Mannosylerythritol lipids: Searching for production and downstream routes

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Resumo

O trabalho apresentado visa a produção, caracterização, recuperação e aplicação de um biosurfactante produzido por *Moesziomyces antarcticus,* manosileritritolípidos (MEL).

Na fase de produção, foi estudado o efeito na produção de MEL, ácidos gordos e biomassa celular do uso de diferentes fontes de carbono, incluindo açúcares (D-glucose e D-xilose) e um óleo biológico (óleo de soja), bem como da adição da fonte de azoto a diferentes níveis. A concentração mais elevada de MEL foi alcançada após 18 dias de bioconversão, 19.4 g/l, quando 40 g/l de glucose foi usada inicialmente como substrato, e 20 g/l de óleo de soja foram adicionados no dia 4 de cultura.

A caracterização do produto, MEL depois de isolado, ou sobrenadante livre de células, envolveu a determinação da tensão superficial, concentração micelar crítica, balanço hidrofílico-lipofílico, ângulo de contacto e conteúdo proteico.

Já numa perspectiva de recuperação de produto de forma sustentável, e de forma a aumentar a percentagem de MEL isolado, diminuíndo a quantidade de solvente usado, foram realizados testes com diferentes solventes e estratégias. Os melhores resultados foram alcançados combinando diferentes passos, incluindo ruptura das células por sonicação, seguida por extracção com solvente (84.86 ± 9.21%) ou liofilização (81.41 ± 1.72%).

Por último, avaliou-se a eficiência do MEL na formulação de um detergente e a adição de surfactante a um detergente comercial aumentou a percentagem de remoção de óleo de soja e de chocolate, previamente aplicados num tecido de algodão de 51.78 para 68.18% e de 86.45 para 91.73%, respectivamente.

Palavras-chave: Manosileritritolípidos, caracterização de produto, recuperação de produto, aplicação

Abstract

The present work aims to study the production, characterization, downstream and applications of a biosurfactant produced by *Moesziomyces antarcticus,* mannosylerythritol lipids (MEL).

In the fermentation phase, different carbon sources were explored, including sugars (D-glucose and D-xylose) and a biological oil (soybean oil); also the influence of the nitrogen source addition was analysed. The effect of the different conditions studied in the fermentation was assessed through the final concentrations of MEL, fatty acids and cell-dry weight obtained. The highest MEL titre was achieved after 18 days of bioconversion, 19.4 g/l when 40 g/l of D-glucose was used as initial substrate and 20 g/l of soybean oil added at day 4 of culture.

Characterization of the product, MEL and cell-free supernatant was performed, in terms of surface tension, critical micelle concentration, hydrophilic-lipophilic balance, contact angle and protein content.

Following a perspective of an efficient sustainable product recovery from culture broth, with an increased percentage of isolated MEL, a strategy of decreasing the amount of solvent used was followed. The best results were achieved using a combination of different steps, including firstly a cell disruption by sonication followed by solvent extraction (84.26 ± 9.21) or lyophilisation (81.41 ± 1.72%)

Finally, the efficiency of addition of MEL in the formulation of a detergent was evaluated. The addition of MEL to a commercial detergent increased the removal percentage of soybean oil and chocolate, previously applied into cotton clothes from 51.78 to 68.18% and 86.45 to 91.73%, respectively.

Keywords: Mannosylerythritol lipids, product characterization, downstream, application

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Abbreviations

ANOVA – Analysis of variance
BSA - Bovine Serum Albumine
CFU – Colony Forming Unit
CMC – Critical micelle concentration
DCW – Dry cell weight
FA – fatty acids
GC – Gas Chromatography
Glu - Glucose
HLB – Hydrophilic-lipophilic balance
HPLC – High performance liquid chromatography
M. antarcticus **–** *Moesziomyces antarcticus*
MEL – Mannosylerythritol lipids
NMR – Nuclear Magnetic Resonance
PYCC – Portuguese Yeast Culture Collection
rpm – rotations per minute
SO – soybean oil
SS – sodium sulfate
STPP - sodium tripolyphosphate
TLC – Thin layer chromatography
Xyl - Xylose

1. Introduction

1.1. Motivation

When we propose ourselves to a new challenge, generally we bring with us the will to know and learn more and that implies a research of all the things that are underlying the concerned challenge.

Surfactants are compounds with a huge industrial interest, especially due to the wide range of applications in which they can be used, and high efficiency in lowering surface tension and very low CMC [1]. However, the use of surfactants have some disadvantages: their presence in the environment and slow biodegradation may have negative impact in the ecosystem, some of the surfactants and respective degradation products are actually toxic for natural life, which triggered the interest to search for other options more environmental friendly [2, 3]. To answer this challenge, biosurfactants can have an important role [1].

The production of biosurfactants can be achieved by chemical transformations of compounds of biological nature or tension active molecules produced by nature, namely by microbial cells, the latter are designated as microbial biosurfactants. Those are chemically very different from conventional surfactants and therefore offer a range of properties, such as ability to act in wider ranges of pH, temperature and ionic strengths, to form micelles and other self-assembling structures of different shapes and dimensions, and so on.

The most widely-used carbon sources for the production of biosurfactants are oils, in particular soybean oil, sunflower oil and olive oil. However, the use of these substrates has some drawbacks due to the fact that all of them are part of food and feed supply chain, the price of vegetable oils and the difficulties associated with biosurfactants recovery from oily broths [4]. Thus, and thanks to the continuous studies carried out, it may be possible to replace these carbon sources by more sustainable ones, such as the more cost effective renewable agro industrial residues. This opportunity to use lignocellulosic materials, to produce products with high added value, such as microbial biosurfactants is now starting to raise more and more interest in the researchers [4, 5].

The possibility to obtain a microbial biosurfactant with a lower environment impact on its production, due to the use of lignocellulose materials, with lower negative impacts in the ecosystem upon its use and discharge and a wider range of tension-active properties, as raises interest to know more about these biosurfactants, methods for their production, downstream and performance when applied. This thesis is focused in a particular biosurfactant, mannosylerythritol lipids (MEL), and search for strategies able to increase their production and recovery efficient.

Actually, there are already several different reports about surfactants, biosurfactants and in particular about MEL. MEL is a glycolipid capable of reducing the surface tension due its amphiphilic molecular atructure, with a hydrophilic moiety comprised by mannosylerythritol and a hydrophobic moiety comprised of two lipidic chains .Different structures of MEL may arise, according to the acetylation degree of the mannose ring, being MEL-A diacetylated in C4 and C6, MEL-B and C monoacetylated either in C6 or C4, respectively and MEL-D completely deacetylated in these two positions [6].

MEL, as other biosurfactants, can be used for various purposes, with commercial or potential applications in the cosmetic, medical industries (as antimicrobial agent) and environmental industries [7].

To use MEL for the different purposes mentioned above, different levels of purity are required, which can be achieved at the cost of downstream processes. These processes designed to recover and purification of the product are expensive, representing around 60% of the total production costs. In this way, it is important to determine in advance the degree of purification required and market value product [8].

All the studies on MEL confirm that this molecule is one of the most promising biosurfactant, not just because of the high yield at which can be obtained and excellent surface tension, but also because its wide range of potential applications, supported on the diversity of biochemical functions and its biocompatibility. Nevertheless, the whole costs behind its production and purification are still a barrier that needs to be overcome so this potential product can enter in more competitive markets.

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1.2. Aims of study

The global aim of this project was to explore the potential of a biosurfactant, MEL, produced from a low concentration of D-glucose, described in the literature as one of the most successfully used water-soluble carbon source for bioproduction. It is also reported that soybean oil is the best substrate for MEL production however, it is use is hardly sustainable.

The main objectives of the thesis are (i) as an attempt to increase MEL concentration, study the use of the carbon source, (ii) characterize the product of interest obtained from basic fermentation conditions, (iii) improve the recovery and downstream processes of MEL and (iv) analyse the efficiency of this biosurfactant in a real application.

The concept developed in this thesis is based on four big chapters:

1. Fermentation conditions: study the influence of nitrogen and carbon sources to produce MEL. Different concentrations of D-glucose and D-xylose as well as a small boost of soybean oil as feeding were tested. Also the addition of $NaNO_3$ in different days was studied in order to understand its importance for the fermentation process.
2. Product characterization: using the basic fermentation condition, MEL was confirmed by thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) spectroscopy. The biosurfactant and the cell-free broth were characterized by some of the main properties that are used to classify these compounds and the surface tension, the critical micelle concentration, the hydrophilic-lipophilic balance, the contact angle and the protein content were measured.
3. Downstream processing: the use of water-soluble carbon sources make this step easier because when vegetable oils are used the removal of the residual oil after cultivation and the purification of MEL from oil are expensive. Liquid-liquid extraction with solvents is the most reported method to recover this glycolipid however, due to the expensive costs associated with their use and the negative impact that they have

to the environment, different alternative strategies were proposed in this thesis in order to try to make this stage of the bioprocess more sustainable.

4. Applications: as a biosurfactant, MEL has a whole panoply of applications. The use of this biosurfactant in a formulation of a detergent was tested and its efficiency was evaluated in accordance with the weight removal percentage of soybean oil and chocolate that were applied in cotton clothes.

Based on these stages, the experimental strategy was designed to uncover the possible answers to research questions.

1.3. Research question

1) How the addition of carbon source influences the productivity and yield of MEL?
2) How the addition of nitrogen source influences the production of MEL?
3) Is MEL cell excretion efficient to deliver MEL into fermentation broth bulk?
4) Is the fermentation broth, after removal of yeast cell, enriched in MEL at amounts high enough to be directly used as a biosurfactant formulation in applications?
5) How much can the MEL produced in this work decrease the surface tension of an aqueous solution?
6) What is the envisage routes for MEL downstream and purification from the fermentation broth?
7) Can this biosurfactant be used as a detergent for cloths cleaning?

1.4. Research strategy

To achieve the proposed goals and answer the research question it was necessary to define a strategy. Outline this strategy is the start to develop the necessary work. First of all some work was performed in order to optimise the fermentation´s conditions:

1) Microorganism: To develop the present work *Moesziomyces antarcticus* PYCC 5048^T was the yeast used to produce mannosylerythritol lipids as it is described as one of the main MEL producers;
2) Carbon source: It was used D-glucose as preferential carbon source, although D-xylose and soybean oil were also used for some assays; it was used feeds of the carbon source to increase the final concentration of the product;
3) Nitrogen source: Sodium nitrate ($NaNO_3$) was the nitrogen source used; its influence was also object of study, by changing the fermentation time points at which it was supplemented to the culture.

Thereafter, some assays were performed to assess were MEL is mostly located during at the end of the fermentation. Namely if it is an extracellular product efficiently excreted to the bulk of liquid fraction of the solution or if it is an intracellular or cell associated product.

With both of these types of studies, the best conditions were known and it was possible to go through the next step of this thesis that includes the search for MEL downstream following different techniques and strategies. Namely,

4) Temperature conditions were studied: the influence of overheating the cells was studied in order to verify if temperature can be used to enhance MEL mobilization to the liquid bulk without breaking the cells and thus increasing number of impurities/compounds from which MEL need to be isolated;
5) Use of solvents: different solvents were tested in order to verify if they can extract the product of interest without breaking the cells, again following a strategy to avoid increasing number of impurities/compounds from which MEL need to be isolated;
6) Different disruption methods were also assessed in an opposite strategy that aims MEL increasing recovery by breaking first the cells, then presumable to extract a MEL crude with higher content, but more impure;
7) Use of resins: different kind of nonpolar resins were tested in order to verify if they are able to adsorb MEL, a non-ionic surfactant.

To conclude, and knowing all the potential of biosurfactants in many different applications, the ability of MEL as a detergent to clean cloths was also tested. With this laboratory strategy, the removal percentage of different compounds, used to soiled up cotton cloths, was investigated with the final purpose to compare the increase in washing efficiency brought by MEL.

2. Literature review

2.1. What are Surfactants and Biosurfactants?

Our daily routine includes the utilization of lots of products which contain surfactants in their composition, like cosmetic and pharmaceutical products.

Surfactants are those compounds able to reduce surface and interface tensions and also have the ability to form micelles and/or vesicles at very low concentration. They are capable of form tightly packed structures which are called monolayers if they occur at the air-water and the oil-water interface or monolayers and aggregates if they occur at the solid-water interface [1].

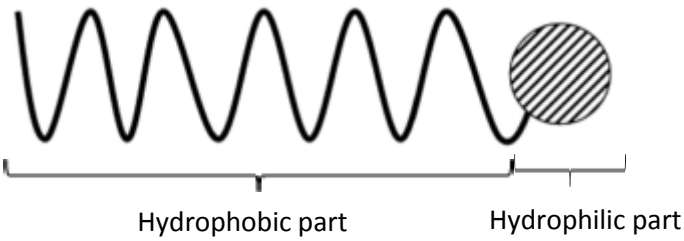

Figure 1 - Schematic illustration of a surfactant

As it can be seen in the figure above, surfactants have at least one polar head group, the hydrophilic part, that will be oriented outward toward the aqueous phase (taking an aqueous solution as example) and at least one hydrophobic tail that prefers to be in an apolar environment [9].

The market for such products is already huge and it tends to increase in the future. However, surfactants can be non-biodegradable and impact negatively the environment by their extended action and by their degradation products or some chemical petroleum accumulated by them, maybe toxic for the environment [10, 11]. Therefore, there is a call for an alternative way to replace the chemical synthesized compounds with biological products, called biosurfactants, which are produced from biological sources, by chemical reaction of those or as metabolites of microbial or plant activity.[12]

Specifically, microbial biosurfactants, when compared to chemical surfactants, are more environmental friendly, are recognized as fine chemicals with high-value applications and remarkable characteristics such as low-toxicity, high biodegradability, resistance to extreme temperatures and pH, beneficial surfactant properties and antibiotic or bioactive effects.[13]

There are several microorganisms that can produce different biosurfactants with different structures depending on their producing mode, the raw material used and the fermentation conditions.

About 2000 different amphoteric structures of biological origin have been described. Usually, the biosurfactants have a molar mass between 500 and 1500 Da. Generally these microbial surfactants are divided in several categories according to their microbial origin and chemical composition [11]. There are anionic, neutral and cationic biosurfactants; the anionic's are used in most detergent formulations, the neutral's are very effective in removing oily soil and the cationic´s are the ones that contain amine groups [1]. These compounds are amphipathic with both hydrophobic and hydrophilic

moieties; the hydrophobic moiety has long-chain fatty acids and the hydrophilic moiety can be a carbohydrate, cyclic peptide, amino acid, phosphate carboxyl acid or alcohol.

Biosurfactants can be considered “green” alternatives to synthetic surfactants, despite they have to handle with some limitations regarding commercial production [14]. On the other hand, biosurfactants exhibit an unique chemical structure and a huge structural diversity including, for instance, glycolipids, lipopeptides, polymeric surfactants, phospholipids, among others that will be presented in Table 1.

As shown in Table 1 there are a diversity of biosurfactants although there are major groups that even been more exhaustively studied such as the rhamnolipids, surfactins and related lipopeptides, the serrawettins, trehalose lipids, sophorolipids and mannosylerythritol lipids.

Rhamnolipids, primarily a crystalline acid, is composed of β-hydroxy fatty acid connected by the carboxyl end to a rhamnose sugar and they trigger interest owed to their broad range of applications in various industries, namely in bioremediation and enhanced oil recovery, for pharmaceutical and therapeutic ends and in cosmetic and cleaning products [15]. It is reported that, when used in high concentrations, rhamnolipids can be potentially toxic to natural vegetation [16] however, it is also reported that these compounds own a biodegradable nature [15, 17].

Surfactins are a group of structurally similar lipopetides produced by some *Bacillus* species with exceptional emulsifying and foaming characteristics [9]. These compounds exhibit antimicrobial, antiviral and anti-inflammatory properties [18]. The chemical structure of surfactin is composed of seven amino acids that are bonded to the carboxyl and hydroxyl groups on long chain fatty acids (C_{13}-C_{15}). It has a wide range of applications, including the therapeutic and environmental ones; however its production is fought due to the high costs of production and low yields [19].

Serrawettins are non-ionic biosurfactants secreted through extracellular vesicles on solid media and composed of fatty acids and amino acids. These biosurfactants play an important role in anti-tumor and anti-nematode activities [20].

Trehalose lipids or trehalolipids are glycolipids containing trehalose hydrophilic moiety and they have gained increased interest for their potential applications in a number of fields including bioremediation (especially for microbial enhanced oil recovery), biomedical industry, agricultural use, food industry, paper industries and in detergents or cosmetics; it also has a role in environmental applications [9, 21].

Sophorolipids are probably one of the most promising biosurfactants which are produced by non-pathogenic yeast strains; they have a hydrophilic part that consists of the disaccharide sophorose and a hydrophobic part that is made up by a terminal or subterminal hydroxylated fatty acid linked to the sophorose molecule. These biological surfactants are biodegradable and they can be used for pharmaceutical and medical ends, in nanotechnology [22].

Mannosylerythritol lipid is also a very studied and reported biosurfactant with applications in a huge range of industries. As this thesis is focused in MEL, all informations inherent to its production and application will be further explored in the following sections.

Table 1 - Examples of the more commnon biosurfactants and their origin

Head Group	Biosurfactant	Microorganism	Substrates	References
Fatty acids	Fatty acid	*Corynebacterium lepus*	Kerosene, Alkanes	[9, 23, 24]
Neutral lipids	Neutral lipid	*Nocardia erythropolis*	Hexadecane	[9, 24]
Phospholipids	Phospholipid	*Thiobacillus thiooxidans*	n-Alkanes	[9, 23]
Lipopeptides	Viscosin	*Pseudomonas fluorescens, P. libanensis*	Glycerol	[9, 23, 24]
	Serrawettin	*Serratia marcescens*	Glycerol	[9, 24]
	Surfactin	*Bacillus subtilis, Bacillus pumilus A*	Glucose	[9, 23-25]
Glycolipids	Pentasaccharide lipid	*Nocardia corynebacteroides*	n-Alkanes	[24]
	Rhamnolipid	*Pseudomonas aeruginosa, Pseudomonas sp.*	Alkanes, pyruvate, citrates, fructose, glycerol, olive oil and glucose	[9, 23-25]
	Rubiwettins	*Serratia rubidaea*	Glycerol	[24]
	Sophorolipids	*Torulopsis bombicola, C. batistae, C. lypolytica, C. bombicola, T. apícola, T. petrophilum, C. bogoriensis*	Vegetable oils; fatty acids; alkanes	[9, 23-25]
	Trehaloselipids	*Rhodococcus sp., Arthrobacter sp., R. erythropolis, N. erythropolis*	n-Alkanes, nonalkanes	[9, 23]
	Cellobiolipids	*Ustilago zeae, Ustilago maydis*	Coconut oil	[9, 23, 26]
	Mannosylerythritol lipids	Genus *Moesziomyces* and *Ustilago*	Soybean oil, glucose, n-Alkanes, Oliec acid, Olive oil, Sunflower oil, Sugarcane juice	[9, 25, 27]
Polymeric	Emulsan	*Acinetobacter calcoaceticus*	n-Hexadecane	[9, 23, 25]
	Biodispersan	*A. calcoaceticus*	Soap stock	[9, 23]
	Alasan	*A. radioresistens*	n-Alkanes	[9, 23, 25, 28]
Siderophore	Flavolipids	*Flavobacterium*	Glucose	[9, 29]

2.2. How is the industry of surfactants?

Worldwide, the industry of surfactants includes more than 500 suppliers and more than 3500 types of chemically different compounds, along with the huge number of applications, markets and customers make the industry really complex [30]. According to data disclosed in 2015, the most important surfactant-consuming area is Europe (25% of total consumption), followed by North America (United States and Canada [22%]), and China (18%).

The highest growth rates in consumption are expected for China and Asia, followed by western Europe and North America. Actually, the most important consumers of surfactants are distributed through Europe, North America and China representing 25%, 22% and 18% of total consumption, respectively [30].

Globally, between 2015 and 2020, the surfactants market is estimated to grow at a CAGR of 5.3% and 5.5% by volume and value, respectively, which can be translated in a volume of 24,037 ktons and a value of USD$42,120 million in 2020. In terms of them charge, the anionic surfactants lead the global market with a volume of 7,686 ktons in 2014 [31].

The concerns about the environment are increasing the use of biosurfactants instead the use of the synthetic ones [12]. However, and up to now these biological surfactants cannot compete with chemically synthesized compounds available in the market due to their high production costs (chemical surfactants can be three to ten times cheaper than the biological ones) [32]. The commercial realization of the biosurfactants, sometimes restricted by the high production costs, can be equipoise by optimized production conditions provided by utilization of the cheaper renewable substrates and application of efficient multistep downstream processing methods [33].

The global biosurfactants market was 344 Mtons in 2013 and is expected to reach 462 Mtons by 2020, growing at a CAGR of 4.3% from 2014 to 2020; in terms of value is expected to reach USD 2.2 bn in 2018 which represents a growth rate of 3.5% per annum since 2011 (USD 1.7 bn) [34].

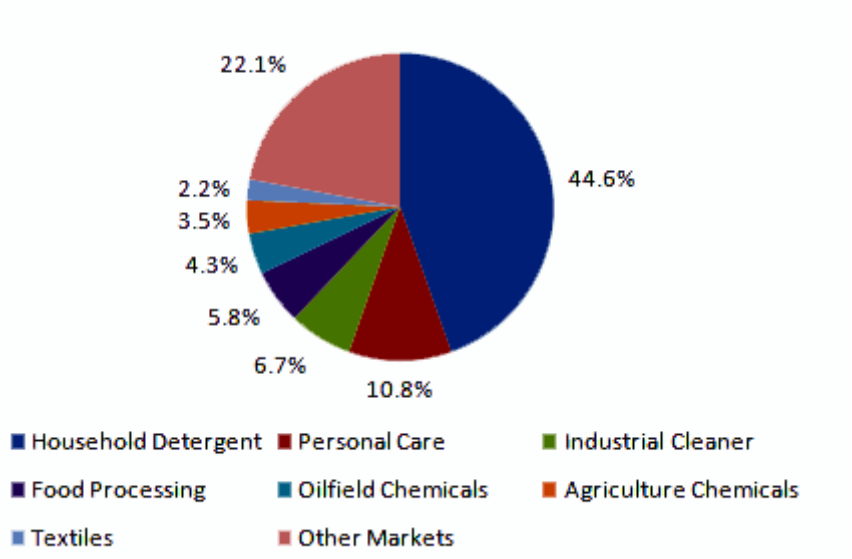

Figure 2 - Biosurfactants market volume share, by application in 2013 [32]

Research efforts have been made to increase the production yield, reduce the costs with the raw material and optimize the growth conditions to increase competiveness of biosurfactants and so contribute to a more sustainable economy [32]. One strategy that can be used to manage and overcome this obstacle is using alternative sources of nutrients with lower costs like reuse some industrial, agro-industrial or the oil-containing wastes; also many food and refinery industries can generate large quantities of waste that can be used [33]. Besides this, the valorisation through microbial transformation of these wastes can also contribute to reduce the pollution caused by the waste disposal in landfills which is also a social and actual problem [35].

In the case of mannosylerythritol lipids which are the aim of this work, lignocellulosic materials, as a renewable carbon source, have been the subject of small number of studies by the Portuguese iBB-IST/LNEG [4, 13], while the majority of the studies uses vegetable oils as carbon source [4].

2.3. Mannosylerythritol Lipids

2.3.1. Which is the composition of MEL and how can it be produced?

Mannosylerythritol lipid (MEL) is a glycolipid biosurfactant, which were first described in 1956 by Boothroyd[7]. This amphiphilic molecule contain a hydrophilic group, 4-O-β-D-mannopyranosyl-meso-erythritol or 1-O-β-D-mannopyranosylerythritol and a hydrophobic unit, the fatty acid and/or acetyl moiety [6, 27].

According to the degree of acetylation and fatty acid length, there are different structures of MEL, due to the number and position of the acetyl group on mannose, the number of acylation in mannose, the fatty acid chain length and their saturation [6]. Therefore, MEL can be designated as MEL-A if it is diacetylated at C4 and C6 of the mannose ring, MEL-B or MEL-C if it is monoacetylated at C6 or C4, respectively and MEL-D if it is completely deacetylated [7]. MELs can be distinguished by thin layer chromatography (TLC) since the elution occurs in accordance with the chemical composition [36].

