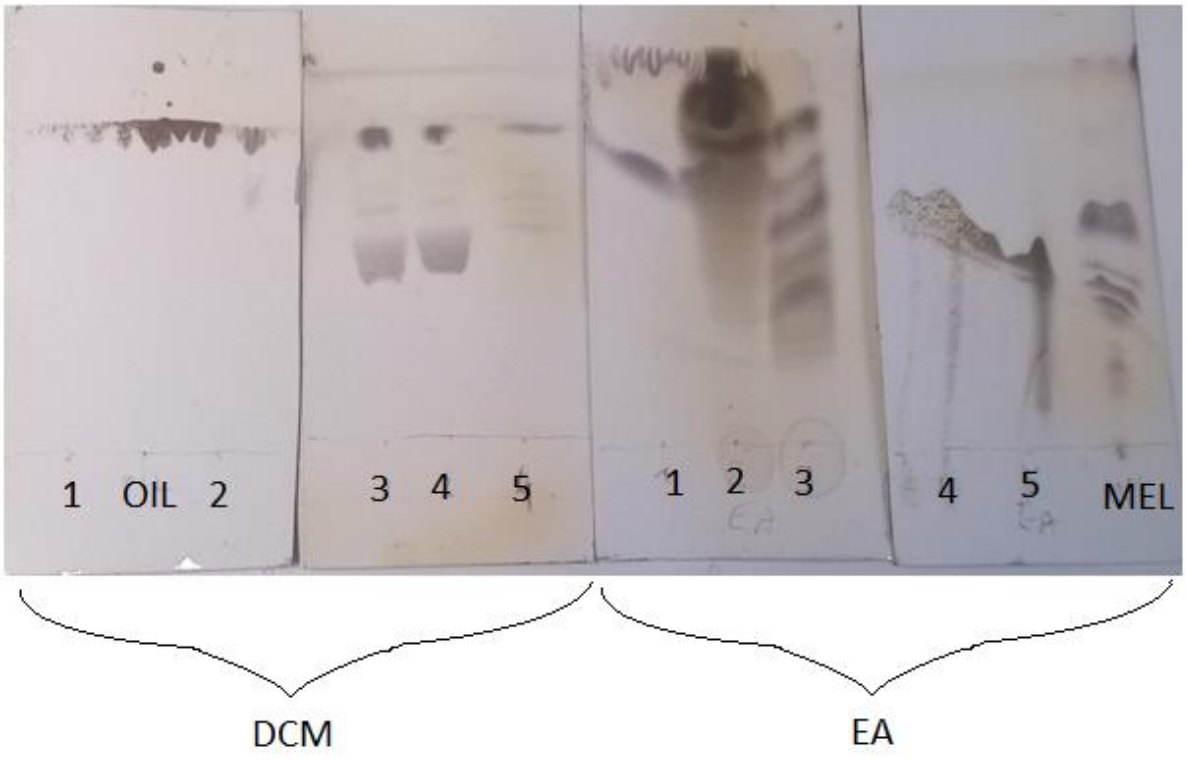

Figure 10.13 Two-step DCM/EA microcolumn separation - TLC analysis of collected fractions of eluted compounds

As can be seen from the TLCs depicting the collected fractions, DCM successfully separated the oil from the mix, with some leakage of MEL-A visible on TLC lanes 3 and 4. However, free fatty acids were not removed from MELs very efficiently using ethyl-acetate. Namely, despite the fact that ethyl-acetate would move fatty acids at a faster rate along the column compared to MELs, the MELs already moved by the DCM would come out simultaneously with the fatty acids, preventing good separation. Due to this, an alternative strategy was sought for.