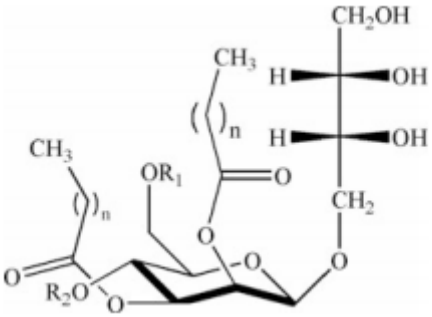

Figure 3 - Chemical structure of glycolipids produced by yeast strains of the genus *Moesziomyces*, mannosylerythritol lipids (MEL). MEL-A (diacetylated): R1=Ac, R2=Ac; MEL-B (monoacetylated at C6): R1=Ac, R2=H; MEL-C (monoacetylated at C4): R1=H, R2=Ac; MEL-D (deacetylated): R1=R2=H; n=6-10 [7]

Mannosylerythritol lipids are a glycolipid class of biosurfactants produced by a variety of yeasts and fungal strains that exhibit excellent interfacial and biochemical properties which was firstly produced by *Moesziomyces antarcticus* T-34 [37]. It produces mainly MEL-A, along with smaller amounts of MEL-B and MEL-C. Subsequently another MEL-producing fungi were identified. Currently, *M. antarcticus, M. aphidis, M. rugulosus* and *M. parantarcticus* are the MEL producers able to deliver higher titres and productivities, producing preferably MEL-A followed by MEL-B and MEL-C [27]. MEL-D is a homolog of MEL without acetyl groups which was prepared by enzymatic synthesis from MEL-B by *Pseudozyma tsukubaensis* or *Ustilago scitaminea*. For example, using *M. antarcticus* and *M. aphidis,* titres of 140 g/l [38] and 165 g/l [39], respectively are reported using high concentrations of soybean oil. It is important to notice that the close phylogenetic relationship between the *Pseudozyma* species with the monotypic genus *Moesziomyces* suggests that the former represent anamorphic and culturable stages of *Moesziomyces* species and can be recategorized as the genus *Moesziomyces* [40].

As written before MEL can be produced by *Moesziomyces sp* yeast strains but also by *Ustilago sp* strains; although the first one promote higher specificity and higher yields of MEL [7].

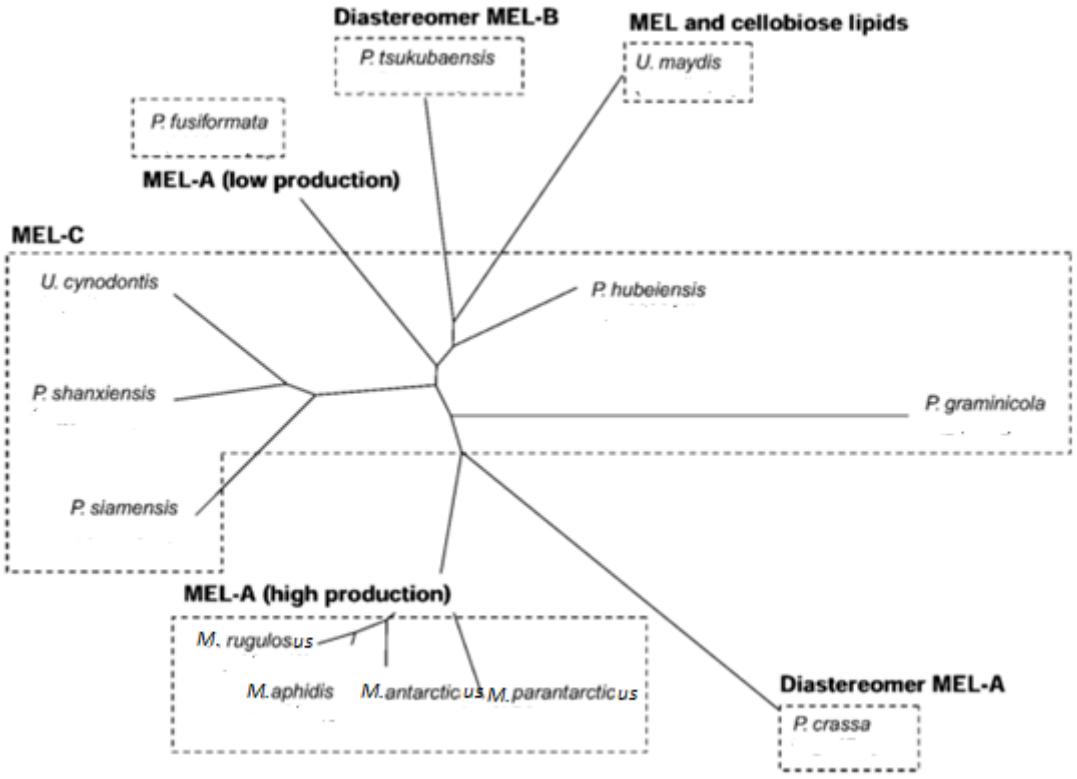

Figure 4 - Molecular phylogenetic tree constructed using ITS1, 5.8 S rRNA gene and ITS2 sequences of the genus *Moesziomyces* and *Ustilago*. Adapted from [41]

2.3.2. Which are the cultivation conditions for MEL production?

The composition of seed culture medium and substrate supply influence the production of MEL by the main culture [36]. Different conditions are justified with the need to adapt the biosurfactants for different applications. In this way there are a huge number of studies [13, 39, 42] that have been developed in order to improve and optimise the process intensification. The carbon source, the nitrogen source and the environmental factors are parameters which influence the production.

The carbon source has significant influence on cell growth and MEL production. Although most of the scientific articles identify the soybean oil as the best substrate for MEL production, when not completely consumed, the presence of this compound in the fermentation broth at the end of the fermentation makes MEL isolation and purification more challenging [38]. To improve the efficiency of MEL production, the use of water-soluble carbon sources instead of vegetable oil would thus be highly desirable with glucose and glycerol as the most successfully ones for bioproduction [9, 38]. The yield of MEL from glucose as carbon source is considerably lower in comparison with soybean oil, but as other advantages, the price of glucose is lower (and can be even lower if glucose-based renewable substrates are considered, e.g. lignocellulosic materials) and the costs involved in the purification processes are also potentially lower [38]. When soybean oil is used, the removal of the residual oils is required. MEL recovery from broth containing vegetable oils requires several steps of liquid-liquid extraction with organic solvents to achieve high purity level and still at very low yields because in the end of the bioconversion process biosurfactant and vegetable oil byproducts, such as free fatty acids coexist. On contrary, the use of water soluble carbon sources should lead to a simplified downstream process with high degree of purity and recover yield and after a simple extraction with ethyl acetate, the purity of MEL produced from glucose is clearly higher than that produced from soybean oil.

Secondly, and also important, is the nitrogen source used in the culture. Nitrogen plays an important role in the biosurfactant production medium as it is fundamental for microbial growth. There are different nitrogen sources that, according to some studies carried out, can be used for biosurfactants production such as urea, peptone, yeast extract, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract and malt extract [35]. In the case of mannosylerythritol lipids, *Rau et al* 2005 reported that the highest yield is obtained when sodium nitrate is used instead of ammonium nitrate or ammonium sulfate which result in a low final pH (NH_4Cl and $(NH_4)_2SO_4$ are acidic nitrogen sources) and a low MEL yield [36].

The production of MEL is also influenced by environmental factors that can affect yield and titres, and so some process conditions should be considered, such as: pH, temperature, aeration or agitation speed are also important.

Concerning the temperature, it is optimal for growth and MEL production within a range between 25 and 30 ºC; the values of pH can differ between 4 and 8; to ensure that the production of MEL occurs with the best aeration conditions, the fermentation broth should not exceed 1/5 of the total volume of the flasks; the best agitation speed is checked at 150 rpm. [13, 36, 43-46].

2.3.3. What are the changes in the production process by using lignocellulose as carbon source?

As previously mentioned, oils are the most used substrates for the production of glycolipids especially because of the high yields that is possible to obtain; also glucose and glycerol are typically studied [27]. However, soybean oil compete directly with the food value chain and it is obtained through land cultivation, so its intensive production might have a remarkable environmental impact [47]. Furthermore, when soybean oil is used as carbon source, recover MEL from oily broths tends to be difficult and it requires downstream processes with more successive steps and more energy or solvent intense [4, 38].

In a search for more carbon source sustainable alternatives, there has been an increase in lignocellulosic biomass processing research [48], considered a substrate of enormous biotechnological value [49] and that be converted in value-add products such as sources for microbial fermentations and chemicals [5].

Lignocellulosic materials are wood, agricultural and forest residues, agro-industrial and municipal solid wastes and comprises about 40-50% cellulose, 25-30% hemicellulose and 15-20% lignin and other extractable components [4, 50, 51].

Cellulose, the major structural component of plant cell walls, is a glucan polysaccharide containing large reservoirs of energy that provide real potential for conversion into biofuels [5, 50].

Hemicellulose are repeated polymers of pentoses, hexoses and a number of sugar acids and they can be highly branched with a lower degree of polymerization than cellulose. Depending on the source, its structural and chemical composition can vary while their properties remain similar [5, 50].

Lignin is the smallest fraction and, generally the most complex polymer comprised of variously linked aromatic alcohols. It fills the gap between and around the cellulose and hemicellulose complexion with the polymers. Lignin is present in all plants biomass and it is considered as a byproduct or as a residue in bioethanol production process [5, 50].

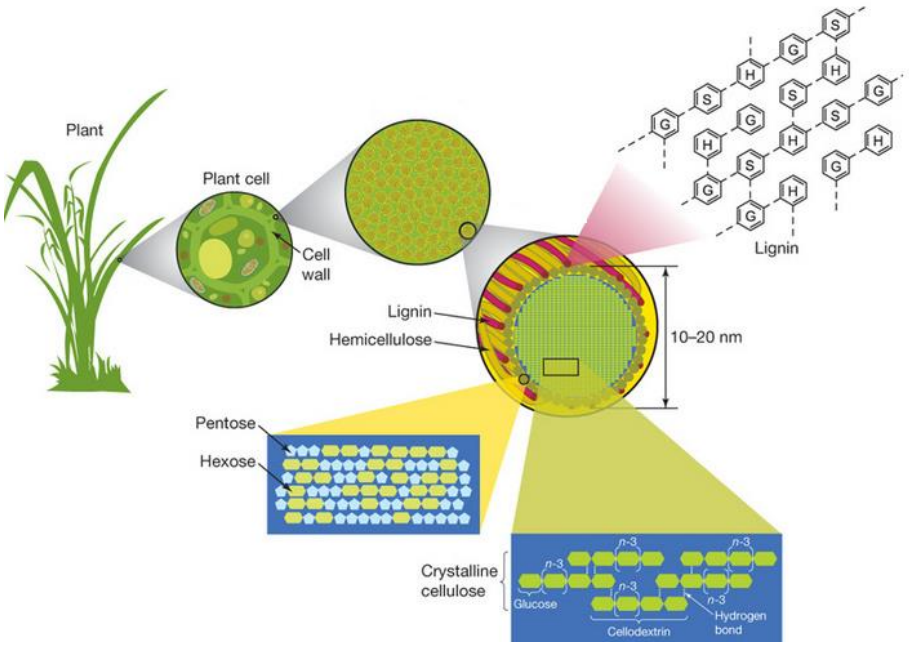

Figure 5 - Diagrammatic illustration of the structure of lignocellulose. Adapted from [52]

Lignocellulosic biomass can be converted into bio-products such as bio-ethanol or bio-fuels and it has been cropping up more and more with the increasing costs of fossil fuels and their greenhouse effects. So, the world needs other alternatives, alternatives that effectively combat the environmental pollution, the global warming and the future of oil production [5, 53].

Lignocellulosic or agro-industrial biomass are potential substitutes since they are cheaper and contain carbohydrates that can be converted into simple sugars like glucose and then fermented in ethanol [50].

However, in order to be used, lignocellulosic biomass requires a pre-treatment, an enzymatic hydrolysis and a fermentation process.

Pre-treatment can be biological, physical, physico-chemical or chemical and it is responsible for breaking down the lignin barrier to recover cellulose. The pre-treated cellulosic biomass is then subjected to enzymatic hydrolysis, an effective and economical method to achieve fermentable sugars under mild and eco-friendly reaction conditions; these fermentable sugars are ready to be used for fermentation processes [50, 51].

Some studies were carried out by the Portuguese iBB-IST/LNEG [4, 13] in order to use lignocellulosic materials as carbon source for the production of mannosylerythritol lipids by *Moesziomyces* yeasts [4]. This substrate can be a good alternative since its use has a clear benefit for the environmental impact and recover MEL become easier and cheaper without compromising the final product [4].

2.3.4. How can the product be characterized? (Surface tension, Critical Micelle Concentration (CMC), Hydrophilic-lipophilic balance (HLB)

Biosurfactants are biological compounds known because of its surface active properties leading to the reduction of surface tension and interfacial tension. Surface tension corresponds to a phenomenon between a liquid and a gas in which the surface of the liquid acts like a thin elastic sheet; if the phenomenon occurs between two liquids, like water and oil, it is called interfacial tension. The SI units for the surface tension are Newton per meter (N/m) [54].

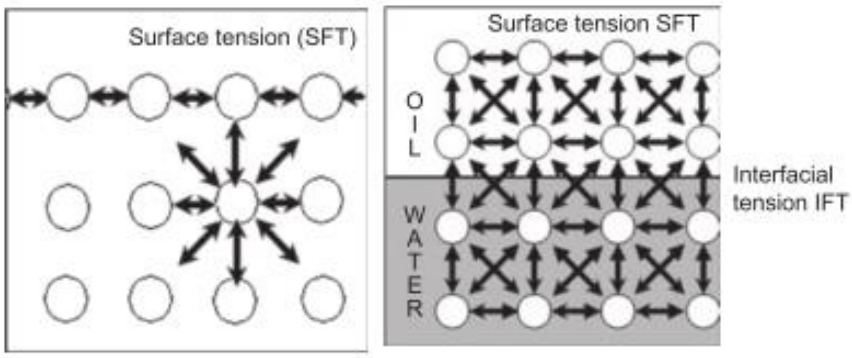

Figure 6 - Pictorial representation of functional properties of biosurfactants, surface and interfacial tension [55]

To measure the surface tension the most frequently used technique is the ring tensiometry, usually known as Du Nouy Tensiometer [56]. In this method, a thin wire is inserted below the interface and held on horizontal; then the ring is pulled up through the interface and a liquid meniscus is formed whilst the measured force increases until a maximum value. At this point the liquid meniscus break and the surface tension value is known [57]. Usually it is used a ring of Platinum-iridium because this alloy is optimally wettable due to its very high surface energy, it is chemically inert and easy to clean [58].

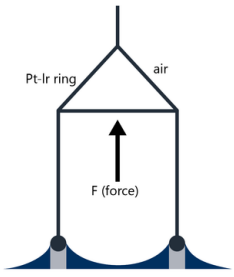

Figure 7 - Schematic diagram of the Du Nouy ring method [58]

Although this is the most widely used method, there are others like the drop volume method, the inclined method, pendent drop method, among others [57]

With a low concentration of biosurfactants, their molecules tend to arrange on the surface; however, as they are added, the surface tension of the solution starts to decrease and become saturated, leading to the formulation of micelles. At this point, the critical micelle concentration (CMC) is known [59]. As written before, biosurfactants are capable of reducing the surface tension, for example, while for water, this value is around 72 mN/m, for a biosurfactant aqueous solution, this value decrease for 35 mN/m or values even lower [9].

Table 2 - Surface tension of some biosurfactants

Biosurfactant	Surface Tension (mN/m)	References
Trehaloselipid	25-30	[21, 24]
Sophorolipid	33	[22, 60]
Rhamnolipid	<30	[15, 17]
Lipopeptides	27	[19, 24]
Surfactin	27-32	[9, 24]
Serrawettin	28.8-33.9	[24]
Fatty Acids	<30	[24]
Mannosylerythritol lipids	<30	[6, 9]

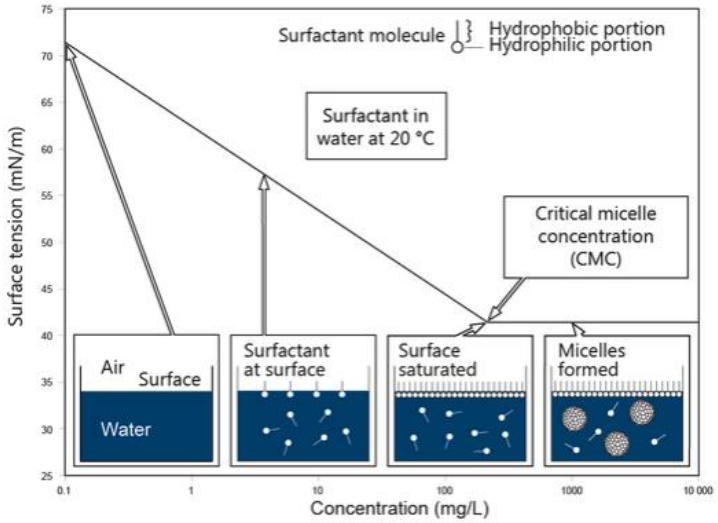

Figure 8 - Surface tension progress of a surfactant solution according to the concentration [61]

Apart from the surface tension and the CMC, biosurfactants can also be characterised by the Hydrophilic-Lipophilic Balance system (HLB). HLB was firstly introduced in the late 1940s by William Griffin and it is based on the fact that all surfactants combine in one molecule both hydrophilic and lipophilic groups [62].

With this method, it is possible to know the proportion between the weight percentages of the two groups and, in the case of non-ionic biosurfactants, it allows you to know what behaviour should be expected from that product [62].

HLB values may be calculated and a low value (e.g. 4) means that the biosurfactant tends to be oil soluble, in other words a water-in-oil emulsifier and a high value (e.g. 16) means that the biosurfactant tend to be water soluble, i.e. a solubilizer [62, 63]. So, HLB may be calculated with the following formula [62]:

$$HLB = \frac{E}{5}$$ **Equation 1**

Where, E represents the mass (or weight) percentage of oxyethylene content. HLB can varies between 0 and 20 and that corresponds to a completely lipophilic/hydrophobic molecule or a completely hydrophilic/lipophobic molecule [63, 64].

According to the value of HLB obtained it is possible to assign different applications for the non-ionic biosurfactants. Generally this division is made as follows:

Table 3 - Application of biosurfactants depending on HLB range (adapted from [63]).

HLB range	Application
4 – 6	Water in oil emulsifiers
7 – 9	Wetting agents
8 – 18	Oil in water emulsifiers
13 – 15	Detergents
10 – 18	Solubilizers

2.3.5. How can MEL, a biosurfactant, be used?

Mannosylerythritol lipids are biosurfactants with a wide range of applications within many diverse areas, some of which will be approached below.

MEL can be used for cosmetic purposes and it can even be a potential skin care material since it is known that MEL exhibits moisturizing properties. It has structural similarity to ceramides and also forms liquid crystals, property that facilitates its penetration into the intercellular spaces. By this way, MEL may be an alternative for higher priced ingredients, like natural ceramides being a cost-effective skin care ingredient [9, 27, 65].

Still within the scope of cosmetic applications, MEL can also be used to repair damaged hair. Ceramides, which are present in the cuticle of hair, protect and repair hair fibres that are exposed to so different environmental impacts; this characteristic is common also in MEL capable of repairing fine cracks on the surface of artificially damaged air, as electron microscopy showed, in the same way as ceramides do it [27, 66].

MEL can be also used as an antimicrobial agent, especially against gram-positive bacteria since the minimum inhibitory concentrations are low. However, this property can be enhanced in the future with some chemical modifications in the sugar moiety [9, 34].

MEL, as well as another biosurfactants, has a promising future in environmental industries. MEL can be used in the process of biotreatment, by enhancing the emulsification of hydrocarbon in water

and it can also be used for the degradation of petroleum compounds instead of chemical synthetic surfactants and thus reduce the environmental pollution [7, 67].

This biological surfactant is capable of inhibit ice agglomeration. Ice slurry systems are finding wide applications as environmental friendly cold thermal storage units, especially as air conditioners. However, sometimes the ice particles tend to agglomerate, blocking the pipeline, which in turn results in a decrease of efficiency. So, MEL can be used as it gets adsorbed on the ice surface and therefore suppresses the agglomeration of ice particles [6, 68].

This biosurfactant also can be used for medical purposes, showing antitumor activity because it can induce cell differentiation and apoptosis in human leukaemia cells; MEL also possesses antioxidant activity and it is proposed in treatment of diseases caused by dopamine metabolic dysfunction like schizophrenia [69, 70].

Surfactants are water-soluble surface-active agents that lower the surface tension of water and possess wetting, emulsifying, detergency and dispersing properties that enable the removal of dirt from the fabrics. They are the most important ingredient of laundry detergents however accumulation of these materials in the environment imposes adverse effects on aquatic life. Despite this, a search of the literature shows that the role of biosurfactants as substitutes of chemical surfactants in laundry detergents has rarely been explored [69, 71].

In accordance with the variety of applications mentioned above, it is easy to understand that mannosylerythritol lipids is a biosurfactant with a huge potential that has been and will continue to be exploited because it gathers all conditions to become more and more attractive for industrial purposes.

2.4. How can biosurfactants be extracted and purified?

Every step involved in the production of a biosurfactant has influence on the obtained final product. Beside a high yield and productivity of the production process, the subsequent downstream of the product is economically crucial.

The downstream processing involves steps of recovery and/or purification that represent around 60% of the total production costs, which makes it quite expensive [55].

In order to minimize these costs, it is important to choose the best extraction method for each biosurfactant that depends on its ionic charge, solubility in water, whether the product is cell bound or extracellular.

Apart from the problems with costs, some recovery techniques require the use of solvents and that can be a problem, because if on one hand some applications need a high level of purity, on the other hand, some of these solvents are toxic and harmful to the environment. So, it is necessary to combine both aspects and then decide which is the best technique to use [72].

The most common methods used for biosurfactant recovery include solvent extraction, adsorption followed by solvent extraction, precipitation, foam fractionation, ultrafiltration and adsorption-desorption [8].

A strategy that includes a combination of several steps may be desirable in order to obtain a high degree of purity. Typically, the downstream process comprises three steps divided in *recovery* to remove solids, to reduce volume and to release target product; an *intermediate purification* to remove impurities and to reduce volume and a *final purification* to remove remaining impurities and liquids. This can be schematized as follows:

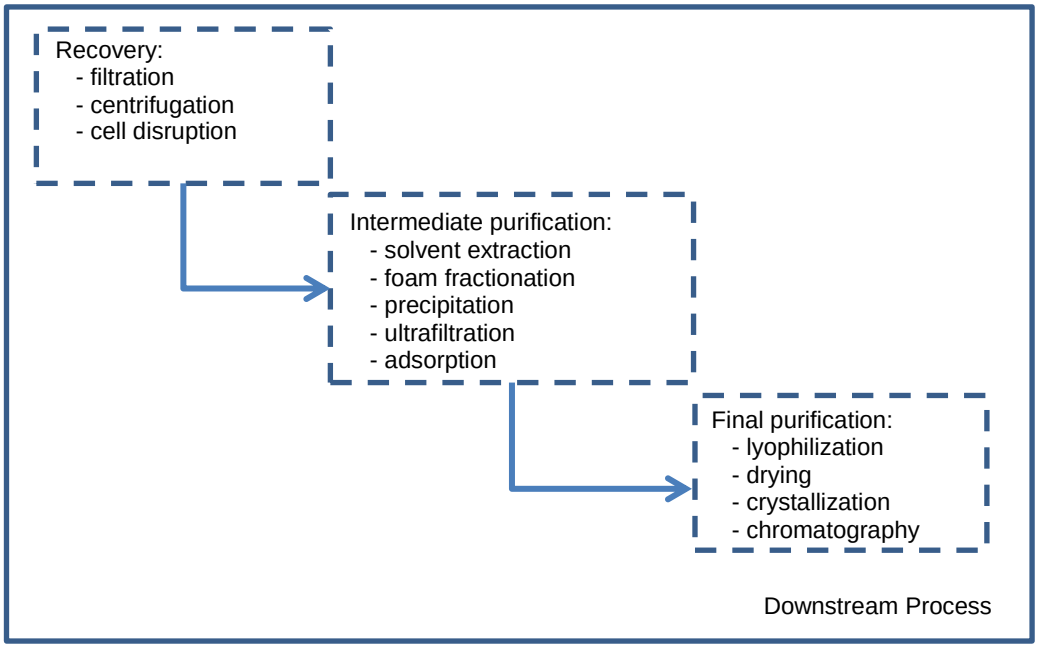

Figure 9 - Downstream stages and the most common used unit operations used

Firstly, and depending on where the product of interest is mostly found, filtrate or centrifugate (if it is extracellular) or disrupt the cells (if it is intracellular) is important to concentrate the desired biosurfactant as it reduces the working volume and consequently the associated costs. In centrifugation, the centrifugal force allows the insoluble biosurfactant to get precipitated; in filtration, the differences between the molecular diameters allows some of them to get through the membrane (permeate) and some of them to get retained by the membranes (retentate); in case of working with intracellular products, it is necessary to disrupt the cells which can occur by sonication, with temperature [8, 73, 74].