10.6.4 Multi-step “microcolumn” separation with Methanol and Ethyl-acetate

After it was observed that methanol successfully elutes MELs and fatty acids, leaving triacylglycerols unmoved, a two-step process was proposed (with an optional intermediate washing step):

1. Methanol would be used to remove fatty acids and MELs from the column, with oil remaining
2. Acetone could optionally be used as an eluent to remove oil from the column, if the only one column was to be used
3. After MeOH evaporation, samples from the first column would be eluted with Ethyl acetate, to gradually separate fatty acids from the MELs.

This strategy is illustrated in Figure 10.14.

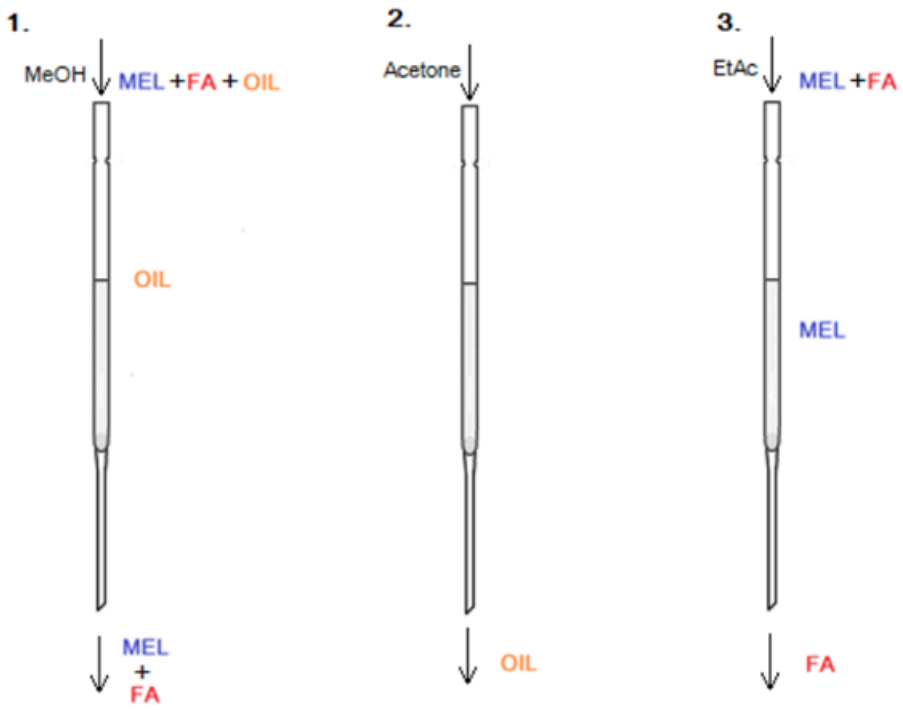

Figure 10.14 Proposed two-step column separation for MELs: 1 - MeOH as eluent, 2 - Acetone as eluent (optional, if column is to be reused), 3- Ethyl-acetate as eluent

10.6.4.1 First step - Methanol as eluent

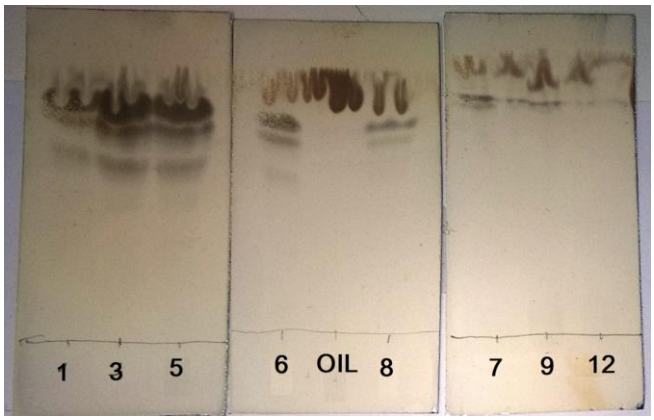

Figure 10.15 TLCs of samples from microcolumn, with SBO as standard (TLC eluent: Chloroform/MeOH/H_2O)

On the picture above are TLCs developed using the standard TLC eluent blend (Chloroform/MeOH/H_2O) of the samples collected during column separation with Methanol as the eluent in the column. However, the standard TLC eluent setup prevents a clear distinction to be made between SBO and fatty acids. In order to prove the absence of oil from the samples, a TLC was performed with samples 1 and 5, along with Oil, using pure chloroform as eluent. This strategy for an alternative TLC eluent was based on findings presented in Figure 8.2. The results are presented below, in Figure 10.16. These results prove that oil was absent from the collected samples and remained in the column.