Secondly, the intermediate purification is when the substantial part of the impurities is removed. Solvent extraction is a technique that is based in the solubility of the hydrophobic moieties of biosurfactants in some solvents. It can be used different solvents like chloroform, methanol, ethyl acetate, dichloromethane, butanol, pentane, hexane, acetic acid and isopropanol [55]. This method has some disadvantages because organic solvents are quite expensive and that imply huge sums of money and, as written before, using organic solvents is harmful to human health and to the environment [73]. Therefore, to use biosurfactants for industrial applications, it´s crucial to find another options, in other words, inexpensive solvents with low toxicity [11].

Precipitation is also a method that involves using solvents as well as acetone, ethanol or ammonium sulphate or an acid. In the case of acid precipitation, biosurfactants become insoluble at

low pH values. In contrast to what happens with solvent extraction, this method is cheaper and can be efficient in crude biosurfactant recovery [11].

Foam fractionation is a downstream processing technology which can be used as an early recovery step and, in opposition to the methods previously described, no solvents are needed [11]. This technique is particularly pertinent when biosurfactants are produced because it is created by the stirring and aerating required to supply the needs of oxygen in aerobic fermentations [18]. Foam fractionation is so based on the formation of foam that rises to the top of the liquid surface and then, the emerging foam column is collected into a separate vessel where it is mechanically or by low pressure collapsed [75]. As this method needs a huge volume of the fermentation broth and it is especially useful in continues recovery procedures, it must be operated in bioreactor vessels with excess headspace. Foaming as a recovery and concentration method for biosurfactants has some disadvantages since that unpredictable formation of foam can occur and, in these cases, the use of chemical antifoams can be costly, can the reduce the oxygen transfer rate and may exert adverse effects on the cell's physiology [18].

In ultrafiltration processes, the formation of micelles or vesicles are needed, that occur at concentrations above the critical micelle concentration (CMC). Cultures are then concentrated, in an ultrafiltration system that retains the surfactant molecules in the form of micelles and allows to pass into the permeate small molecules such as salts, amino acids and other small metabolites; however, the hydrophobic impurities, such as oil or fatty acids, cannot be separated because they remain solubilized in the micelles. To disassociate the molecules and recovery the biosurfactant, solutions of organic solvents are used, particularly methanol and acetone that are able to destabilize surfactant micelles [73, 76, 77].

To recover the biosurfactants, adsorption techniques can also be performed and they are mostly conducted by hydrophobic polymeric resins that interact with the hydrophobic moiety of the biosurfactant. Amberlite XAD resins are often used to adsorb the molecules and the desorption is achieved using organic solvents such as ethyl acetate or methanol due to the differences of polarity. Using resins bring some problems because, in most of the cases, huge amounts of resin is needed which increases the costs and, however, it can be regenerated, this is also an expensive step [8, 78]. In some cases, wood-based activated carbon is also used, based on kinetic studies of biosurfactant production and in these situations the desorption of biosurfactants occurs with acetone [79].

Finally and since one of the key parameters to choose the best extraction method for each case is the obtained purity level, the final product can be lyophilized, dried, crystallized and analysed by thin layer chromatography (TLC) [80].

In case of MEL, its isolation and purification is difficult when hydrophobic substrates, such as vegetable oils, are used due to the several complex extraction and purification steps that result in a cost increase of the overall process to obtain pure MEL but with low yields [81]. Rau, et al [78] reported different downstream processes to recover MEL from a fermentation broth with high concentrations of soybean oil. The use of different organic solvents in liquid-liquid extractions achieved a purity level of 100% w/w however with a reduced recovery yield (around 8% w/w); also adsorption on commercial resins (Amberlite XAD-16, XAD-7 and XAD-4) and a heat treatment to the

cell suspension were tested. For the first case, the resins did not show to be able to specifically adsorb MEL however a yield of 93% w/w with a purity of 87% w/w were achieved for the second case when a cell suspension was submitted to a temperature of 110ºC during 10 minutes [78].

The use of a physical method before solvent extraction is also reported [82]. Here, the fermentation broth was centrifuged and the resulting supernatant is extracted with ethyl acetate (1:1 v/v). A complete separation of residual soybean oil and fatty acids is achieved by using a solvent mixture of n-hexane, methanol and water (1:6:3, v/v) followed by a multiple extraction with n-hexane. To obtain a purify MEL fraction, the water/methanol phase is distillate and lyophilize.

Another method reported [78] combines the use of ethyl acetate and a preparative chromatography column filled with silica gel using chloroform and acetone as eluents and a yield of 79% w/w for a purity level of 100% w/w was reached. This method is essential used to purify small amounts of MEL and the separation of different MELs is possible; however, due to the substantial loss of product, the costs for the downstream process tend to increase if applied at an industrial scale [78]. As an alternative, the use of hydrophilic substrates as sugars can facilitate MEL recovery and make the process more sustainable [38].

3. Material and Methods

3.1. Cell cultivation

3.1.1. Microorganisms and maintenance

Moesziomyces antarcticus PYCC 5048^{T}, provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal, is cultured for 3 days at 30ºC on yeast malt agar medium that consists on yeast extract (3 g/l), malt extract (3 g/l), peptone (5 g/l), D-glucose (10 g/l) and agar (20 g/l). Cultures were kept at 4ºC and renewed every 2 weeks and glycerol stocks were stored at -70ºC to recover the cultures when necessary.

3.1.2. Medium and cultivation conditions

The production of MEL started with the preparation of the inoculum, which has been prepared as described elsewhere [13] (3 g/l $NaNO_3$, 0.3 g/l $MgSO_4$, 0.3 g/l KH_2PO_4, 1 g/l yeast extract, 40 g/l D-glucose) and incubated at 27°C and 250 rpm for 48 hours. The inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of the medium described above.

To start the fermentation, 10% (v/v) of the inoculum was transferred and incubated (Aralab, Agitorb 200) for 14 days at 27°C and 250 rpm.

A reference condition (here named α) uses an initial concentration of 40 g/l of D-glucose as carbon source with feeding at day 4 of 40 g of D-glucose per liter of fermentation broth [13] was considered for *M. antarcticus.*

When started, the time of addition of nitrate was also evaluated (addition at day 0 or 4) as well as the combination of D-glucose with a feed of hydrophobic substrate, soybean oil.

All media were sterilized in an autoclave (AJC, Uniclave 88) at 121ºC and 1 bar for 20 minutes.

3.1.3. Growth and biomass determination

To follow cell growth, samples of 1 mL were taken to quantify the biomass (cell dry weight). After centrifugation (Sigma, Sartorius 1-15P) at 13000 rpm for 5 minutes, the supernatant was collected and the pellet, after being washed twice with deionized water, was dried at 60 °C for 48 hours. The supernatant was kept at -20 ºC for further analysis and the dry biomass was weighed to build the growth curve.

3.1.4. MEL isolation – solvent extraction

To obtain the product of interest, an isolation procedure was carried out that consists on a liquid-liquid extraction with ethyl acetate. Typically an equal volume of ethyl acetate and fermentation broth

were added together in separator funnels, the mixture was shaken vigorously under manual agitation and then it was allowed to settle in two distinct phases.

The organic phase was separated and an extra equivalent volume of ethyl acetate was added to the remain aqueous phase for a new extraction. This process can be repeated two or three times depending the visual aspect of the collected organic phases. The aqueous phases were discarded. The collected organic phase, enriched in MEL (since it was known that MEL has more affinity for the ethyl acetate instead of water) were added together and the solvent was then evaporated and collected in a rota vapour (BUCHI, Rotavapor R-3) under vacuum.

3.2. Analytical tools

3.2.1. MEL analysis through GC-FID

Isolated MEL or MEL in biological samples were lyophilized to remove water and then submitted to transesterification as described below. After, gas chromatography (GC) is used to quantify the methyl esters (C7-C18) content and so, indirectly, determine MEL concentration considering the specificity of MEL chains size.

Broth samples of 3 ml were lyophilized (Christ, Alpha 1-2 LD plus) for 48 hours, because the following transesterification does not work in presence of water.

For the transesterification reaction, acetyl chloride (0.5 µl) was added, under stirring, to pure methanol (10 ml) previously cooled down, which generated a water free HCl/methanol solution.

The freezing-dried samples were weighted and mixed with 2 ml of the solution described above, using a solution of heptanoic acid 4% (v/v) as internal standard and incubated (Aralab, memmert) for 1h at 80 °C for reaction into methyl esters.

The organic phase (1 µl), after extraction with hexane (1 ml), was injected in a GC system (Hewlett-Packard, HP5890), equipped with a FID detector and a HP-Ultra 2 column. The oven was programmed from 140 °C and temperature raised to 170 °C at 15 °C/min, to 210 °C at 40 °C/min and to 310 °C at 50 °C/min; nitrogen gas was used at a flow rate of 25 ml/min.

The concentration of MEL was obtained through the amount of C8, C10 and C12 and the concentration of fatty acids through the amount of C14, C16 and C18.

3.2.2. Sugar quantification by HPLC

To follow the sugar profile, more specifically, to quantify D-glucose and D-xylose, samples of supernatant were measured in a high liquid performance chromatography (HPLC) system equipped with an auto sampler (Hitachi LaChrom Elite L-2200) equipped with a UV detector (Hitachi LaChrom Elite L-2400) coupled to a (Chromolith Performance RP-18) endcapped column.

The supernatants were diluted in a proportion of 1:2 with a solution of sulphuric acid (0.05 M) and centrifuged (Sigma, Sartorius 1-15P) at 13000 rpm for 1 minute to remove some protein that may have precipitated. The supernatant was collected and diluted (1:10) once more in the same sulphuric acid

solution (0.05 M) The samples with a total dilution of 1:20 were then analysed and sulfuric acid was used as mobile phase.

3.2.3. Surface tension and Critical Micelle Concentration

To determine the surface tension, aqueous solutions of MEL were prepared (0.0001 mg/ml, 0.001 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.05 mg/ml, and 0.1 mg/ml).

These solutions and all the procedures involved in this analytical tool were performed using glass material to avoid interference with the properties of the solutions, that could change the surface tension; plastic materials can adsorb some molecules.

The surface tension was measured in a tensiometer (Kruss, Reagente 5), using the ring method. In this method, a platinum ring was introduced in the solution and then the ring was pulled up through the interface until it was observed a liquid meniscus. After this, the ring was continuously and slowly pulled up until the meniscus break. At this moment, the surface tension value was collected.

The values of surface tension decrease with the increase of concentration until stabilize, which corresponds to the critical micelle concentration.

Each assay was repeated three times with a difference between them at least of 1 mN/m, above or below, and the final value of surface tension corresponds to the average of them.

It was also measured the values of surface tension of supernatant samples and deionized water. For supernatant, it was measured directly and after being concentrated; solutions with less 25%, 50% and 75% of water were analysed. To easier the writing and reading of this thesis the supernatant will be designated as supernatant β, and the concentrated ones will be designated as β-25, β-50 and β-75, respectively.

3.2.4. Contact angle

The contact angle (kruss, DSA25) is a technique used to characterize the supernatant and it measures the angle formed by the intersection of the liquid solid interface (in this case it was used “Parafilm M”).

Samples of supernatant β, β-25, β-50 and β-75 were analysed.

3.2.5. Thin layer chromatography (TLC)

Samples of culture broth were extracted with ethyl acetate, as described earlier. To TLC analysis, the organic phase was recovered, evaporated, dissolved in methanol and eluted using a solvent system of chloroform/methanol/water (6.5:1.5:0.2). To reveal the compounds a solution of α-naphtol sulfuric acid (1.5 g of naphtol, 5 ml of ethanol, 4 ml of water and 6.5 ml of sulfuric acid) was sprayed and the plate was charred at high temperatures.

3.2.6. Silica gel column chromatography

In order to separate fractions of MEL-A, MEL-B and MEL-C, MEL dissolved in a solution of chloroform/acetone (7:3) was eluted through a silica gel column chromatography. A solution of chloroform/acetone (7:3) was used as eluent except for MEL-C in which the eluent proportions were changed to 5:5.

TLC technique was used in order to confirm the separation of different MELs and some fractions were analysed through nuclear magnetic resonance (NMR).

3.2.7. Nuclear magnetic resonance (NMR)

For NMR spectroscopy analysis (Bruker, Magnet System 300 MHz/54mm UltraShield), the samples were evaporated and re-suspended in 450 µl of deuterated chloroform ($CDCl_3$). For the obtained 1D ^1H NMR spectra, chemical shifts are referenced to that of external chloroform, which is designated as 7.24 ppm and the peaks were compared to the ones mentioned in literature [83].

3.2.8. Determination of protein concentration

Protein concentration was determined by the Bovine Serum Albumine (BSA) Assay Kit. Diluted BSA standards were prepared from 20 µg/ml to 2000 µg/mL, for calibration curve.

25 µl of each sample and 175 µl of protein assay reagent were added to the blank wells, and then the plate was incubated for 30 minutes at 37ºC. The absorbance was read at 562 nm in a microplate reader (Thermo Scientific, Multiskan Go).

The calibration curve obtained is represented in Figure 10.

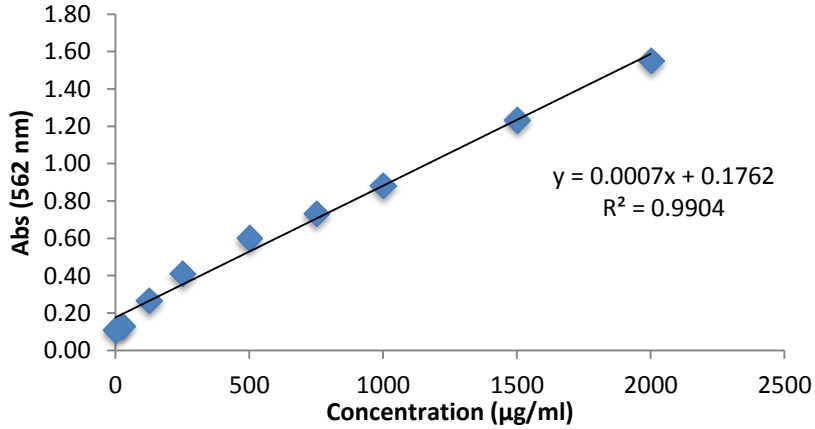

Figure 10 - Calibration curve of the pierce BCA protein assay method from 20 to 2000 µg/ml at 562nm. It has 0.0007 slope and 0.1762 intersection.

3.3. Downstream processes

For all the processes described, samples of 10 ml of a 14 days fermentation were taken and centrifuged (Eppendorf, centrifuge 5810 R) at 10000 rpm and 4ºC for 5 minutes in order to separate the cells from the supernatant. The cells were used as described below and the supernatant was frozen.

The viability of cells was calculated using the following expression:

$$\frac{CFU}{ml} = Nº\ of\ cells\ \times Dilution\ factor \times V\ inoculated \qquad \textbf{Equation 2}$$

3.3.1. Alcoholic solvent extraction

The pellet was washed with different solutions of isopropanol (25%, 50% and 75%), methanol and with only water deionized.

The samples were vortexed and filtrated under vacuum. The weighed filter papers were stored in petri plates for drying and the filtrates were placed on the rota vapour (BUCHI, Rotavapor R-3) to remove the alcoholic solvent.

All the samples were then lyophilized (Christ, Alpha 1-2 LD plus) and MEL was analysed through GC-FID (Hewlett-Packard, HP5890), as explained above.

3.3.2. Solvent extraction without cell disruption

Cells were re-suspended in 10 ml of solvent aqueous solution, some biphasic and some monophasic. One aliquot was taken and diluted to measure CFU and the remaining mixture was vortexed for 2 minutes and centrifuged (Eppendorf, centrifuge 5810 R) to separate the organic fraction from the cells. After evaporate the solvents, MEL was analysed through GC-FID (Hewlett-Packard, HP5890) after transesterification.

3.3.3. Solvent extraction with cell disruption

Cells were exposed to different disruption techniques, heated in autoclave (AJC, Uniclave 88), sonicated (Bandelin, Sonopuls) and mixed with glass beads.

In the first case the cells were autoclaved for 10 minutes at 100ºC.

In the second case the cells were re-suspended in 2 ml of water and sonicated in 3 cycles of 30 seconds interleaved with breaks of 60 seconds. After sonication, the suspension was centrifuged (Eppendorf, centrifuge 5810 R) to separate the water from the cells.

In the third case, 5 ml of NaOH and 1 ml of glass beads, with a diameter of 0,5 mm, were added to the cells and vortexed for 2 minutes followed by centrifugation to separate the phases.

All the phases described earlier were extracted with ethyl acetate and after evaporate the solvent, the samples were lyophilized (Christ, Alpha 1-2 LD plus), transesterified and analysed through GC-FID (Hewlett-Packard, HP5890) to quantify MEL.

3.3.4. Cell disruption

To study the effect of sonication the cells, different conditions were tested. In this case, beside the cells, re-suspended in 3 ml of water, also the fermentation broth was sonicated (Bandelin, Sonopuls). Different times of sonication were also tested including 3 cycles of 30, 15 and 5 seconds with breaks of 60 seconds between each cycle.

After sonicate, all the phases were separated by centrifugation (Eppendorf, centrifuge 5810 R), lyophilized (Christ, Alpha 1-2 LD plus) and analysed through GC-FID (Hewlett-Packard, HP5890) to quantify the biosurfactant.

3.3.5. Polymeric XAD resins

Different types of Amberlite XAD were used as adsorbent. XAD-4 is a crosslinked aromatic polymer; XAD-7 can adsorb non-polar compounds from aqueous systems; XAD-16 is a hydrophobic polyaromatic.

Adsorption tests were carried out as follows: 4 ml Amberlite beads were added to 10 ml culture suspension. After 24h of stirring (150 rpm), the beads were separated by vacuum filtration and rinsed with 5 ml of Mili-Q water. The phases were separated in a separating funnel and the beads were extracted by the addition of 10 ml EtAc followed by 10 ml of MeOH. The organic solvents were evaporated and all the phases were analysed in terms of MEL by GC-FID (Hewlett-Packard, HP5890).

3.4. Applications

3.4.1. Application of MEL in the formulation of a detergent

Pieces of dry cotton cloth (Piriuki) were cut into 5 x 10 cm and each piece was stained with 0.25 ml soybean oil or 0.5 ml chocolate. The pieces were stored at room temperature overnight and weighted precisely before washing. The stained cotton cloths were washed in an Erlenmeyer containing approximately 1 g of detergent in 50 ml of tap water under stirring (Aralab, Agitorb 200) (300 rpm) and with a set temperature of 30ºC for 30 minutes. After washing, the pieces were rinsed in 100 ml of distilled water twice, and dried at room temperature to a constant weight.

The same procedure was used for washing the pieces with a commercial detergent and a mixture of biosurfactant in commercial detergent.

It was used a set of formulation containing 0.7 g of sodium tripolyphosphate (STPP), 0.3 g of sodium sulfate (SS) and 0.025 g of MEL in a total volume of 50 mL diluted (solution A) with an

aqueous solution of sodium tripolyphosphate and sodium sulfate (solution B) until final concentrations of 0.01 mg/ml, 0.02 mg/ml and 0.1 mg/ml that correspond to a concentration below the CMC, the CMC and a concentration above the CMC, respectively.

	$V_{Solution\ A}$ (mL)	$V_{Solution\ B}$ (mL)	Final Concentration (g/L)
Above CMC	1	49	0.01
CMC	2	48	0.02
Below CMC	10	40	0.1

The removal percentage of the soybean oil or chocolate was calculated as follows:

$$Oil\ or\ choc\ removal\ (\%) = \frac{weight\ after\ overnight(g) - weight\ after\ dried(g)}{weight\ after\ overnight(g)} \times 100 \qquad \textbf{Equation 3}$$

3.5. Statistical Analysis

For statistical analysis, analysis of variance (ANOVA) was used and the post hoc Tukey's test was applied by aid of SPSS software (v. 24.0; IBM Corporation). The significance level for all analyses was $p<0.05$.

4. Results and discussion

4.1 Fermentation

4.1.1 Objectives and strategy

This section includes results obtained and the correspondent discussion for the performed assays in order to evaluate the ability of *M. antarcticus* to produce MEL without using or reducing the use of vegetable oils alone, such as the soybean oil, and implementing the alternative use of sugars, D-glucose and D-xylose and combined with vegetable oils.

These alternatives, in the context of the use of lignocellulosic residues, of which D-glucose and D-xylose are the main constituent monomers, have been reported as an alternative to the use of soybean oil [13]. Oils have the advantage to increase the final titre of MEL, showing high efficiency however it is known the impact of soybean oil in cultures once MEL production from vegetable oils is hardly sustainable due to environmental impact, competition with food chain, the price of the substrate and the difficulties associated with the recovery of biosurfactant from fermentation broths containing oil. The use of sugars and lignocellulosic materials are a sustainable alternative to the production of MEL, which in combination with lower amounts of hydrophobic vegetable oils, may reduce the amount of oil required, and thus to potentially represent, still a valid, but different approach to produce the microbial surfactant, possibly at higher titres.

The nitrogen source also plays an important role in the fermentation process and its efficiency for the production of MEL was analysed although its impact to the environment is not yet known. The addition of $NaNO_3$ in different days, at the beginning of the fermentation or after 4 days was studied due to the fact that a nitrogen limitation improves the lipid profile. For *Ustilago maydis,* which is a phytopathogen fungus that also produces MEL, is reported that a limitation on the nitrogen available, strongly induce the expression of enzymes. This lack of nitrogen also increased the cell size, which was reported to happen due to the accumulation of lipids.[84]

Different growth conditions were studied in order to increase the titre because the higher it is, the easier will be the downstream processes to perform. The efficiency of each condition was assessed on the basis of the titre and sugar consumption.

First of all, different concentrations of D-glucose, which acts as carbon source, were tested with initial values of 40 g/l or 80 g/l with different feeds at day 4.

Besides this, the addition of nitrate was also studied in order to understand if its addition at the beginning or at day 4 influences the production of the biosurfactant and its final titre.

Combinations of D-glucose and soybean oil feeds were also tested, to boost the titres of MEL because it is already known that these substrates are capable of produce large amounts of the glycolipid. Once more, the addition of $NaNO_3$ was tested.

Finally, D-xylose was tested as alternative sugar carbon source to the use of D-glucose. For assays with D-xylose lower sugar concentrations (40 g/l) were always used at the beginning with only one case in which a feed of 80 g/l D-xylose was performed. For fermentations where D-glucose and soybean oil were combined, all the fermentations started using only D-glucose as carbon source at 40

g/l, then feeds of only soybean oil (21 g/l) or mixtures of D-glucose:soybean oil (40 g/l:21 g/l) were carried out at day 4. Note that the carbon available in 21 g/l of soybean oil is roughly the same in 40 g/l of a glucose solution.

4.1.2 Influence of D-glucose

The carbon source plays an important role in the growth and production of biosurfactants by microorganisms with effects that varies from species to species. For *M. antarcticus,* D-glucose is widely used to grow cell or even to produce MEL [13]. However, the utilization of soybean oil is reported to provide higher titres, but comes with the cost of increased problems in downstream due to residual oils.

In this section, the overall goal is to improve MEL titres in sugar-based fermentations, specifically testing alternatives to improve titres achieved with condition α. To do so, different concentrations of D-glucose with feeds (with hydrophilic and hydrophobic sources) were tested to try to increase the final concentration of MEL.

4.1.2.1 Addition of $NaNO_3$ at day 0

With the addition of nitrate at day 0, different concentrations of glucose with different feedings were tested.