Figure 10.16 TLCs of samples from microcolumn and Oil as standard (TLC eluent: Chloroform)

10.6.4.2 Second step - Acetone as eluent (optional)

Acetone was used to remove residual components, mainly oil, from the column, so it could be reused. TLCs were performed until there were no traces of any component, however just a couple of ml of Acetone were needed to clean the column completely.

10.6.4.3 Third step - Ethyl Acetate as eluent

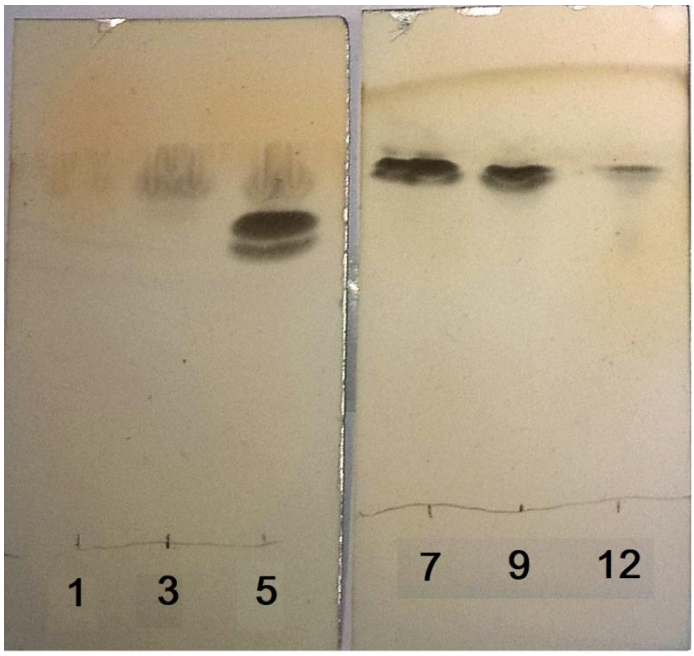

Figure 10.17 TLCs of samples from microcolumn (TLC eluent: Chlor/MeOH/H2O)

On the picture above are TLCs developed using the standard TLC eluent blend (Chlor/MeOH/H2O) of the samples collected during column separation of the sample recovered after the first column, with Ethyl acetate as the eluent.

Finally, after the complete column separation, the remaining solvents were removed from the samples and a TLC with Chlor/MeOh/H_2O was performed. Samples 5 and 9, recovered from the last step of the separation, were used, along with the full initial MELs sample. The results are presented on the picture below.

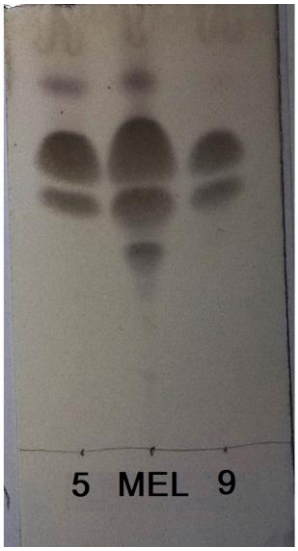

Figure 10.18 TLCs of samples from microcolumn and MELs sample (TLC eluent: Chlor/MeOH/H2O)

It is noticeable that earlier samples collected from the column have traces of Fatty acids, as well as the loss of MEL-D in latter fractions. This might be avoided on larger columns, which could enable easier separation between the components.