When starting with a low concentration of D-glucose (40 g/l), this substrate is almost consumed until day 4 (Figure 11a and Figure 11b), with titres of MEL around 1 g/l. With these initial conditions, different feedings of D-glucose at day 4 were tested (40 g/l or 80 g/l) and for fermentations starting with 40 g/l of D-glucose there is no significant increase in production of MEL. For example, in these two fermentations starting with 40 g/l of glucose, one can note that the addition of 80 g/l of D-glucose at day 4 actually result in a slight increase in biomass, and a more significant increase on long fatty acids.

In contrast, when higher concentrations of D-glucose were used in the beginning (80 g/l), D-glucose is not exhausted and this value is still high after 4 days (Figure 11c and Figure 11d). In these cases different feeding were performed and it is possible to observe that actually the feed of 80 g/l of D-glucose also do not lead to increase in MEL production, which was only observed for values around 7.5 g/l, when to the initial 80 g/l, an additional feed of 40 g/l were added at day 4 (Figure 11c). This experiment dismissed the theory that once D-glucose is kept in the solution MEL production will be favoured. That may be a required condition, but not sufficient alone to increase MEL productions. Also, for the condition that result in MEL higher production, no D-glucose was present in the fermentation media after day 14 of fermentation. Therefore, the existence of D-glucose in the fermentation media, pressing metabolic fluxes for MEL production, may be more important in the first part of the fermentation, around day 4 to 10, but less important towards the end of the fermentation where the cell may already had accumulated the resources to make MEL. Note that towards the end of the fermentation with higher MEL production, when no more D-glucose is available in the

fermentation broth, actually is observed a decrease of long fatty acids concentration. An additional note is that the condition with less MEL production, it was the one when an overall higher amount of D-glucose was added, reaching values of 120 g/l of this substrate in the fermentation and such higher amounts of substrate may have negative contributions. All together, the results obtained indicate that while the amount and regime of glucose added, as well as the maintenance of its values in the media at a certain level in early times of the fermentation 4-5 days can be important to support MEL production; these factors alone are not enough to boost MEL production.

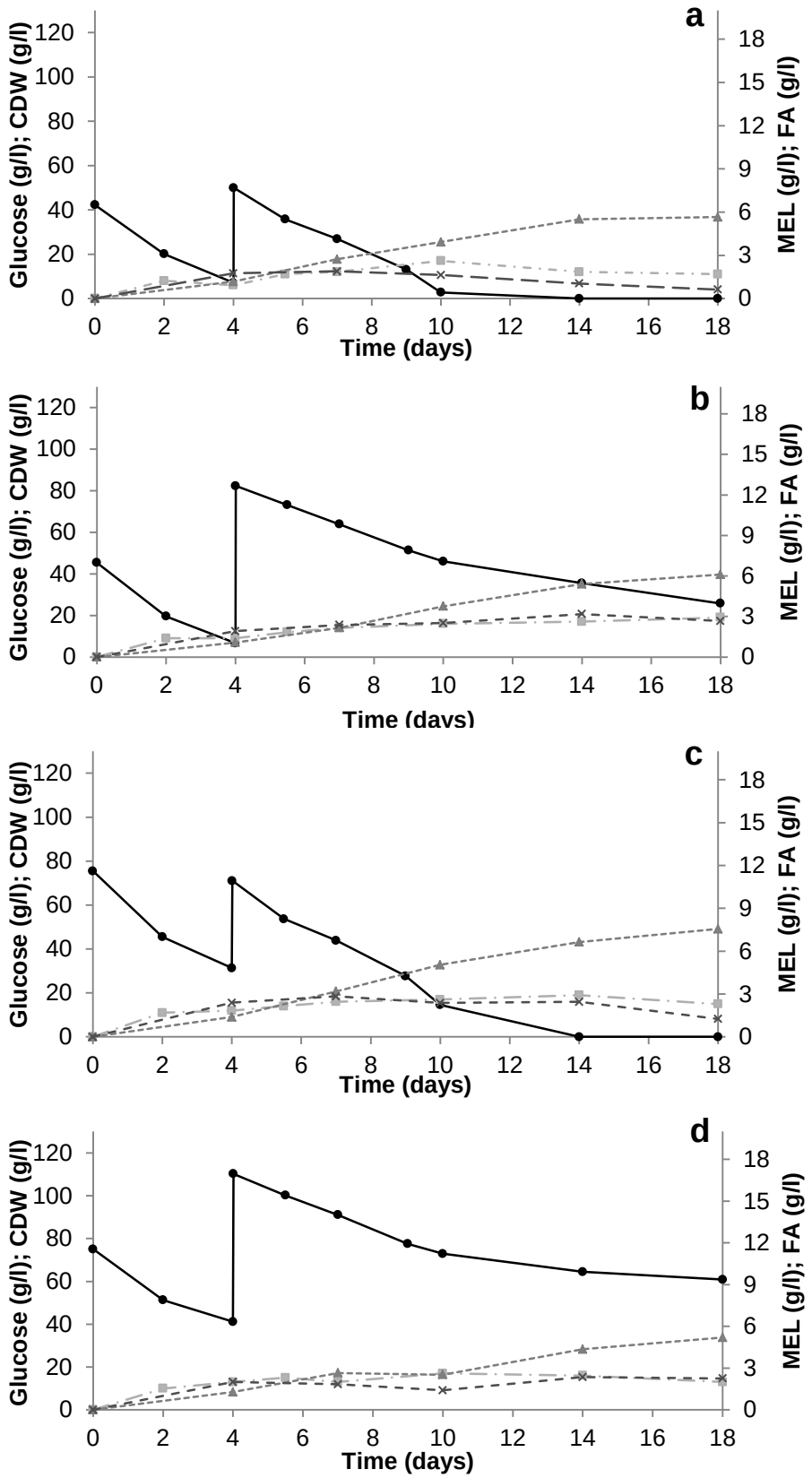

Figure 11 -Pulsed fed-batch cultivations of *M. antarcticus* PYCC 5048^{T} with addition of 40g/l of D-glucose (a and b) or 80g/l of D-glucose (c and d) at day 0; D-glucose feeding of 40g/l (a and c) or 80g/l (b and d) at day 4. $NaNO_3$ added at day 0. *Circles* D-glucose, *triangles* MEL, *squares* biomass, *crosses* fatty acids.

4.1.2.2 Addition of $NaNO_3$ at day 4

The same experimental conditions previously assessed with addition of $NaNO_3$ at day 0 (starting the fermentation with 40 g/l or 80 g/l D-glucose and to a further addition of 40 g/l or 80 g/l D-glucose at day 4) but the addition of nitrate took place at day 4. Note that the inoculum was prepared with nitrate, and therefore some residual nitrate (around 0.3 g/l) is present from the beginning to end of fermentation.

A first comparison of MEL titres at day 4 allow to evaluate whether in the short term there is an influence on MEL production by addition or not of nitrate at day zero. When the fermentation starts with a lower concentration of D-glucose (40 g/l) (Figure 12a and Figure 12b), the titre of MEL obtained after 4 days is, in both cases, relatively low, at a value of 0.71 g/l and 1.21 g/l, respectively.

Comparing the final titre when different feedings at day 4 were evaluated (40 g/l and 80 g/l D-glucose):

- for the cases starting with the lower D-glucose, a higher value of MEL concentration was obtained (approximately 8 g/l instead of 6 g/l). However, in the first situation (Figure 12a) the higher value of MEL was reached at day 14 and this value remained constant in contrast with the second situation (Figure 12b) in which the concentration of MEL never stopped to raise until the day 18. This might happened because with a feed of 40 g/l of D-glucose (Figure 12a), the carbon source ended up, which did not happen with a feed of 80 g/l (Figure 12b).
- for the cases starting with higher concentration of D-glucose (80 g/l) (Figure 12c and Figure 12d) did not make a significant difference with final values of MEL titre of 7.78 and 6.29 g/l, respectively. In the cases that fermentation started with an initial concentration of 80 g/l of D-glucose, the fermentation ended without any glucose in the medium (Figure 12c and Figure 12d).

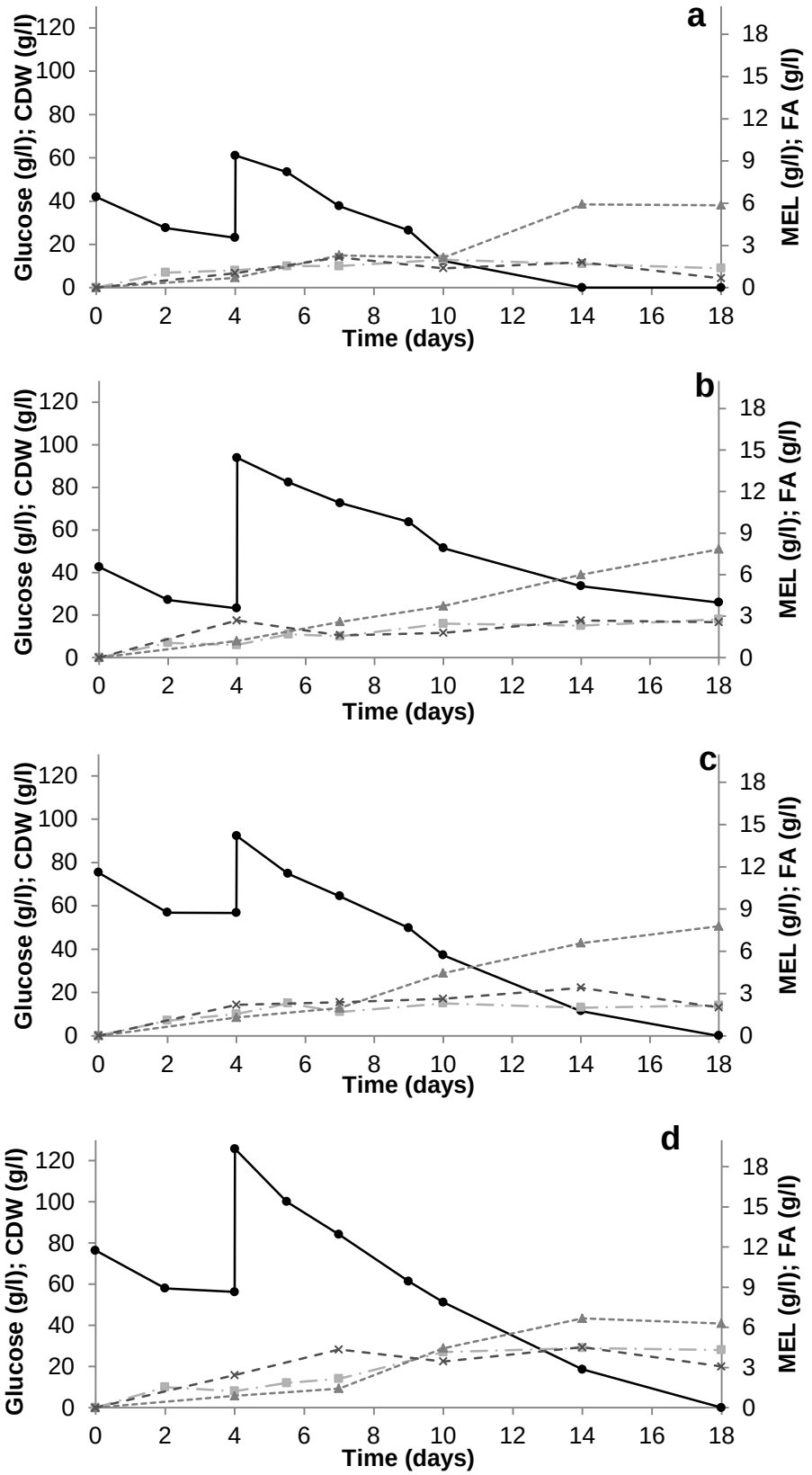

Figure 12 -Pulsed fed-batch cultivations of *M. antarcticus* PYCC 5048^{T} with addition of 40g/l of D-glucose (a and b) or 80g/l of D-glucose (c and d) at day 0; D-glucose feeding of 40g/l (a and c) or 80g/l (b and d) at day 4. $NaNO_3$ added at day 4. *Circles* D-glucose, *triangles* MEL, *squares* biomass, *crosses* fatty acids.

This experimental work also investigates whether the time of addition of nitrate influences the results obtained (Figure 11 and Figure 12).Whereas the previous section report results with addition of nitrate at the beginning of the fermentation, the current section discusses results obtained when the nitrate is only added at day 4. Note however that a residual amount of nitrate (around 0.3 g/l) is carried out from the inoculum.

Analysing the different situations it is possible to conclude that the addition of nitrate at day 4 always results in higher titres of MEL at day 18, however these increases are low (just an increase of 3.5% when comparing Figure 11a and Figure 12a). Still is worth to note that there are some differences to mention. While the same final MEL titre was obtained, regardless the time point for nitrate addition, when 40 g/l of D-glucose was added at day 4, instead of day 0, for conditions in which 80 g/l of D-glucose was also added at day 4.

Moreover, looking at the profiles over time, there are some differences, especially at the level of substrate consumption, since in some cases it is completed depleted (Figure 11a, Figure 11c, Figure 12a, Figure 12c and Figure 12d) and in other it remains in the medium after 18 days of fermentation (Figure 11b, Figure 11d and Figure 12b) which means that large amounts of D-glucose cannot be consumed when introduced in the medium. So, nitrate supply at day 0 increased sugar consumption rate until day 4, when the feed occurred (Figure 11d and Figure 12d). On the one hand, when the nitrate is added at the beginning, the glucose consumption rate and the titre of MEL are higher until day 4; however, after the feed, the nitrogen source was already almost depleted and this might be the reason for a significant reduction of carbon source consumption rate and incomplete sugar assimilation until day 18 (Figure 11d). On the other hand, when the nitrate is added at day 4, the consumption of glucose in the first days (before the addition) is lower and consequently the production of MEL is also lower. However, after the addition of $NaNO_3$ at day 4, the concentration of D-glucose decreased drastically and the production of MEL increased. This resulted in a total sugar assimilation until the end of the fermentation (there was no D-glucose in the medium at day 18), rather than what was previously described, and in a higher MEL titre (Figure 11d and Figure 12d).

For example, in Figure 11c the consumption rate of glucose until day 4 was higher when compared with Figure 12c, when the nitrate was added at day 4

The best results, considering the final concentration of MEL, were achieved with an initial concentration of D-glucose of 40 g/l and the addition of $NaNO_3$ and a feed of 80 g/l of D-glucose at day 4. For this case, a final value of 7.84 g/l of MEL was reached (Figure 12b).

4.1.3 Influence of D-xylose

4.1.3.1 Addition of $NaNO_3$ at day 0

If a renewable alternative source, as a lignocellulosic material, is considered as a substrate for a bioprocess one must bear in mind that the sugar composition of such materials contemplates other important fractions than cellulose (from which D-glucose is derived after hydrolysis), which is the

xylan, a heteropolymer composed mainly by D-xylose units. Thus, the previous section tested conditions were applied in cultures of *M. antarcticus* using D-xylose as substrate.

Once more the addition of $NaNO_3$ was evaluated but this change in the conditions, did not result in significant differences in the final concentration of MEL obtained, that was around 6 g/l in both cases (Figure 13a and Figure 14). The other situation, in which a feed of 80 g/l of D-xylose occurred (Figure 13b), the titre of MEL obtained after 18 days of fermentation increased significantly, reaching values of 10 g/l. Using D-xylose as carbon source and without adding any kind of oil, these last conditions were the ones that allowed to obtain the highest values. It is also important to mention that, in this case, the nitrate was added at the beginning of the fermentation.

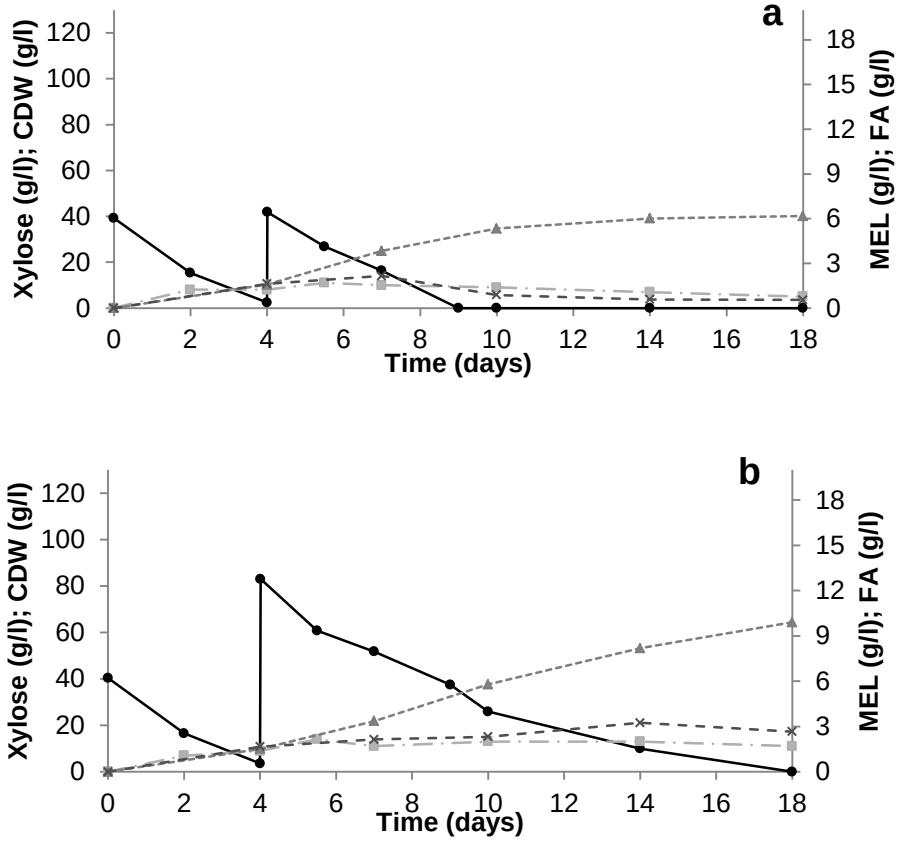

Figure 13 - Pulsed fed-batch cultivations of *M. antarcticus* PYCC 5048^T with addition of 40g/l of D-xylose (aandb) at day 0 and D-xylose feeding of 40g/l (a) or 80g/l (b) at day 4. *Circles* D-xylose, *triangles* MEL, *squares* biomass, *crosses* fatty acids.

4.1.3.2 Addition of $NaNO_3$ at day 4

The alternative addition of $NaNO_3$, after day 4, was also tested using D-xylose as substrate. So, if comparing the results present in Figure 14 with the D-glucose cultures represented in Figure 12a, the changes were not striking, since the titre of MEL obtained in both cases was almost the same (around 6 g/l).

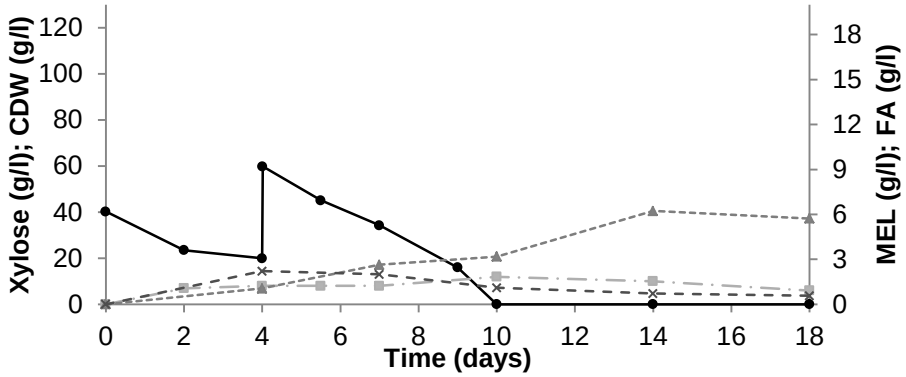

Figure 14 - Pulsed fed-batch cultivations of *M. antarcticus* PYCC 5048^{T} with addition of 40g/l of D-xylose (a) at day 0 and D-xylose feeding of 40g/l (a) at day 4. *Circles* D-xylose, *triangles* MEL, *squares* biomass, *crosses* fatty acids.

4.1.4 Influence of soybean oil

4.1.4.1 Addition of $NaNO_3$ at day 0

One of the main objectives of this experimental work was trying to achieve the high concentrations of MEL reported in many articles about this theme without using or reducing the concentration of oil used, not just because the downstream is more difficult when vegetable oils are used but also because soybean oil, that is the most used, is an integral part of the food sector for humans.

So, three different mixtures of D-glucose, as sugar, and soybean oil were tested; all of them started with a low concentration of D-glucose (40 g/l) (Figure 15 and Figure 16). Once more, also the addition of nitrate was evaluated. The concentration of soybean oil used (21 g/l) represents the equivalent amount of carbon present in 40 g/l of D-glucose.

In the first situation (Figure 15a), $NaNO_3$ was added at the beginning and at day 4, a feed of D-glucose (40 g/l) and soybean oil (21 g/l) was performed; after this feeding the concentration of MEL increased sharply until day 14, when it suffered a decline that match with the end of D-glucose in the medium. Since, it still had oil in the medium, MEL continued to be produced until a maximum value of 19.39 g/l at day 18.

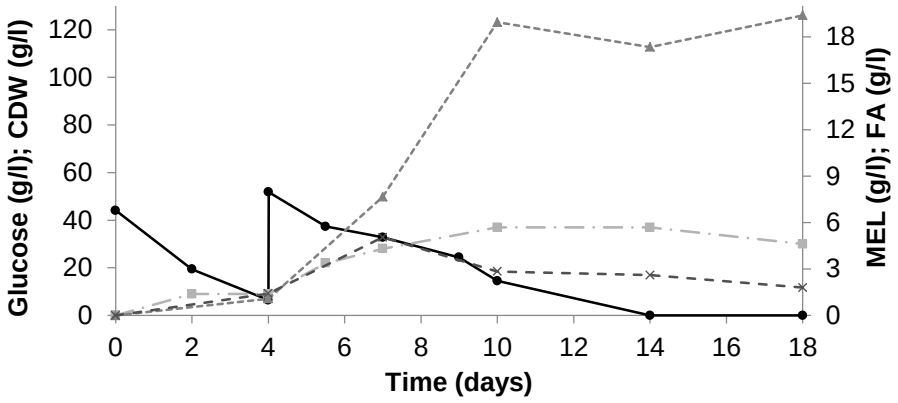

Figure 15 -Pulsed fed-batch cultivations of *M. antarcticus* PYCC 5048^T with addition of 40g/l of D-glucose and D-glucose feeding of 40g/l supplemented with a feeding of soybean oil (21 g/l) at day 4. *Circles* D-glucose, *triangles* MEL, *squares* biomass, *crosses* fatty acids.

4.1.4.2 Addition of $NaNO_3$ at day 4

The high value of MEL titre obtained with the addition of soybean oil and initial addition of $NaNO_3$ was also obtained using the same conditions but with the addition of nitrate only after 4 days (Figure 16a). In this case, the concentration of MEL increased drastically until day 10 and after that, the increasing slowed down until the end of the fermentation, when a value of 19 g/l of MEL was achieved.

A third situation was performed in order to evaluate the production of MEL without D-glucose feed, which means that at day 4 only soybean oil was added to the medium (Figure 16b). In this case, the nitrate source was also only added at the fourth day. Comparing with the cases described above (Figure 15 and Figure 16a), this condition was the one that resulted in lowest titres of MEL by the end of the fermentation, around 13.11 g/l at day 18. Therefore, it is possible to conclude that despite the importance of soybean oil to increase the concentration of MEL obtained, the presence of D-glucose, boosts MEL production.

The influence of soybean oil in the medium can also be evaluated through the comparison between a situation when only D-glucose was used with a situation of a mixture of D-glucose and soybean oil (Figure 11a and Figure 15). As expected, the use of soybean oil as substrate increased the final titre of MEL, with a difference of around three times the concentration when only D-glucose was used; however also the concentration of fatty acids follow this increase which is a disadvantage so far as it more challenging to collect MEL with low level of impurities. In Figure 15 it is possible to verify that after the addition of the hydrophobic substrate, the concentration of MEL increased sharply until day 10. At this time, D-glucose was still present whereas in the situation without soybean oil (Figure 11a), the sugar was almost depleted which means that in the presence of both substrates, the hydrophobic vegetable oil interferes in the consumption rate of the sugar hydrophilic substrate which remains in the medium until day 14. Despite the absence of the carbon source after 10 days when only D-glucose was used, the concentration of MEL increased until day 14 and only establish from here whereas when also soybean oil is present this value achieved a maximum at day 10 and remains more or less constant until the end.