10.7 Use of sugar beet processing waste in medium formulation

Sugar beet is the main source of sugar in the European food industry. This culture contains significant amounts of sucrose which is partially extracted during processing. However, in this process, substantial amounts of by-products and waste materials are generated, which pose an environmental danger due to their high residual carbohydrate content.

The ability of several MELs producers to metabolise sucrose was reported by Morita et al. (Morita et al. 2009b). The ability to use sucrose in MELs fermentation would enable the use of many waste materials containing this carbohydrate, mainly agricultural and food residues.
A by-product from sugar beet processing was tested, which was kindly gifted from the Faculty of Technology Novi Sad, Serbia. Sugar beet molasses is a viscous residue obtained from the refinement process, and it was tested to be used in a role of a hydrophilic source of carbon in MELs production.

10.7.1 Materials and methods

M. antarcticus was used for these tests, and cell maintenance, progression and the cultivation protocol are all discussed in other chapters of the thesis.

10.7.1.1 Molasses treatment

Molasses was provided by the Faculty of Technology, Novi Sad, Serbia, and originated from the Crvenka Sugar Factory, Serbia. Initial content of moisture was 10% and 51% of fermentable sugars (in dry weight). It was diluted and sterilised in an autoclave prior to fermentation.

For protein removal, the molasses solution diluted 10:1 with Milli-Q water was centrifuged for 10 minutes at 6000 rpm. The liquid phase was used for precipitation with Na_2HPO_4 and $Ca(OH)_2$ (0.15 M). After a mild centrifugation (3000 rpm for 2 minutes), the liquid phase was removed. For samples treated with sodium phosphate, a part of the solution was treated with EDTA (99.5%, Sigma-Aldrich), after which the precipitate was removed in a similar manner. Laccases were used to degrade polyphenol compounds, following a protocol from literature (Asadgol et al. 2014).

10.7.2 Molasses as substrate

Analysis revealed that the molasses contained roughly 50% (w/w d.w.) of small carbohydrates, mostly sucrose. It was diluted and used as a basis for the medium. For comparison, sucrose and glucose were used as a carbon source, combined with vegetable oil (SBO) using the established feed strategy of 40 $g.L^{-1}$ of sugars on day 0 and 20 $g.L^{-1}$ of oil added on day 4. Samples were periodically collected and used for determination of fermentable sugars, extracellular protein, MELs and lipids. These graphs are presented in Figure 10.19.

The culture in glucose and sucrose flasks formed beads on day 9 and 8, respectively, which dissipated before the fermentation ended. The culture fed with sucrose performed better than glucose in terms of the production of MELs, indicating a possible stimulative effect on the cells of either the disaccharide or fructose released during the hydrolysis of the sugar. The molasses was inhibitory to the culture, reducing drastically the final product concentration in the broth. Results for profiles of extracellular protein indicate that the molasses contained a significant amount of protein, and this shifting the C/N ratio in the medium could be blamed for poorer performance in this case. This is further confirmed by the lack of effect on biomass

development (initial concentration of biomass for the culture of molasses is high due to precipitate from the substrate. The concentration of free extracellular protein drops in the first part of the fermentation, as the culture manages to degrade and metabolise some of them and starts to rise in the second half of the process due to secretion of enzymes.