Analysing all the cases described until now, it is possible to notice that the presence of soybean oil is crucial to improve the production of the biosurfactant, even though having sugar in the medium only improve all the process. With this, one of the main objectives, as described before, was fulfilled.

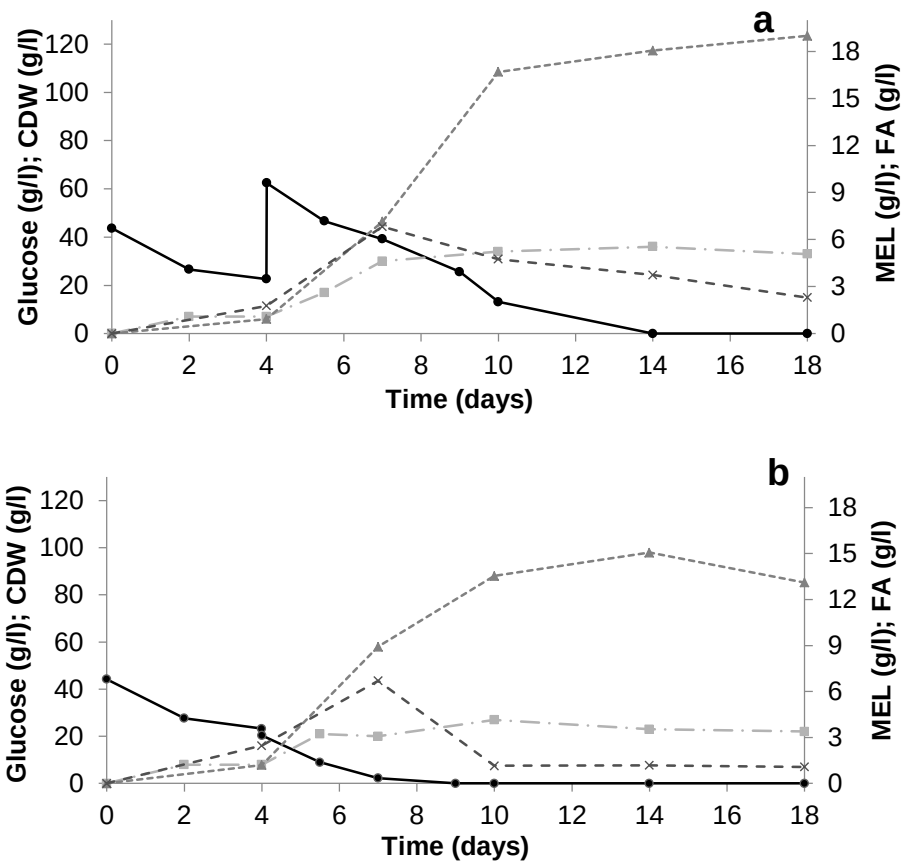

Figure 16 -Pulsed fed-batch cultivations of *M. antarcticus* PYCC 5048^{T} with addition of D-glucose and D-glucose feeding of 40g/l (a) supplemented with a feeding of soybean oil (21 g/l) (a and b) at day 4. *Circles* D-glucose, *triangles* MEL, *squares* biomass, *crosses* fatty acids

4.1.5 Fermentation overview

In the previous sections, profiles of different fermentation conditions were showed in order to analyse the behaviour of the yeast all over the days in terms of cell growth, concentration of the carbon source and fatty acids present in the medium and the concentration of MEL.

Moreover, some important considerations can be taken and explored about the results obtained by the end of the fermentation, and those are resumed in the present section.

Firstly, Figure 17 shows the titres of MEL obtained, after 18 days of fermentation, for all the conditions tested and generally it is possible to conclude that the addition of the nitrogen source in different days does not influence significantly the production of the biosurfactant; still for conditions where the second D-glucose addition was 80 g/l and not 40 g/l, an increase in final MEL titres was observed, suggesting the need of a late nitrate addition to effective use a small part of such carbon

into MEL production. Slightly higher concentrations are obtained when a total of 120 g/l (40 g/l : 80 g/l or 80 g/l : 40 g/l) D-glucose is added to the system than when only 80 g/l (40 g/l : 40 g/l) D-glucose were used; however, further increase to 160 g/l (80 g/l : 80 g/l) D-glucose actually result on a decrease of MEL titres. The results shown also allow the comparison between the carbon sources and some conclusions can be taken such as the fact that using high concentrations of glucose does not increase the concentration of MEL in a significant way which means that with the conditions used, the yeast cannot consume all the substrate introduced in the medium and that results in an expenditure of this component without achieving better results. The use of D-xylose (40 g/l : 40 g/l) at lower sugar feeding provides similar MEL titre than when D-glucose was used in the same feeding regime, and interestingly, to achieve higher MEL titres increasing D-xylose (40 g/l : 80 g/l) in the addition at day 4 to 80 g/l is achieved even with addition of nitrate only at day 4.

However, when different carbon sources types are compared, the results are different because it is proved that addition of soybean oil, even if at lower values than usually reported in the literature, boosts MEL production when compared to an exclusive use of hydrophilic carbon sources. At this point, it is interesting to analyse these two kinds of substrate because in one way, soybean oil is described as the substrate leading to higher MEL titres and productivities, in other way it has the disadvantage to be hardly sustainable due to the increasing prices of this hydrophobic substrate and the recovery of MEL from oil-containing broth is hindered when vegetable oils are used.

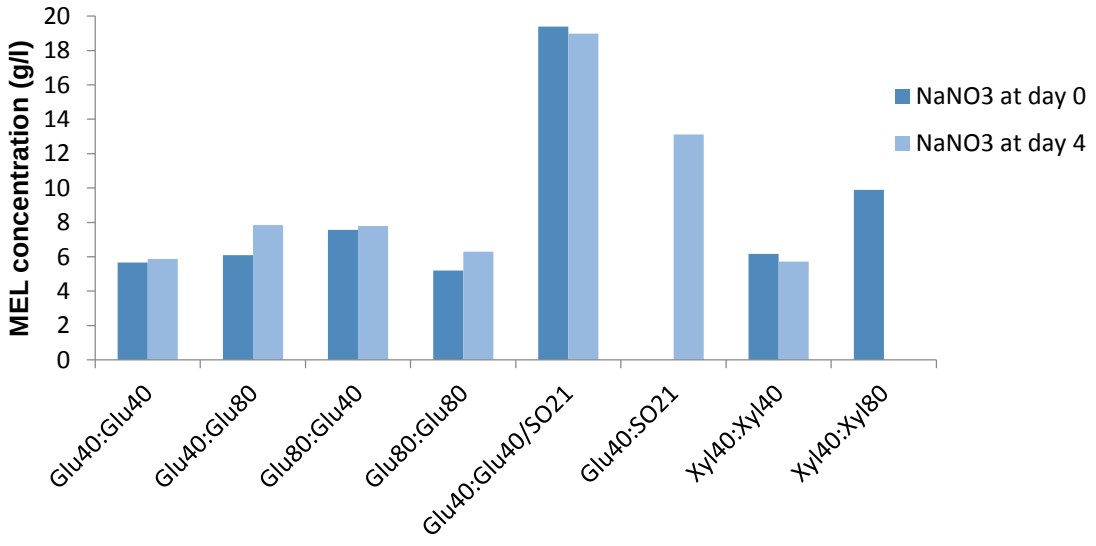

Figure 17 - MEL titre at day 18 of all conditions tested

It was also evaluated MEL yields and productivities for the different conditions tested (Table 4). Analysing the yield results it is possible to conclude that increasing the concentration of the hydrophilic substrates (D-glucose or D-xylose) did not lead to an improve of the yield (for example, for condition α it was achieved a yield of 0.09 g/g which was the same value obtained with the same starting conditions but a feed of 80 g/l at day 4) which means that some of the carbon source introduced in the fermentation medium was not even consumed and, consequently, an unnecessary expense of

resources happened. The addition of $NaNO_3$, as the nitrogen source, in different fermentation moments did not also result in differences for the obtained yield. The highest values were reached when a feed of a hydrophobic vegetable oil (soybean oil) occurred with yields of around 0.20 g/g, the double of the ones obtained for D-glucose and D-xylose.

In terms of productivity, this parameter did not rise significantly with an increase of the substrate concentration and, once more, only the feeding of soybean oil brought better results, corresponding to a maximum value of 1.24 to a feed of 21 g/l to α condition.

Table 4 - MEL maximum concentrations, yields and productivities in 18 days cultivation of *M. antarcticus*

		MEL maximum concentration (g/l) (day of fermentation)	$Y_{MEL/S}$ (g/g)	Productivity (day 14) (g/l.day)
Addition of $NaNO_3$ at day 0	Glu40:Glu40	5.66 (18)	0.09	0.39
	Glu40:Glu80	6.10 (18)	0.09	0.39
	Glu80:Glu40	7.56 (18)	0.09	0.48
	Glu80:Glu80	5.20 (18)	0.08	0.31
	Glu40:Glu40/SO21	19.39 (18)	0.20	1.24
	Xyl40:Xyl40	6.17 (18)	0.11	0.43
	Xyl40:Xyl80	9.90 (18)	0.11	0.59
Addition of $NaNO_3$ at day 4	Glu40:Glu40	5.92 (14)	0.10	0.42
	Glu40:Glu80	7.84 (18)	0.12	0.43
	Glu80:Glu40	7.78 (18)	0.10	0.47
	Glu80:Glu80	6.67 (14)	0.06	0.48
	Glu40:Glu40/SO21	18.99 (18)	0.21	1.29
	Glu40:SO21	15.06 (14)	0.24	1.08
	Xyl40:Xyl40	6.23 (14)	0.11	0.45

With the results obtained it was possible to characterize the fatty acid profiles present on MEL for the different conditions tested and in this way comparing the composition of the lipidic moieties when different substrates were used.

It is reported that using soybean oil as substrate to produce MEL, the lipidic moieties are mainly comprised of C10, in majority, and C8 fatty acids [85] whereas using hydrophilic substrates, such as D-glucose or D-xylose, C10 and C12 are the major components of the lipidic moieties [13, 85].

Figure 18a and Figure 18b show the profiles obtained for the different conditions tested. Firstly, it is possible to establish that the profile is quite similar for each carbon feeding regime, regardless the time at which the nitrogen source was added (at the beginning or after 4 days of fermentation), with exception for the condition in high concentration of glucose, at a total value of 160 g/l, (80 g/l : 80 g/l) was fed into the fermentation

The fatty acids profiles obtained show that the acyl groups of MEL are mainly composed of C10 and C12 chains when sugars are used as substrate however such profiles are slightly different when soybean oil is also introduced in the medium, with a higher contribution of C10; indeed for feeding regimes combining the use of D-glucose with soybean oil led to MEL lipidic chains profiles where C10 accounts for more than 50% of the total fatty acids associated to MEL. It is interesting to notice that when soybean oil was feeding the amount of C8 increased and a C12 higher percentage was

reached. In the other cases that soybean oil and D-glucose were fed the percentages of C8 also increased however these values do not exceed the amount of C12 which is responsibility of the D-glucose that is also present in the medium.

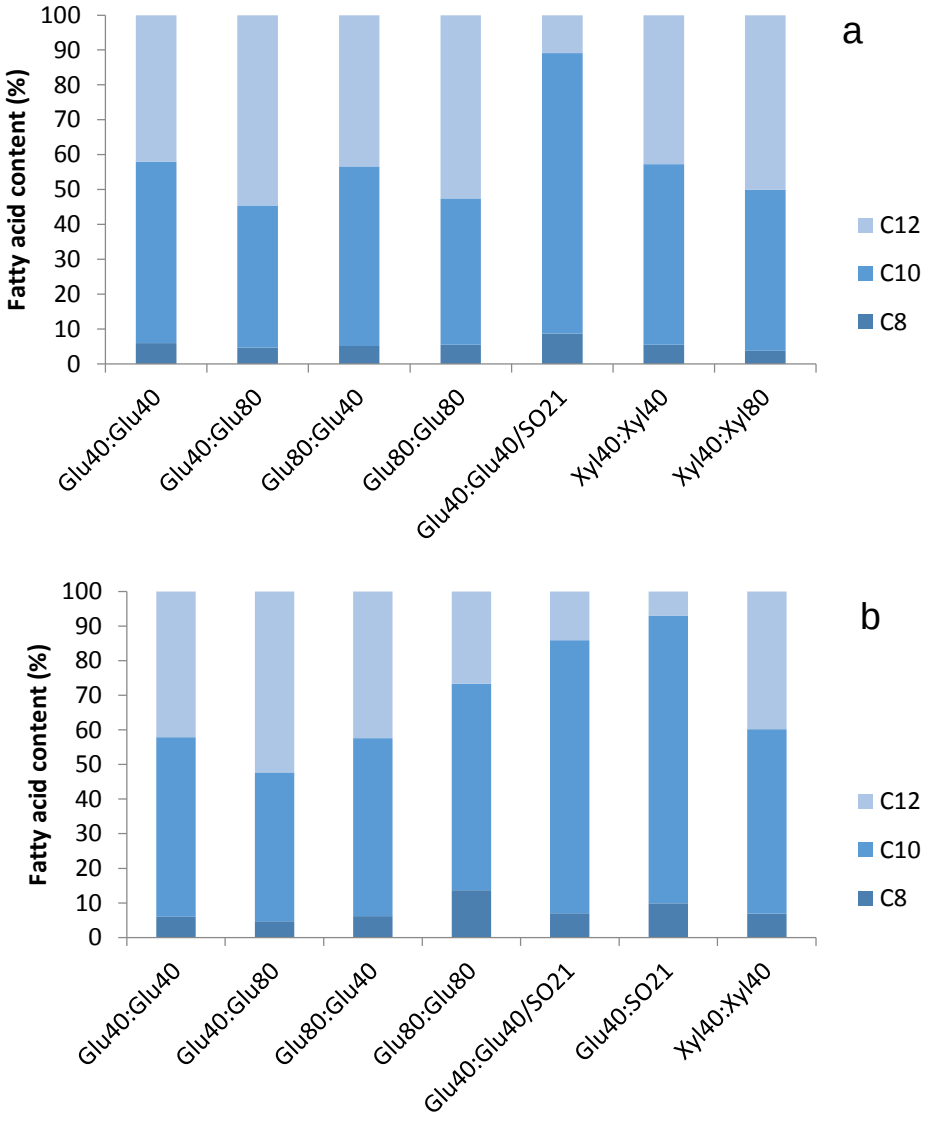

Figure 18 - Fatty acid profile of culture broth at day 18 for all conditions tested with additions of the nitrate source at day 0 (a) or at day 4 (b).

As explained before, the addition of nitrate influences the sugar consumption rate, which is higher in the presence of D-xylose than of D-glucose. The results obtained suggest that when the nitrogen source is added, the sugar consumption rate improves, contributing to a better use of the D-glucose or D-xylose added as substrate. Therefore an additional addition of the nitrogen source at day 4 when the pulse is given may be desirable. However, an extra nitrate feed will increase the C/N ratio and that can impact MEL production.

Analysing the results obtained (Table 5) when nitrate was added at the beginning, the sugar consumption rate decreases after 4 days of fermentation, most probably due to the lack of $NaNO_3$. Rather, when nitrate is only supplied at day 4, the sugar consumption rate increases from here, supporting what is written above that the presence of nitrogen in the medium is important in order to improve the sugar consumption.

The simultaneous sugar feeding and supply of $NaNO_3$ at day 4 allow higher consumption rates proving that the presence of nitrate favour D-glucose or D-xylose assimilation.

Table 5 - Sugar consumption rate before and after the feeding of hydrophilic or/and hydrophobic substrate at day 4 for all conditions tested.

	$NaNO_3$ at day 0		$NaNO_3$ at day 4	
	Before feed at day 4 ($g.l^{-1}dia^{-1}$)	After feed at day 4 ($g.l^{-1}dia^{-1}$)	Before feed at day 4 ($g.l^{-1}dia^{-1}$)	After feed at day 4 ($g.l^{-1}dia^{-1}$)
Glu40:Glu40	8.79	7.23	4.70	7.22
Glu40:Glu80	9.67	6.11	4.85	6.60
Glu80:Glu40	11.03	8.46	4.66	8.68
Glu80:Glu80	8.47	6.30	5.02	10.53
Glu40:Glu40/SO21	9.42	5.22	5.26	7.13
Glu40:SO21			5.25	6.02
Xyl40:Xyl40	9.20	8.55	5.08	8.63
Xyl40:Xyl80	9.21	8.52		

Considering a carbon mass balance between the carbon fed to the system for each fermentation conditions tested, as D-glucose, D-xylose or soybean oil and the carbon present in either the MEL or biomass, one can obtain the results present in Figure 19. The results suggest that most of the carbon present in the medium cannot be converted into neither MEL or biomass, most probably a significant fraction of the carbon will be converted in CO_2 as a result of all the cell functions, and some relatively part will be taken to build up extracellular proteins. However when soybean oil is used the conversion is more sustainable.

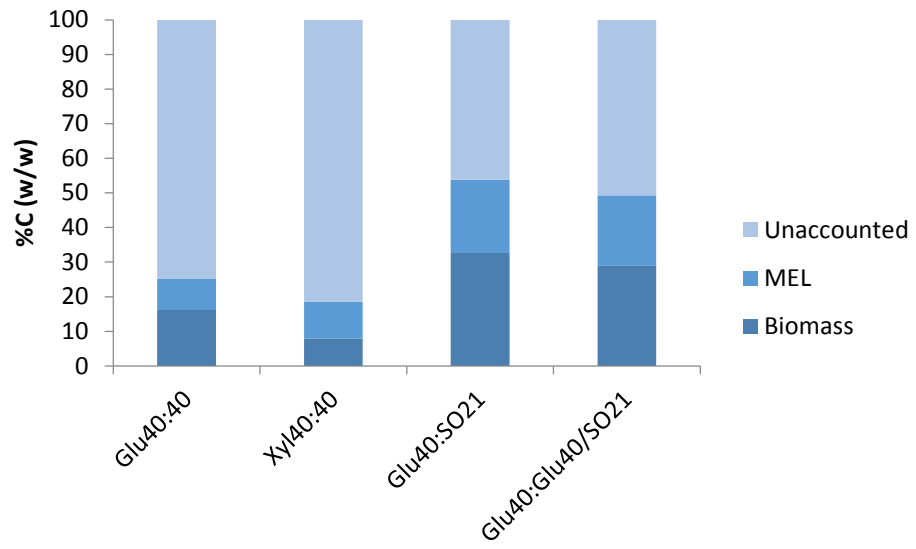

Figure 19 - Weight percentage of carbon distributed by biomass and MEL

4.2 Product characterization

4.2.1 Objectives and strategy

In section 4.1, different growth conditions of *M. antarcticus* were studied in order to improve yield, productivity and MEL titre of condition α. The current section is focused on providing information related with product characterization, which is important to define alternative downstream strategies and new MEL applications. Note that there are several reports [36, 38, 85-88] in the literature about MEL characterization, but as discuss MEL is not a single molecule, but a family of molecules and the number of reports on MEL properties, when produced using sugars as carbon source is relatively scarce.

A first question particularly important concerning the definition of a downstream route is the localization – intracellular, extracellular or cell bound – of the product of interest, MEL. Many studies were performed using soybean oil as substrate and they are consistent in refer that this glycolipid biosurfactant is an extracellular product [89, 90]. This thesis is focused on the use of MEL produced from water-soluble carbon sources and, in these conditions, it was aim of study to understand if MEL is intracellular or extracellular and being extracellular if it is in the supernatant or trapped in the cell wall or cell clusters, as the soybean oil in the fermentation broth may have an effect to help MEL to move on from cell vicinity into the fermentation broth bulk.

A second question, important for definition of applications of this surfactant, relates with the surface tension and critical micellar concentration of sugar produced MEL. The estimation of these properties provides indications of surface-active properties of the surfactant produced and compared with the ones for vegetable oil produced MEL. These properties are crucial to access the potential success of this glycolipid in applications such as the use in laundry detergent formulations [6]. To provide information on the structure of the sugar based MEL obtained and using thin layer

chromatography (TLC), a qualitative analysis was performed, allowing to identify the type of MEL produced (MEL-A, -B and –C) [13]; The indication on the MEL structure obtained by TLC, was then validated by nuclear magnetic resonance (NMR) [13]. Such information and the information on lipidic chains side of MELs allows to estimate the hydrophilic-lipophilic balance, which is used to classify emulsifier compounds, as an indicator of the behaviour that may be expected for the product [64].

The tests were performed for product (MEL or cell-free broth) recovered from cultivation condition α, the standard condition for all sections in this thesis.

4.2.2 Intracellular or extracellular product

MEL has been described as an extracellular product in vegetable oil-based fermentations [37, 90] and gene cluster includes a transporter (MMF 1) to secrete MEL produced. Regarding the difference in fermentation media due to a shift of hydrophobic to hydrophilic carbon source, MEL localization in the system was studied to understand whether MEL produced is intracellular or extracellular-located and if MEL is produced extracellularly, if it can be found in the fermentation broth supernatant solution bulk and/or if remains adsorbed in the cell wall or vicinity.

In the current study, it was established that after a simple centrifugation of the fermentation broth, 66% of total MEL was present in the cell pellet while only 31% was actually in the supernatant, Figure 20, for samples collected after 14 days of fermentation.

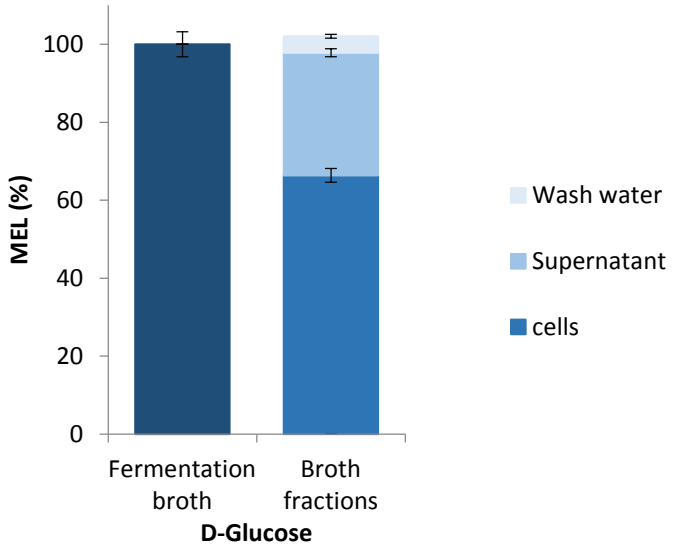

Figure 20 - Total percentage of MEL in a sample of the fermentation broth and how is it divided between cell pellet, supernatant and wash water after broth centrifugation from a fermentation using an initial concentration of 40 g/l of D-glucose and a feed at day 4 of 40 g/l of D-glucose.

Observing Figure 20, it is inconclusive to claim that MEL is an intra or extracellular biosurfactant. So, a further study was performed to gain more insights about MEL cellular localization. Thus, a process of culture broth sonication was followed, a physical method in which high frequency sonic

waves generate intense local shock waves equivalent to thousands of atmosphere pressure, and, depending on conditions used, may cause cell disruption [91].

Usually this technique is used to release intracellular materials because a higher degree of disruption causes increased breakdown of the cells. In this work, sonication use is envisaged for cells breaking, if that is required to retrieve high amounts of MEL, but also when used less intensively as a potential physical means to disassemble MEL from cells when MEL is located extracellularly, but somehow yet bound to the cells. The advantage of maintaining intact cells was to avoid further contamination of MEL with intracellular products, namely fatty acids.

In this work different sonication conditions were tested, from less to more aggressive conditions and the efficiency on MEL mobilization to the solution bulk was quantified in parallel to cell's viability analysis to indicate whether cell disruption took also place.

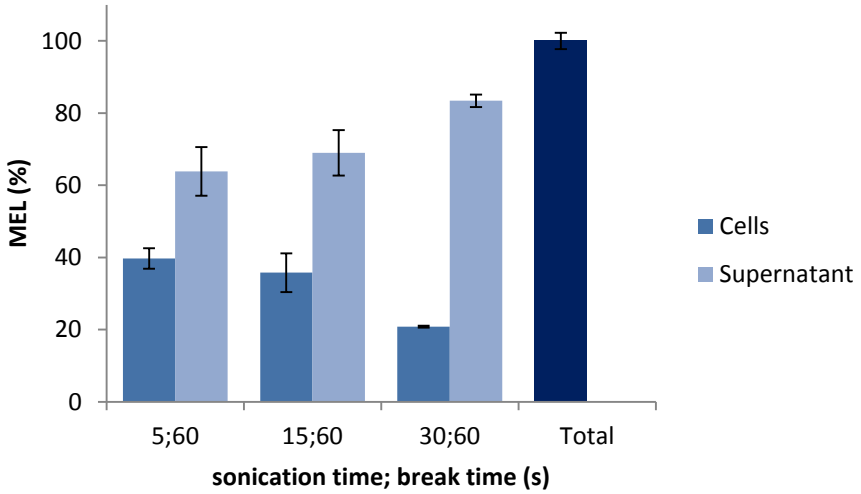

Figure 21 – Percentage of MEL in each phase (cells and supernatant) for the different times of sonication tested.

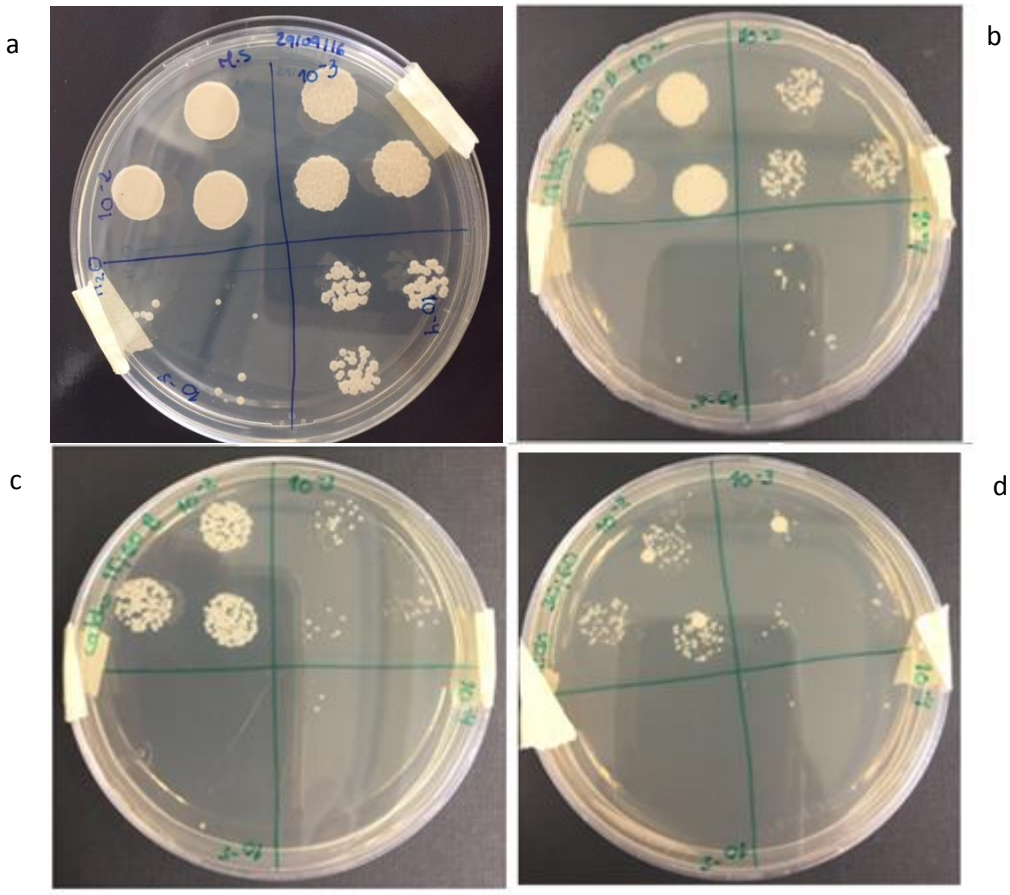

Figure 22 - Different conditions of medium sonication tested, starting with the lowest time of sonication, 3 x 5 s (b), then 3 x 15 s (c) and 3 x 30 s (d) with 60 s intervals in all conditions; (a) is the control sample without sonication.

Within the conditions tested, for the control a viability of $1.37 \times 10^7 CFU's/mL$ was reached and values of $2.55 \times 10^6 CFU/mL$, $2.33 \times 10^6 CFU/mL$ and $1.47 \times 10^5 CFU/mL$ were obtained for respectively 5, 15 and 30 s of sonication, suggesting that for the sonication conditions there are always a large fraction of cells that are break down. Actually, even in the lowest aggressive sonication condition, the percentage of MEL increased in the supernatant from a value of 31% (Figure 20) to a percentage of 64% (Figure 21). As the conditions of sonication become more aggressive, the percentage of MEL in the supernatant increases up to 83.4%. Note that for each order of magnitude cell's viability decreases (Figure 22) but a significant increase of MEL in the supernatant is observed, suggestion that, cell disruption is required for full mobilization of MEL.

4.2.3 Mannosylerythritol lipids characterization

MEL is a biosurfactant and has some properties that allow its use in a wide range of applications. The identification of MEL was confirmed by thin layer chromatography (TLC) and by Nuclear Magnetic Resonance (NMR) spectroscopy and the molecule was characterized based on the critical micelle concentration (CMC) and the hydrophilic-lipophilic balance (HLB).

4.2.3.1 Thin layer chromatography (TLC)

The formation of MEL and its components in a fermentation using D-glucose as carbon source was confirmed by thin layer chromatography (TLC). Figure 23 shows the silica plate where the biosurfactant was eluted.

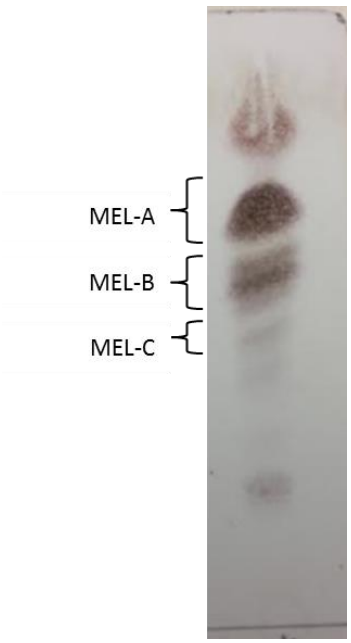

Figure 23 – TLC analysis of culture broth from *M.antarcticus* cultivated in D-glucose, after extraction with ethyl acetate

MEL can be divided in MEL-A, -B and –C according with their elution position in TLC. Based on the TLC obtained, the major component present in a sample of MEL is MEL-A, followed by MEL-B and MEL-C. This information is in accordance with the information cited on the literature, in which MEL-A comprises more than 70% of the total lipids [38]. To identify the bands a small sample of each one was collected and analysed by ^{1}H-NMR.

4.2.3.2 Nuclear Magnetic Resonance (NMR) spectroscopy

After collecting the samples from the chromatography column and verify their position in the TLC plate, they were analysed by nuclear magnetic resonance in order to confirm if the separation of different MELs was successful. The RMN spectra of MEL-A, MEL-B and MEL-C are represented in Figure 24.

Table 6 - Relevant chemical shifts of 1H-NMR data of glycolipids produced by *M. antarcticus* cultured in D-glucose [83].

Functional group	^{1}H-NMR δ (ppm)		
D-Mannose	**MEL-A**	**MEL-B**	**MEL-C**
H-1’	4.71 d	4.69 d	4.77 d
H-2’	5.51 dd	5.49 dd	5.50 dd
H-3’	5.06 dd	4.91 dd	5.09 dd
H-4’	5.24 t	3.82 m*	5.16 dd
H-5’	3.71 m*	3.56 m	3.53 m
H-6’	4.23 m	4.44 m	3.70 m*
***Meso*-Erythritol**			
H-1	3.75 m*	3.76 m*	3.74 m*
H-2	3.68 m*	3.68 m*	3.65 m*
H-3	3.74 m*	3.73 m*	3.79 m*
H-4a	3.86 dd*	3.87 dd*	3.80 m*
H-4b	3.99 dd	3.99 dd	4.03 dd
Acetyl Group			
-CH_3	2.03, 2.10 (s)	2.13 s	2.05 s
Acyl Group			
-CO-CH_2	2.22 m	2.28 m	2.21 m

s-singlet; d-doublet; dd-double doublet; t-triplet; m-multiplet
*Not distinguishable in this scale of 1D ^{1}H-NMR

Figure 24 shows the NMR spectra for MEL-A, MEL-B and MEL-C obtained after analyse some fractions collected from the silica gel column chromatography. MEL-A is diacetylated which is possible to verify with the presence of the two peaks corresponding to the functional group of –CH_3. For MEL-B and MEL-C it is possible to distinguish the differences between the functional groups H-6’ of mannose and the H-4’ of mannose, respectively. This leads to the fact that MEL-B is monoacetylated at C6 and Mel-C is monoacetylated at C4 [13].

MEL-B and MEL-C are monoacetylated however, and as it can be seen in the figure above, the spectrum of MEL-C has two peaks that correspond to the acetyl group which might be due to a contamination in the sample.

After an analysis of the spectrum of total MEL, it was verified that MEL-A represents 68%, MEL-B 13% and MEL-C 15% of the total lipids.

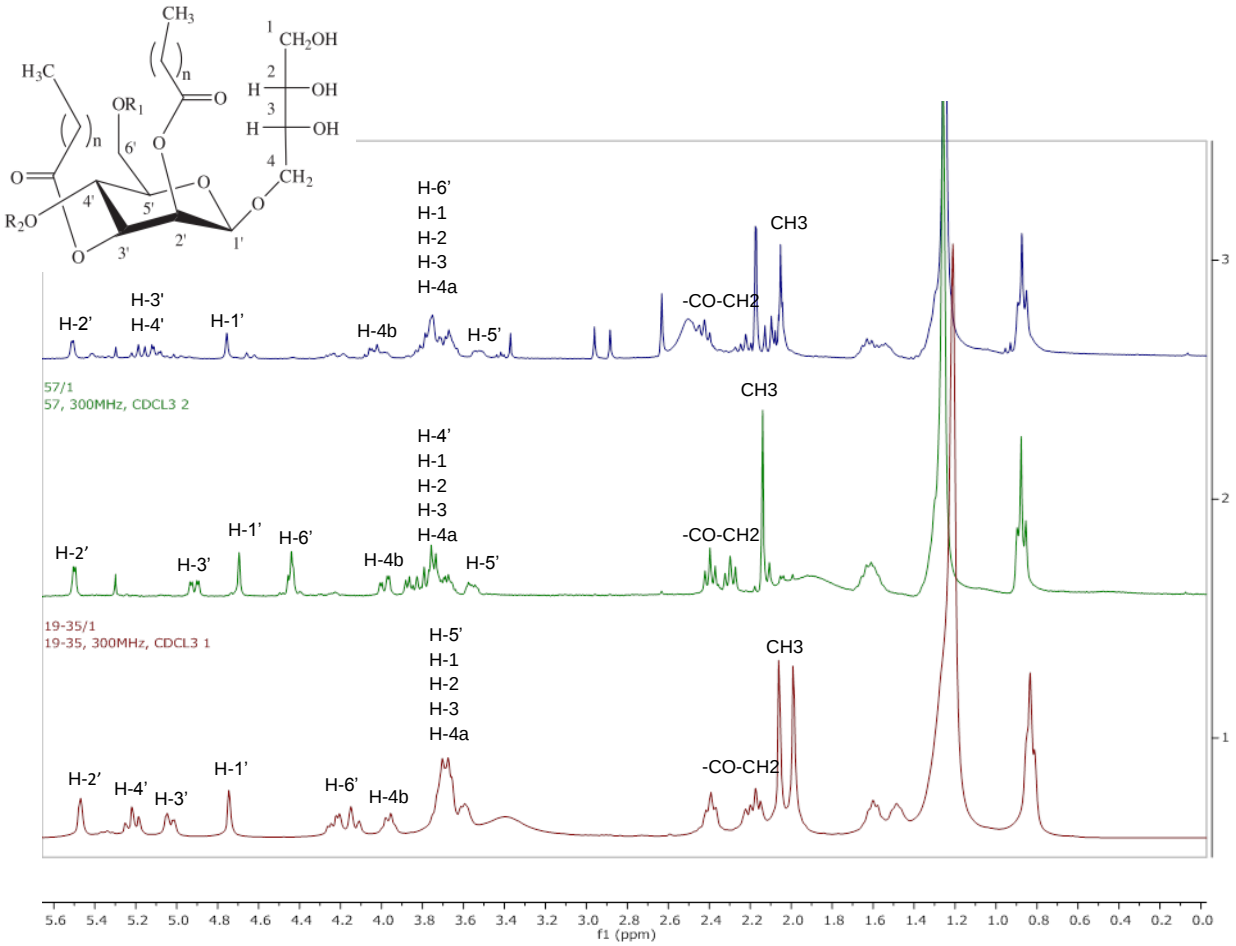

Figure 24 - 300 1H-NMR spectra of MEL-A (red), MEL-B (green) and MEL-C (blue) extracted from D-glucose cultures of *M. antarcticus* PYCC 5048^T. The molecule of MEL is represented on the upper left corner; for MEL-A: R1=R2=Ac; for MEL-B: R1=Ac, R2=H; MEL-C: R1=H, R2=Ac.

4.2.3.3 Critical Micelle Concentration (CMC)

The ability to lower surface tensions, the correspondent critical micelle concentration (CMC) and the formation of stable emulsions are part of the surface-active properties that characterize biosurfactants.

The CMC is obtained when the biosurfactant concentration is so high that biosurfactant molecules start to organize themselves in self-assembling structures like micelles [8]. As these structures balance out hydrophobic and hydrophilic contributions of the surfactant, the surface tension does not reduce further above the CMC, in many processes the CMC specifies the limiting concentration for meaningful use.

The next figure shows the evolution of the surface tension as long as the concentration of MEL increases until the critical micelle concentration is found.

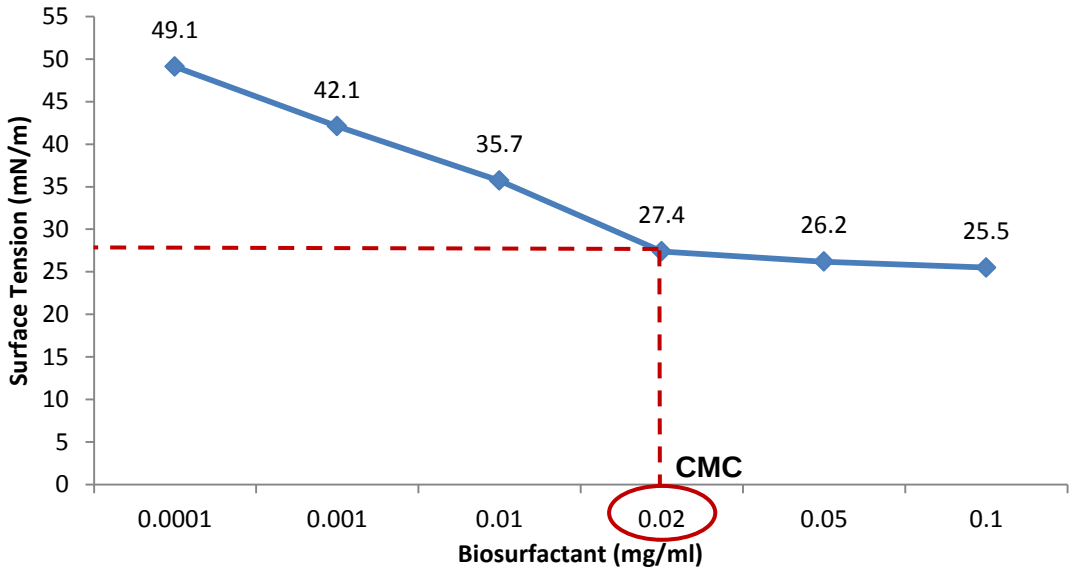

Figure 25 - Surface tension of aqueous solutions of mannosylerythritol lipids (mN/m) as function of concentration (mg/ml) and the correspondent critical micelle concentration. The blue line that joins the values only pretends to facilitate the visualization of the data.

Increasing MEL concentration decreases the surface tension of water until a minimum value of 27.4 mN/m being constant. This value corresponds to a concentration of 0.02 mg/ml, the critical micelle concentration., i.e., this is the minimum concentration of biosurfactant required to give maximum surface tension reduction of water and initiate micelle formation. These values are in accordance with the ones mentioned in the literature since it is known that this biosurfactant can reduce the surface tension of water from 72 mN/m [88] to less than 30 mN/m [6].

The CMC is also a criteria to analyse the efficiency of a biosurfactant because a low value of CMC means that less surfactant is required to decrease surface tension [92].

4.2.3.4 Hydrophilic-lipophilic balance (HLB)

The hydrophilic-lipophilic balance is an important property to characterize the biosurfactants because it allows its distinguish according to the different applications [63].

To calculate the hydrophilic-lipophilic balance it was used the Griffin's method [62], using Equation 1 (HLB=E/5), in which E represents the weight percentage of the hydrophilic content of the molecule. So, E was calculating using the following expression:

$$E = \frac{molecular\ weight\ of\ the\ sugar\ moiety}{total\ molecular\ weight} \times 100$$ **Equation 4**

The molecular weight of the sugar moiety (mannose and erythritol) of MEL and the total molecular weight of MEL were obtained using the information provided on H-RMN spectra for condition α. A total

HLB of 8.4 was reached and, for each type of MEL, and considering a chain length of 10.5, the HLB of MEL-A is 8.2, the HLB of MEL-B and MEL-C is 8.7 and the HLB of MEL-D is 9.3. These values confirm the potential of MEL as an oil in water emulsifier or dispersion agent [63] and they are in accordance with the one reported in literature of 8.8 [88].

Emulsifiers are used in creams and lotions with the role of forming a homogenous mixture, keeping water and oil together; oil in water emulsifiers are especially used in moisturizing products, such as body lotions and day creams [93]. Higher values of HLB, around 16 are traduced in a solubilizer surfactant [62].

4.2.4 Cell-free broth characterization

As reported in the previous section, MEL is highly concentrated in the cells pellet after simple centrifugation. Nevertheless, considering the low CMC of MEL, the concentration of MEL in the supernatant, even with only 30% of the total MEL produced, is well above its CMC. For example for a MEL final titre of 5-6 g/l, the 1.5-2 g/l of MEL dissolved extracellularly in the fermentation broth will be 100 times above the 0.02 g/l CMC previously estimated. Thus, direct use of the supernatant obtained after removal of cells by centrifugation can have interest as product that does not need further purification.

Therefore, some properties, such as the surface tension and the contact angle of the supernatant obtained after centrifugation were tested as described below. Also, the supernatant was analysed to quantify protein, sugars, MEL and fatty acids. A total of 5.51±0.9 mg/ml of protein was determined. The high amount of extracellular protein determined is important to take into consideration when the use of this supernatant as a product is equated. Extracts from *M. antarcticus* cultures have been reported within our group to present xylanase and lipase activity [38, 94].

Regarding the presence of carbohydrate sugars, the condition α presents no sugar content at the end of the cultivation (Figure 11a). The values of fatty acids in the supernatant are low with concentrations of 0.105 ± 0.01 g/l which correspond to values of carbon chains of only C14 with C16 and C18 not being detected. If the goal is to purify MEL from a supernatant fraction, low levels of free fatty acids are beneficial for MEL production.

Finally, the concentration of MEL in the samples of supernatant analysed was determined and a value of 1.69 ± 0.09 g/l was obtained, proving once more that the supernatant has also biosurfactant present and it can be used for many applications.

These tests were performed using the supernatants β, β-25, β-50 and β-75. Where β stands for the supernatant obtained by centrifugation of the fermentation broth operating in conditions α (40 g/l D-glucose and $NaNO_3$ addition at the beginning of the fermentation, followed by a 40 g/l addition at day 4 of the fermentation). β-25, β-50 and β-75 stands for the amount of water removed from the supernatant β, 25%, 50% and 75%, respectively.

4.2.4.1 Surface tension

The surface tension is one of the most important characteristics that distinguish biosurfactants from other compounds. Once the supernatant also has a percentage of MEL, it was aim of investigation to the surface activity abilities

Table 7 - Surface tension (mN/m) for samples of supernatant (β, β-25, β-50 and β-75)

Sample		Surface Tension (mN/m)
Supernatant β		24.5±0.10
Concentrated supernatant	β-25%	24.9±0.07
	β-50%	25.4±0.42
	β-75%	25.9

Analysing the values obtained, the surface tension of the supernatant is similar to the one measured for MEL at critical micelle concentration (section 4.2.3.3). Also different concentrations of supernatant showed a similar behaviour.

These results mean that also the cell-free broth can be used in applications where the ability of biosurfactants to reduce the surface tension of water is needed, such as in formulations for detergents.

4.2.4.2 Contact angle

The contact angle is a property based on the wetting ability of biosurfactants and, in this work, it was evaluated for different samples of supernatant using water as control. The contact angle was measured for the different droplets against a hydrophobic surface, Parafilm M. Mili-Q water was used as control and the results obtained are the ones showed in Figure 26.

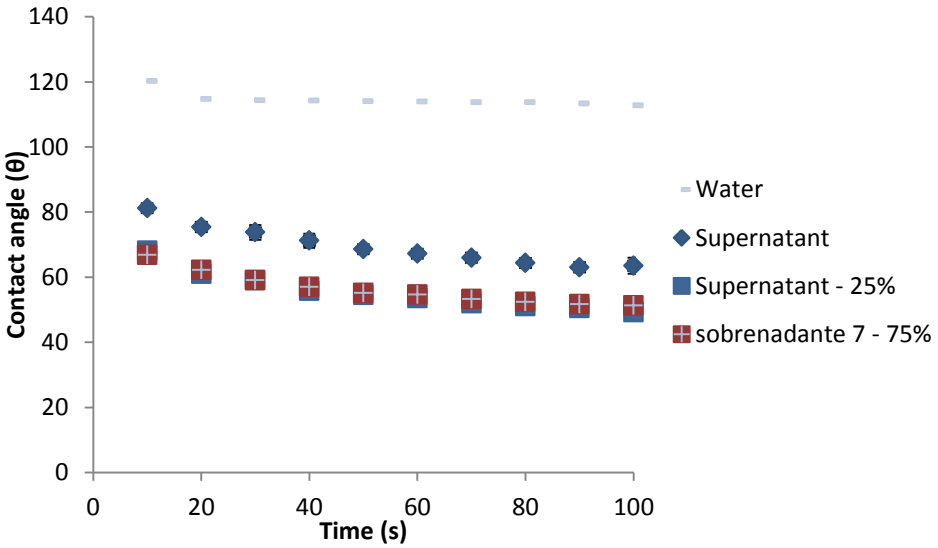

Figure 26 - Temporal dynamics of contact angle of each surfactant droplet placed on Parafilm M.

It is known that the angle between the surface-liquid interface and air-liquid interface of a liquid droplet varies inversely with the strength of the liquid attraction to the solid surface. Therefore, when an aqueous solution containing the biosurfactant is used for this test, lower values of the contact angle reflect an improvement in the extensibility of the biosurfactant solution on the surface [95].

Comparing the results obtained for the samples of supernatant and for the water, in the first case the angle decreased after dropping onto the hydrophobic surface used (Parafilm M), whereas that of the water droplet showed only a slight chance. This means that the supernatant exhibit higher spreading properties onto a hydrophobic surface compared to water.

Additionally, comparing the different samples of supernatant, when the percentage of water decreases the contact angle also decreases, confirming what was written above. However, different concentrations of the supernatant, after removing 25% or 75% of the water, did not show differences on the values of the contact angle over time, reaching a final value of approximately 50º in contrast to one obtained for the supernatant without water removal, which was 63.5º.

In this case, only samples of supernatant were measured however this property could be also evaluated for solutions of MEL as reported in [95]. MEL-A, -B and –C was analysed and results of about 11º were reached which means that the spreading properties are even stronger than in the supernatant.

4.3 Downstream processes

4.3.1 Objectives and strategy

Usually it is stated that 60-80% of the manufacturing costs of biotechnological products are allocated to downstream processing which is often due to the small titres of the target product and the fact that some applications, such as a pharmaceutical product, require a high level of purity [35]. The increasing know-how in these products and all the different work developed in this area, has been a key in the selection of the best recovery operations to try to minimize the cost in these isolation and purification steps [96].