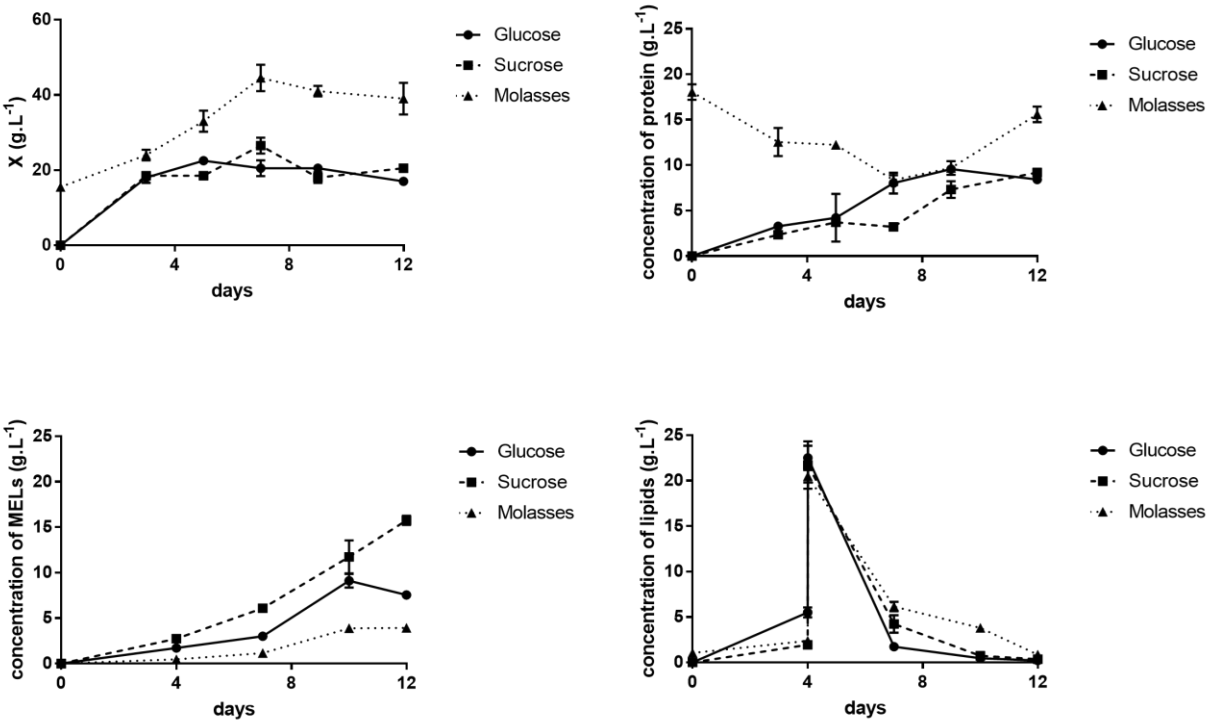

Figure 10.19 Results for concentration profiles for biomass, extracellular protein, MELs and lipids for glucose, sucrose, and sugar beet molasses

To further explore the negative effect of excessive nitrogen in the medium on the culture, several methods for protein removal were used on a solution of molasses (Figure 10.20).

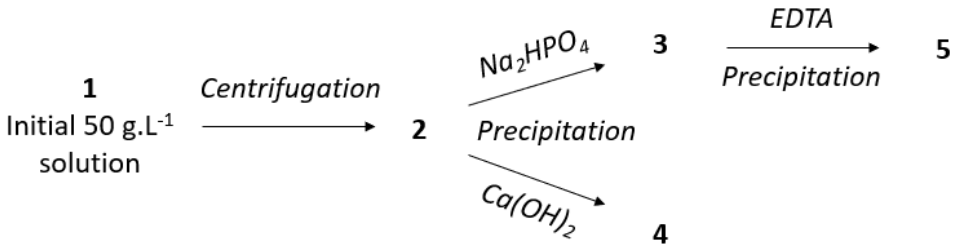

Figure 10.20 Multi-step process tested for protein removal from molasses. Numbers indicate samples collected and tested.

Several techniques for removing excessive protein from the molasses included centrifugation (where a solid precipitate was obtained and discarded), as well as precipitation with three compounds. Analysis of extracellular protein concentration was performed in the solutions after each step. Fermentations were started with these solutions of molasses, which were diluted to a concentration of fermentable sugar of 40 $g.L^{-1}$ and used for medium formulation. Samples were periodically collected to be analysed on GC. These results are presented in Figure 10.21.