There are many studies with different technologies about how to recover biosurfactants. However, and in particular for MEL, this investigation is still in development because, for example in cosmetic and pharmaceutical applications a high degree of purity is required without decreasing the amount of biosurfactant recovered, or in other words, the compromise between a high purity and a high yield, and also a method that may be used at industrial scale, still need to be achieved. The strategies for MEL separation are related with yeast cultivations using vegetable oils as carbon source, what translated into high final MEL concentrations of product, but the presence of residual vegetable oils make MEL extraction and purification more difficult, a problem that is circumvent when D-glucose (or other carbohydrates) are used as carbon source. However, as seen in section 4.2.2 more than 60% of MEL remains associated to the cell pellet after centrifugation, still MEL is reported as an extracellular

product [37]. Generally, the downstream process is facilitated when a given bio-based product is extracellular and is easily separated from cells. Here, strategies were tested to separate MEL from the yeast cells in order to:

i) develop a strategy to mobilize MEL from cells into a liquid fraction and consequent MEL isolation;

ii) to export this strategy to mobilize MEL from cells directly in cultivation broth, enriching MEL concentration in separating layer.

Methods tested included organic solvent extractions and chemical and physical methods. Several strategies were assessed and they are summarized in the diagram below (Figure 27).

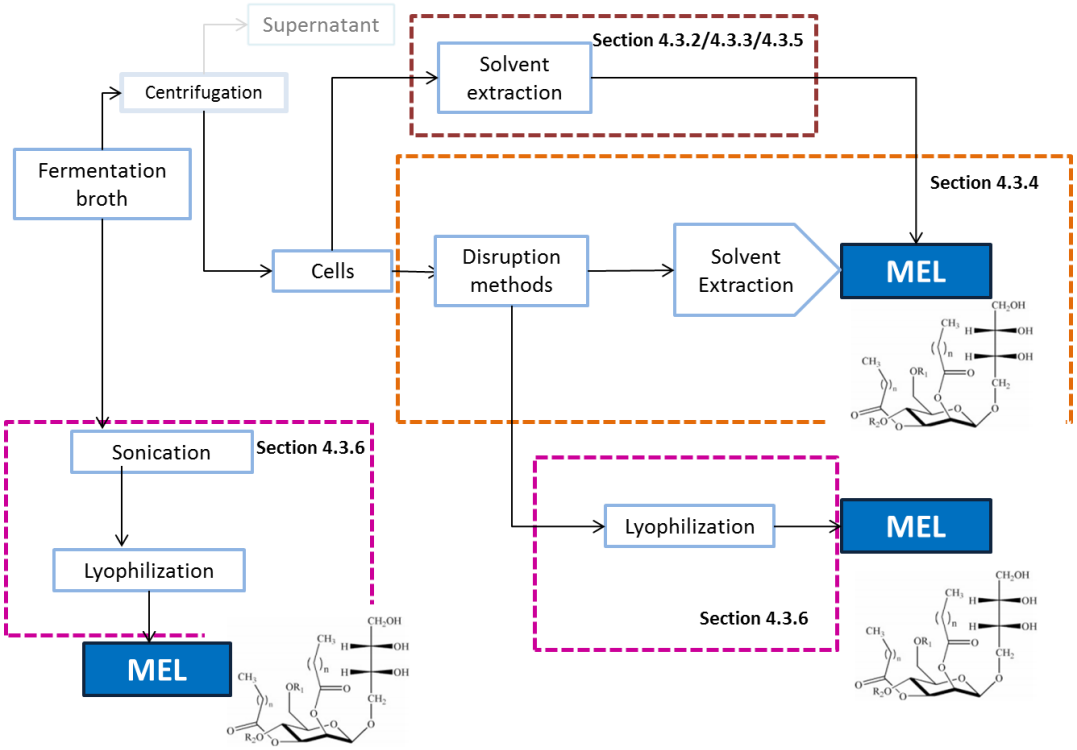

Figure 27 - Diagram representing the strategies of downstream followed, divided by sections.

4.3.2 MEL extraction with different solvents

One of the most common methods used to recover biosurfactants is its extraction with organic solvents. Despite its efficiency, the use of solvents translated into high costs related with their extensive use, their environmental performance, toxicity to general environment but also for cellular viability, and, in some processes, the inexistent solvent reutilization. Note that compatibility of maintenance of cell viability with product extractant phase can be important when process intensification combining bioconversion and product isolation is envisaged and cell integrity (viable or dead) to avoid contamination of an extracellular product with intracellular products.

Different solvents were tested in order to understand their effect in MEL extraction (Figure 28).

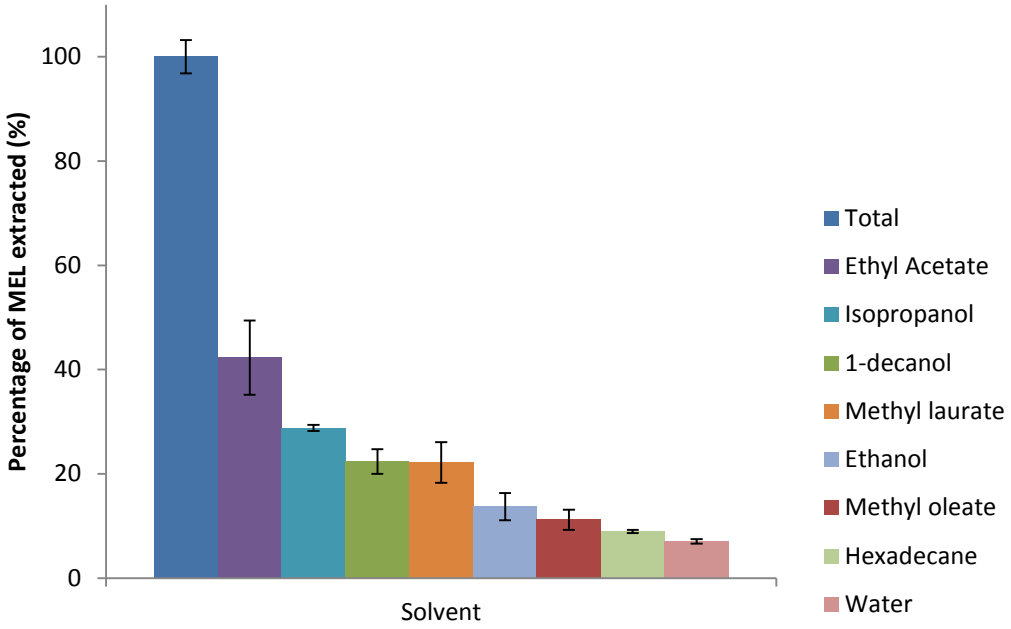

Figure 28 - Percentage of MEL extracted by an aqueous solution of different solvents, 5 mL of each solvent and around 5 mL of aqueous phase. The solutions are all biphasic except with ethanol and isopropanol that are miscible in water.

As it can be seen in the figure above most of the systems of water and solvent used (1:1) with cells showed low capacity to extract MEL; however, ethyl acetate and isopropanol were the best ones, reaching higher percentages of removal, 42.27% and 28.80%, respectively, of the total MEL determined in cell pellet fractions. All the systems used are biphasic except the ethanol and the isopropanol that does mixture.

Ethyl acetate, besides presenting the best results in this study, is also the most common solvent used in extraction due to it favourable characteristics such as its light polarity, immiscibility with water, and it is less toxic than chlorinated solvents.

Although the well-known effectiveness of ethyl acetate in biosurfactants extraction, it was studied, in parallel, the viability of cells after a solvent extraction procedure.

This ability was analysed by inoculating diluted cell's samples and counting colony forming units (CFU) and the obtained results are represented in Figure 29.

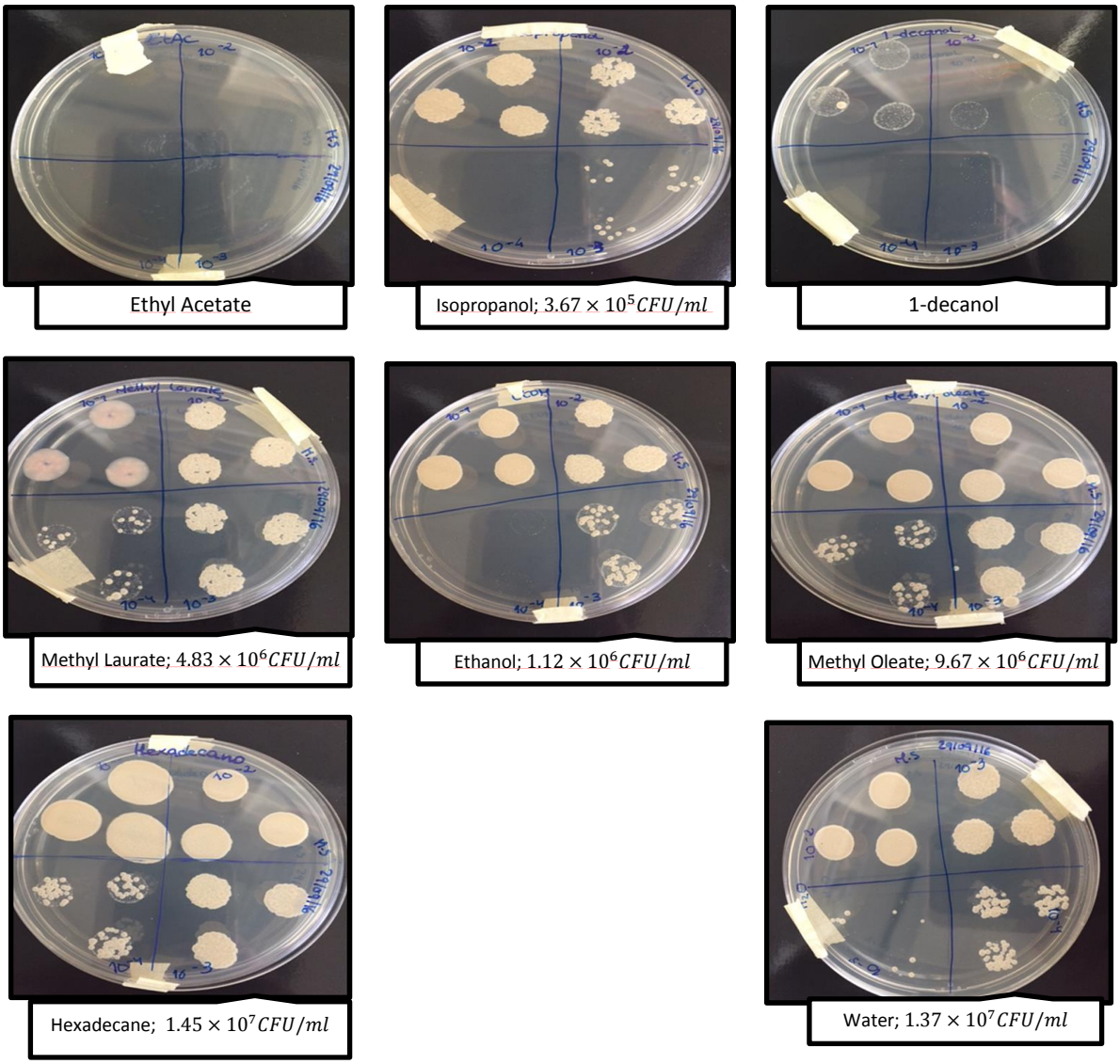

Figure 29 - Different solvents tested. Three drops of 20 µL each were plated on agar medium in order to evaluate the effect of the solvent in the cells growth; colony-forming unit (CFU/ml) based on the average of colonies present in each drop.

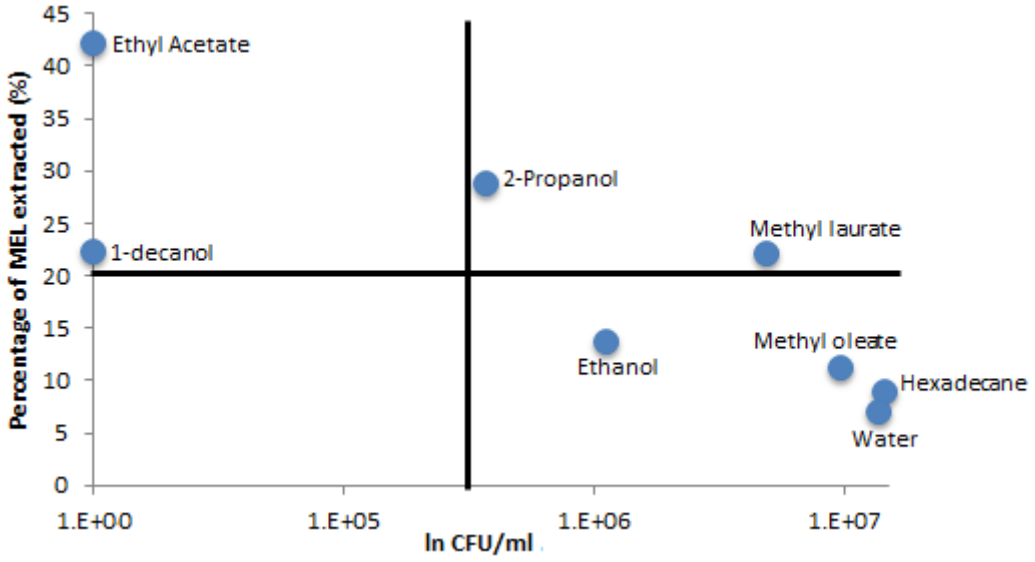

Figure 30 - Comparison between the cells' resistance to the solvents and MEL extraction. The upper left quadrant concerns to solvents that are capable of extracting MEL but affects cells viability; the upper right quadrant concerns to solvents that are capable of extracting MEL without break cells; the lower left quadrant concerns to solvents that affect cells viability but do not extract MEL and the lower right quadrant concerns to solvents that do not affect cells viability and do not extract MEL.

Figure 30 shows the performance of the different solvents, concerning to the ability to not affect cell viability and to extract the biosurfactant.

Based on the solvents tested and on the results showed, in a 2-D goal of extract MEL with less cell viability affectation, isopropanol showed the best results because (after the ethyl acetate) is the one that is capable of removing MEL (despite the removal percentage being lower than ethyl acetate) and does not break the cells which are capable to grow in a plate with agar medium. Comparing the two solvents, the ethyl acetate has the advantage of behaving like a biphasic system and that means that all the debris go directly to the aqueous phase which does not occur when isopropanol is used. That fact can represent a disadvantage to use isopropanol because it requires an additional step to remove the debris and cannot be considered, for instance, for a byphasic cultivation directly with use of solvent for in situ product recovery. In this regard, methyl laurate showed to be capable of extracting MEL without breaking cells however with a low percentage of biosurfactant removal when compared with the other two, representing a total percentage of 22, but generating a two-phase system.

4.3.3 MEL extraction with Ethyl Acetate

First of all, different concentrations of ethyl acetate were tested in order to understand its influence in the extraction of MEL. Bearing in mind that samples of 10 mL were taken, also the extract solutions used had the same final volume in order to perform a ratio of 1:1. The results obtained are summarized in Figure 31. Note that the third bar of the figure below represents an equal condition for the result present in Figure 28 with ethyl acetate.

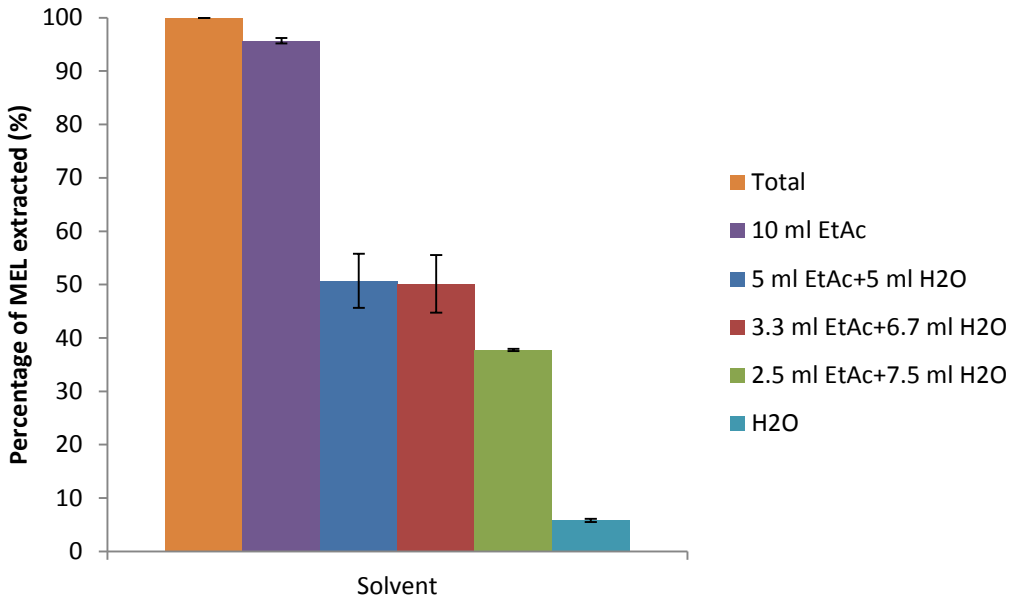

Figure 31 - Percentage of MEL extracted as function of the different concentrations of ethyl acetate used.

The results obtained showed that when the concentration of ethyl acetate is lower, the extraction of MEL decreases drastically from around 95% to around 50%, which means that the extraction is not as efficient as when higher quantities of solvent are used. However, it is important to keep these results in mind and the use of less solvent will be more explored in order to try to achieve a more sustainable process.

Many references use ethyl acetate to extract MEL using equal amounts of solvent and fermentation broth because of its efficiency, as proved in this assay, and more recently as a substitute for tert-butyl methyl ether since this solvent is more toxic [4, 78, 85].

There is not a statistically significant difference between using 5 ml or 3 ml of ethyl acetate as determined by one-way ANOVA with a *p*-value>0.05. This means that this reduction of solvent does not decrease the percentage of MEL extracted and with less amount of ethyl acetate similar results can be achieved.

4.3.4 Cells disruption followed by extraction with ethyl acetate

As stated above MEL is extracellular however it is mostly concentrated in the cell's phase. So, being a goal of this work reduce the amount of organic solvents used, different disruption methods were performed in order to understand if they are able to destabilize the cells and consequently improve the percentage of MEL extracted with lower quantities of ethyl acetate.

Three different methods were tested including the use of an autoclave to evaluate the cells' resistance to high temperatures, the sonication that may facilitate the transfer from the solid to the

liquid phase (in this case the cells were re-suspended in Mili-Q water) and, at least the use of glass beads also with the same purpose.

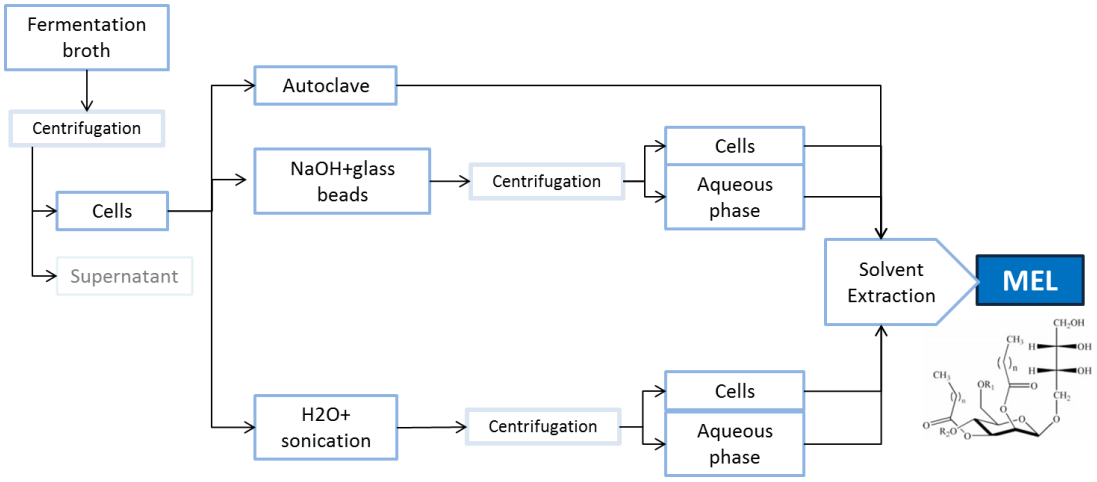

Figure 32 - Diagram representing the strategies of downstream including cells disruption followed by solvent extraction.

The results obtained in each case are represented in Figure 33.

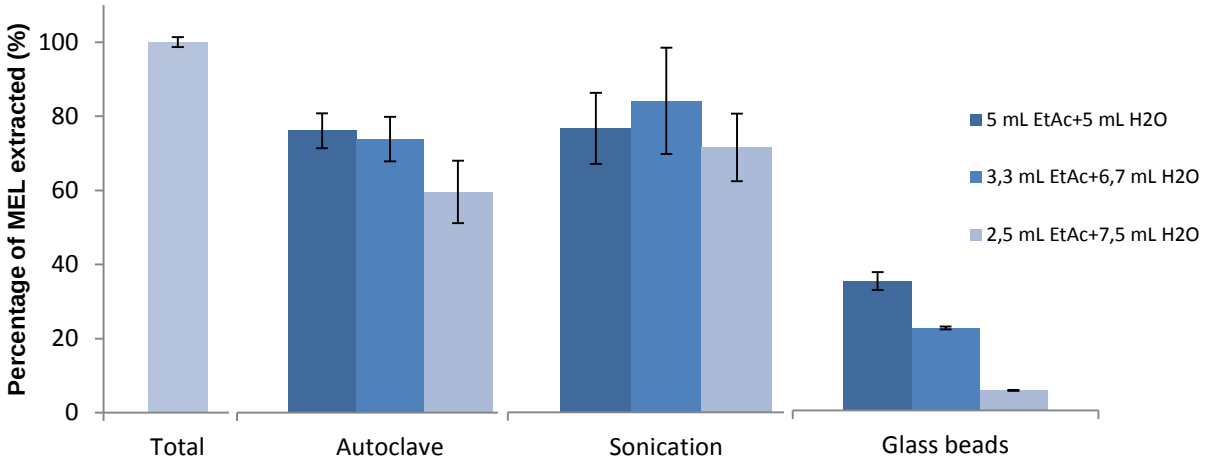

Figure 33 - Percentage of MEL extracted as function of the different concentrations of ethyl acetate used after cell's disruption by autoclave, sonication and the action of glass beads.

Comparing the results obtained for the three different methods of disruption, the use of the autoclave or the ultra sounds increased the removal percentage of MEL; however, the use of glass beads showed not to be efficient. Possibly the mechanical action that the glass beads are exerting on the cells are not strong enough to destabilize the medium and it does not promote such an efficient mobilization of MEL to the aqueous phase. On the other hand and assuming that the glass beads action was efficient, the lower percentage of MEL removed using this method can due to the fact that ethyl acetate is not the adequate solvent to disadsorb MEL from the glass beads.

So, it is possible to verify that two steps of downstream, including cells disruption followed by solvent extraction, perform better results than just one step. In accordance with the tests performed by

ANOVA, there is no statistically difference (*p*-value>0.05) between using 10 ml of ethyl acetate without any disruption technique before the solvent extraction and using just 3.3 ml ethyl acetate after disrupt the cells with the autoclave or the sonicater which means that using these mechanical methods, the amount of solvent can be decreased

The values showed in Figure 33 concern to a total volume of MEL however before sonication the cells were re-suspended in water or in NaOH (1M) before the addition of the glass beads. The aqueous and cell's phases were then separated by centrifugation and both of them were extracted with ethyl acetate.

In Figure 34 is represented the distribution of the total value of MEL by each phase; this kind of result allows understanding if the method was capable of disturb the cells and consequently transferring MEL to the aqueous phase, facilitating product's removal.

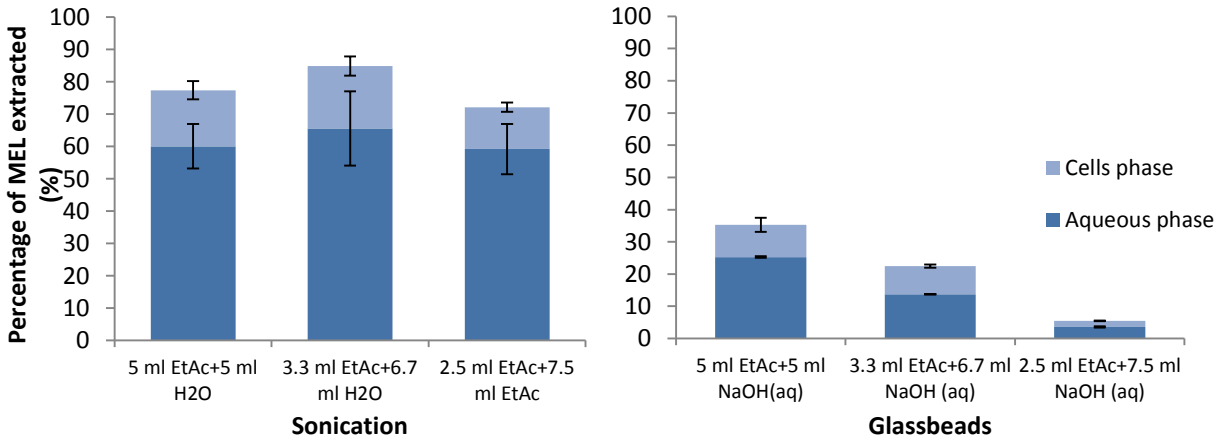

Figure 34 - Percentage of MEL extracted in each phase as function of the different concentrations of ethyl acetate used after cells' disruption by Sonication or using glass beads.