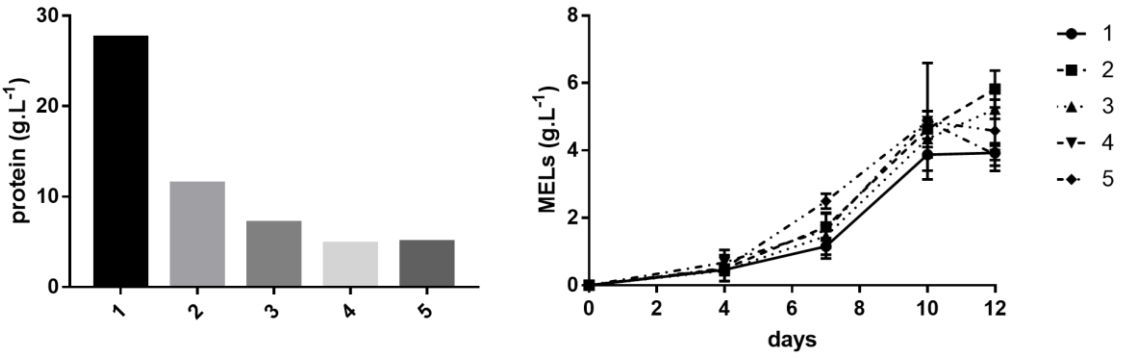

Figure 10.21 Left - Concentration in molasses samples treated by several protein removal techniques; Right – Profiles of MELs concentration when those samples were used in medium formulation

No statistically relevant impact was made on increasing MELs productivity, although significant amounts of protein were removed. Thus, the high presence of polyphenols was the next to blame for the negative impact of molasses on the cell's metabolism. Laccases were used to hydrolase these compounds, and polyphenol concentration in the samples was measured by titration. The samples tested were solutions obtained following the protocol from Figure 10.20.

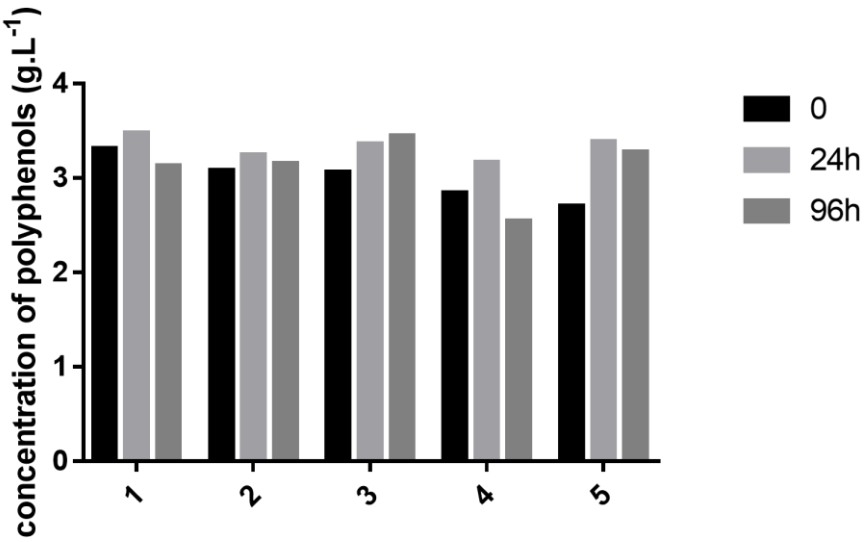

Figure 10.22 Concentration of polyphenols in samples after 24h and 96h of laccase treatment

In conclusion, the laccase treatment was unsuccessful in hydrolysing the polyphenols present in the molasses solution, probably due to interference with some of the compounds in this complex substrate. Tests with molasses were halted at this point, and alternative methods for purification of the substrate (ultrafiltration) were left for future work. Still, sucrose was found to be beneficial for MELs production, and other waste materials containing this sugar should be considered for designing a more sustainable process.