The results reveal that using both methods the percentage of MEL in the aqueous phase is higher than in the cells phase. In the case of the glass beads and, despite the fact that the majority of MEL was transferred to the aqueous phase, this is the method of the three ones with worst results, being the one with lower percentages of MEL extraction, even with the higher concentration of solvent used; after the autoclave or the ultra sounds values of around 80% were reached whereas using the glass beads only percentages of around 35% were obtained.

According with literature, the use of glass beads is a mechanical method with some disadvantages since it is an uneven process, without total ability to disrupt cells and, besides this, separate the beads from the product of interest can be a complex step [91, 97].

4.3.5 MEL extraction with alcoholic solvents

As described in section 4.3.2, isopropanol showed interesting characteristics in MEL removal without breaking all the yeast cells. So, in order to understand the efficiency of this solvent, different assays were performed as described in the following diagram Figure 35.

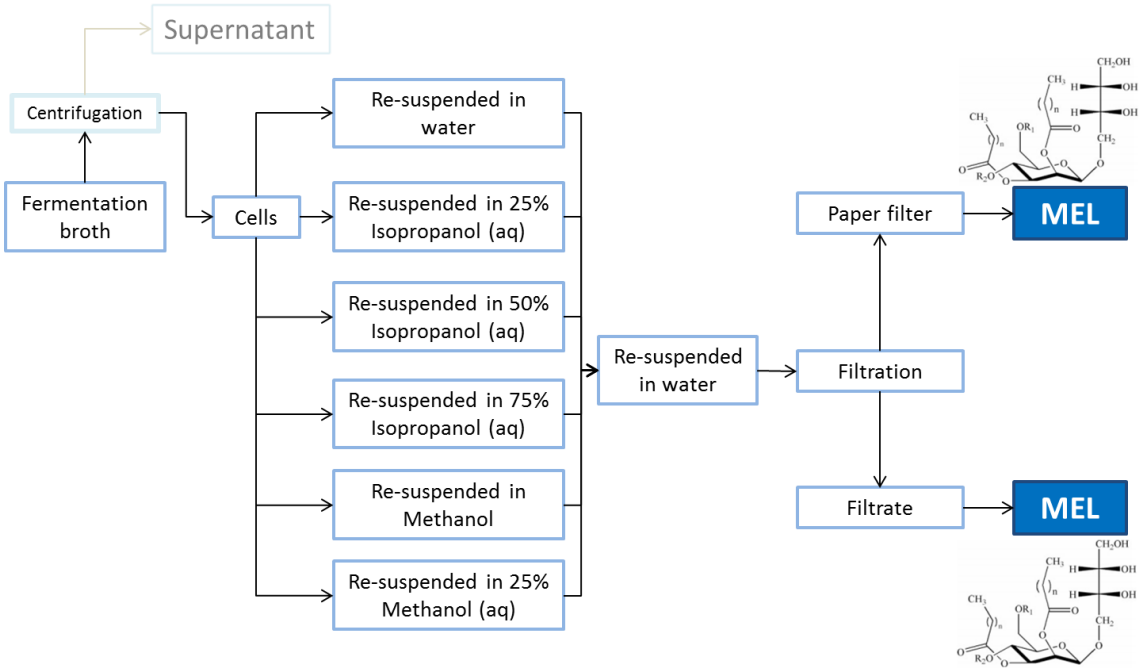

Figure 35 - Diagram representing the strategies of downstream using monophasic systems, alcohols.

Usually it is used biphasic solutions, where the solvent is not miscible with water, such as the ethyl acetate in order to separate the debris, which stays in water, from the solvent phase with the product of interest. In this case, it was used alcohols solutions which mean that just one phase was formed. So, in order to separate the debris from the product a different strategy, and not centrifugation, was used and then the samples were filtrated. The filtrate and paper filter phases were analysed and Figure 36 sums up the results obtained after separation of the two fractions.

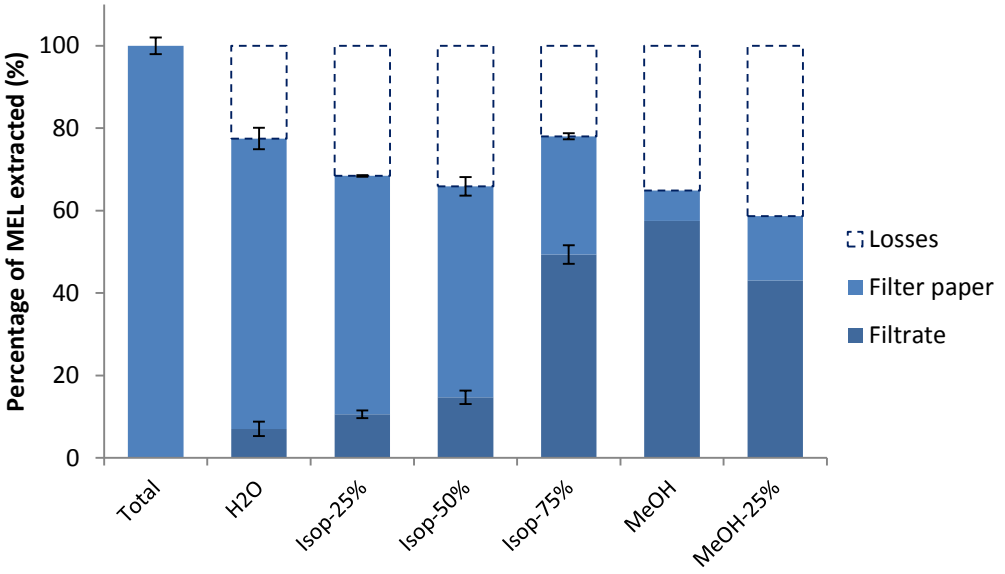

Figure 36 - Percentage of MEL extracted after filtration of different solutions of isopropanol or methanol; the bars are divided into filtrate and paper filter; water was used as control and the total bar corresponds to the total value of MEL obtained in the fermentation.

Based on the results described in Figure 36 is understandable that only with high concentrations of isopropanol, is possible to extract a significant percentage of biosurfactant because when solutions of 25 and 50% of solvent are used, the majority of MEL remains in the cells that do not pass through the filter paper, being retained.

This process also has the disadvantage of having high percentages of losses due to higher number of steps involved and especially in the last unit operation, i.e. filtration, because it is difficult to remove all the retentate that remains in the filter paper.

It is already known that ethanol is not capable of removing MEL (Figure 28) but, besides the use of isopropanol, another alcohol was tested, methanol.

The results obtained showed that methanol can also be used to extract MEL however and, once more, using filtration cannot be the best unit operation because of the significant percentage of losses.

4.3.6 Cells and fermentation broth disruption by sonication

Since the sonication was one of the best methods tested to mobilize MEL to supernatant fraction, the interest in this method increased. Therefore, different samples of the fermentation broth, as well as the cells fraction resulting from a centrifugation and re-suspended in 3 ml of water were submitted to different sonication times. In the end of the sonication the solution was centrifuged and each phase lyophilized for further quantification of MEL (no solvent extraction was used as part of the MEL recovery system). The results obtained are described in Figure 37 and Figure 38.

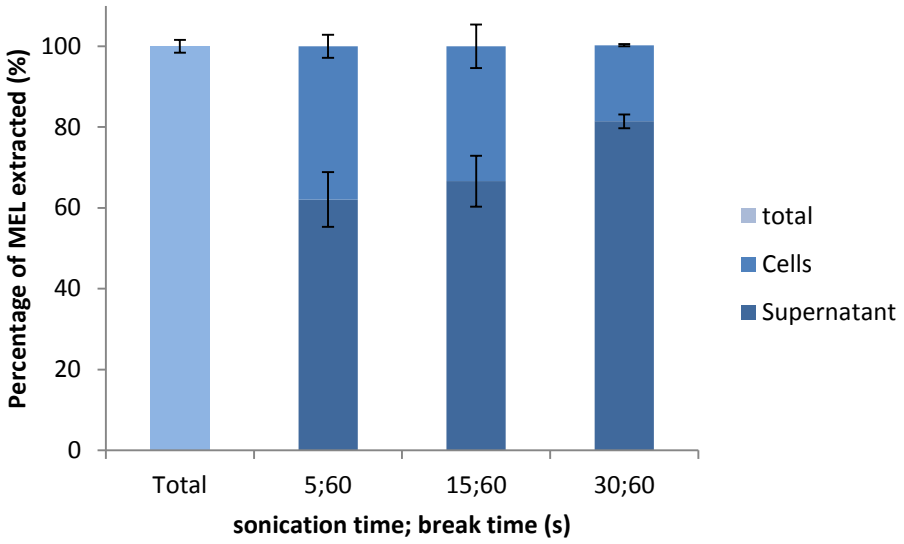

Figure 37 - Percentage of MEL extracted as function of the different times of sonication (30s, 15s, 5s). The fermentation broth was sonicated in 3 cycles always with breaks of 60s. The percentage of MEL was calculated after separation of the supernatant from the cells and lyophilisation.

First of all, and as written before, samples of fermentation broth were sonicated in three cycles of different duration, from 5 seconds to 30 seconds with time intervals between each cycle of 60 seconds. The results showed, once more, the efficiency of the method especially in the most aggressive conditions in which around 80% of product obtained was recovered in the supernatant.

Comparing the results obtained with 15 seconds or 5 seconds of sonication, these were also efficient, reaching values of 66.6 and 62.06% in the supernatant fraction.

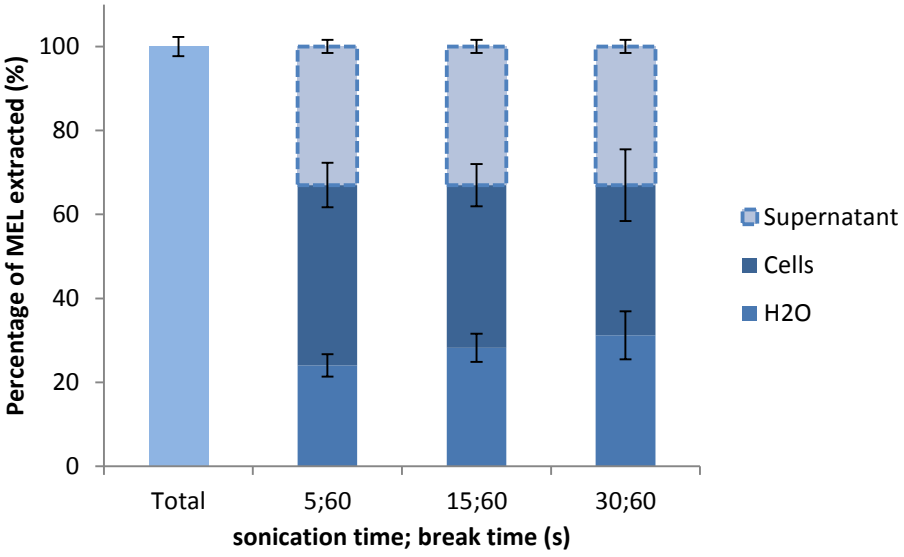

Figure 38 - Percentage of MEL extracted as function of the different times of sonication (30s, 15s, 5s). The cells re-suspended in water were sonicated in 3 cycles always with breaks of 60s. The percentage of MEL was calculated after separation of the cells from the aqueous phase, by centrifugation, and lyophilisation. The fraction of supernatant is represented with a dashed line because it wasn't sonicated and it's always showed in order to verify that the balance can be closed.

Using re-suspended cells after broth centrifugation to sonicate, the differences between sonication cycles were not so notorious and although some MEL was transferred to the aqueous phase, the amount of biosurfactant that remained in the cells fraction was meaningful.

Comparing the results showed in Figure 37 and in Figure 38, sonicate all the fermentation broth during 3 cycles of 30 seconds each reveals to be the most efficient case (around 80% of MEL was recovered), reaching values near the ones obtained with extraction only with ethyl acetate (around 95% -Figure 31).

4.3.7 MEL binding to polymeric XAD resins

Considering the information available in the literature [78], the use of polymeric Amberlite XAD resins for adsorption of MEL was assessed.

Three different resins were used, including XAD-4, XAD-7 and XAD-16. XAD-4 is used to remove small hydrophobic compounds especially in the pharmaceutical sector; XAD-7 is used to recover insulin, metal ions, dry waste and antibiotics and XAD-16 to remove detergents from protein solutions [98].

After stirring the fermentation broth with the different resins for 24h, the broth was filtrated under vacuum and after that, the resins were washed with Mili-Q water, ethyl acetate and methanol.

After evaporate the solvents and lyophilized, the samples were analysed through GC-FID in order to quantify the biosurfactant.

In none of the phases analysed, the results obtained show the presence of either MEL or fatty acids with just trace amounts of MEL in the filtrate (the liquid fraction obtained after filtration) and in the methanol phase and probably, most of the product has remained in the resins and couldn't be eluted by the solvents used.

Comparing these results to the ones mentioned in the literature [78] and, taking in account all the differences between the fermentation conditions, they also couldn't reach a conclusive result since a specific adsorption was not possible by neither of the resins.

4.4 Applications

4.4.1 Objectives and strategy

In sections 4.1, 4.2 and 4.3 it was described the fermentation and downstream processes to obtain the product of interest, mannosylerythritol lipids. These steps are of paramount importance however all of this work aims to obtain the biosurfactant so it can be used in different applications, some of them requiring high levels of purity, such as the pharmaceutical field, and others are not so demanding in terms of purity. In the first case, the pharmaceutical industry, cosmetics and detergents require a high purity grade whereas there are some fields of application with lower purity requirements such as environmental remediation, agriculture, and microbial-enhanced oil recovery

Biosurfactants, due to their characteristics, have the potential to be widely used for various industrial applications, some of them mentioned above. The ability of emulsify or disperse, solubilize, their biodegradability and low CMC make these compounds many attractive in a wide range of areas[34].

In this thesis, the use of MEL in the formulation of a detergent was tested, including different concentrations of biosurfactant and its comparison with a commercial detergent in terms of removal percentage of dirt.

4.4.2 Application of Mannosylerythritol Lipids in the formulation of a detergent

A main application of biosurfactants is its addition in the formulation of a detergent. For this work, small pieces of cotton cloth were stained with chocolate and soybean oil and then washed following the method described on 3.4.1. The percentage of oil or chocolate removed from each cotton cloth was calculated in accordance with Equation 5:

$$Oil\ or\ choc\ removal\ (\%) = \frac{weight\ after\ overnight\ washing(g) - weight\ after\ sample\ dirting(g)}{weight\ after\ overnight\ washing(g)} \times 100 \qquad \textbf{Equation 5}$$

Firstly a set of formulation was performed including sodium tripolyphosphate and sodium sulphate as described in section 3.4.1 and the results obtained are present in Figure 39. Sodium tripolyphosphate was used to reduce the hardness of tap water and sodium sulphate was used as filler.

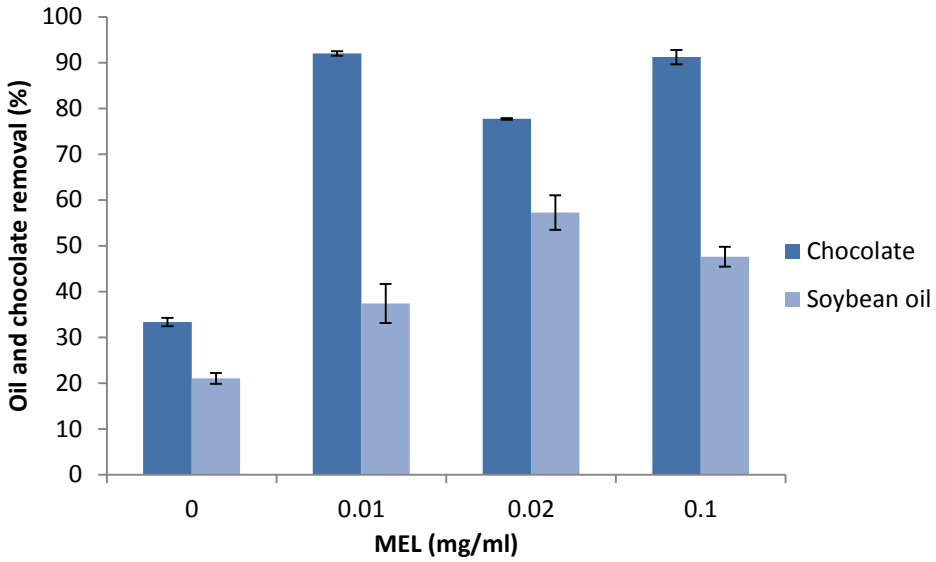

Figure 39 - Weight percentage of oil and chocolate removed in accordance with the different concentration of biosurfactant used in the set of formulation; the dark blue bars correspond to chocolate and the light blue bars to soybean oil.

The results showed in Figure 39 indicate clearly the effectiveness of biosurfactant in oil and chocolate removal.

On one hand, washing the cotton cloths stained with soybean oil, using MEL at CMC (0,02 mg/ml) improves rapidly the removal when compared with its absent; however, this removal is not improved by concentrations above this value which was then selected as the optimum value.

On the other hand, use concentrations of MEL above or below the CMC to wash the cotton cloths stained with chocolate is greater as the weight percentage of removal is higher when compared with the washing solution that contains MEL at critical micelle concentration. Without biosurfactant, the removal was lower as it happened with soybean oil.

Comparing the removal of chocolate with the removal of soybean oil, the first is higher which could happen because chocolate is more hydrophilic which result in a higher dissolution in water, instead of soybean oil that is more hydrophobic.

For both cases, there is a statistically significant difference between the formulation with and without MEL (for all concentrations) as determined by one-way ANOVA with a *p*-value<0.0.5.

Also the comparison between a commercial detergent and a mixture of commercial detergent with MEL was investigated. The results are described in Table 8 and the showed the differences in the cloth before and after washing.

Table 8 - Soybean oil and chocolate weight percentage removal (%) by commercial detergent and by commercial detergent supplemented with MEL.

	Soybean oil (%)	Chocolate (%)
Commercial Detergent	51.78±2.49	86.45±0.28
Commercial Detergent + MEL	68.18±0.31	91.73±1.19

Figure 40 - Example of the action of MEL in cloth´s washing when chocolate was applied. The figure on the left is before washing and the figure on the right is after washing and drying the cloth.

The results mentioned in Table 8 show that the biosurfactant had a significantly positive effect on the performance of the commercial detergent. Once more the removal percentage of chocolate was higher than the removal percentage of soybean oil, in support of what was written before that the removal is easier for hydrophilic contents. Whereas for soybean oil, there is a statistically significant difference between groups as determined by one-way ANOVA ($F(1,2)=21.44$, $p=0.044$), for chocolate there is no statistically significant differences between group means as determined by one-way ANOVA ($F(1,2)=9.38$, $p=0.092$).

5. Conclusions

All the experiences performed in this thesis have been built based on the same fermentation condition, previously nominated as condition α, a sustainable condition based on the use of sugars as carbon source. This condition includes the addition of 40 g/l of D-glucose at the beginning with a feed of D-glucose (40 g/l) at day 4 with titres of 5.7 g/l after 18 days of fermentation.

From here, it was first tested the influence of different concentrations of D-glucose as well as other carbon sources, such as xylose and a boost of a hydrophobic vegetable oil, soybean oil. In parallel, the influence of the nitrogen source was assessed with additions of $NaNO_3$ at day 0 or at day 4.

With a condition that mixes glucose and soybean oil (initial concentration of 40 g/l of D-glucose and a feed of 40 g/l of D-glucose and 21 g/l of soybean oil), better results were obtained and a maximum value of 19.39 g/l was reached after 18 days of fermentation. Even a condition with initial 40 g/l and 21 g/l of soybean oil added at day 4, with total molar carbon equivalent to condition α, resulted in 13.11 g/l of MEL, an improvement of 131.63% in produced MEL, and maintaining a low concentration of residual oils in the end of fermentation.

Secondly, some tests were carried on to try to understand where the biosurfactant is mostly located and samples of pellet and supernatant were analysed. Results show that around 65% of MEL is in the cells fraction, however is not yet possible to conclude if it is intracellular or extracellularly adsorbed in the membrane.

In order to characterize the product, different properties were measured being the surface tension one of the main parameters to assess biosurfactant potential. The surface tension and, consequently, the critical micelle concentration have never been analysed for D-glucose in driven and a CMC of 0.02 mg/ml was established, corresponding to a surface tension of 27.4 mN/m was obtained. This value is in accordance with others obtained for fermentations using soybean oil as carbon source [88, 90].

The ability to lower surface tension is a characteristic common to biosurfactants and it supports for the potential of MEL products for commercial applications.

The surface tension, as well as other properties such as, the contact angle and the protein content were measured for the cell-free broth. Also the supernatant showed to have surface properties with a surface tension of 24.5 ± 0.10 mN/m which proves that also this phase has MEL in its composition and it can be used in many applications. In the case of the contact angle, good results were also obtained with values lower than water which means that the supernatant exhibit higher spreading properties onto a hydrophobic surface compared to water [95]. A protein content of 5.51 ± 0.9 mg/ml was measured. The existence of extracellular protein has to do with the production of enzymes since high values of protein content indicate an increase in the production of enzymes, such as xylanases and lipases [94].

In order to increase the concentration and purity of MEL extracted, different downstream processes were assessed, including solvent extraction with different solvents, cells disruption with mechanical processes followed by solvent extraction and the use of resins. The use of solvents demonstrated the lack of efficiency of this method, with exception to the ethyl acetate and the isopropanol the first one reaching the highest percentage of MEL removal and the second one,

although decreases the removal percentages, has proven to be capable of maintaining the cells integrity. The best results were achieved when disruption methods followed by solvent extraction or lyophilisation were performed with a maximum removal percentage of 84.86 ± 14.45% using sonication followed by extraction with ethyl acetate and 83.41 ± 1.72% using sonication followed by lyophilisation. Using high temperatures (autoclave) as disruption method also good values were obtained (76.09 ± 4.72%). Never before, this kind of downstream processes had been tested with *Moesziomyces antarcticus* and using sugars as the only carbon source. The use of resins Amberlite XAD was tested but the results showed the lack of efficiency of this method in the removal of MEL.

Finally, the application of MEL as a detergent was tested, firstly using different concentrations of MEL in the formulation and secondly comparing its efficiency with a commercial detergent. The removal of chocolate and soybean oil from a cotton cloth was performed and for the chocolate cleaning percentages of around 90% were obtained, even for a concentration of MEL below the CMC. The commercial detergent used was capable of removing around 51.78 ± 2.49% of soybean oil and 86.45 ± 0.28% of chocolate. The addition of 10% of MEL in the commercial detergent solution increased the values in 33 and 6.1%, respectively.

6. Future opportunities

Like in all research studies answers come up with questions. Regarding the production of MEL, it would be important to increase the titres of MEL without using or reducing biological oils, since higher titres ease the downstream processes and the absence of oils reduce the final concentrations of fatty acids, especially C16 and C18 that prevail in the presence of these long chain esters. Also the use of lignocellulosic materials as carbon source has been showing advantages due to the benefits for the environment that this kind of materials has when compared to first generation sugars or vegetable oils.

Other topic that needs further study relates to the downstream processes than need to be developed, especially the disruption methods that showed to be efficient and can be an alternative to the mostly used and efficient method applied until now, the use of solvents, especially ethyl acetate.

Also the supernatant disclosed to have many interesting properties that need to be developed in order to increase its use in different situations. One of these situations, which also need to be further explored, is the application of MEL in the formulation of a detergent and since the supernatant also have lower surface tension would be an interesting possibility to explore. Other applications, such as the use of this biosurfactant in bioremediation assays should be performed in the future.

7. References

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