Universidade do Minho

Departamento de Engenharia Biológica

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Biosurfactants from renewable raw materials

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Aos meus *pais* pelo exemplo que são para mim

e a minha *afilhada* para quem espero ser um exemplo a seguir.

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Abstract

Biosurfactants (BS) can replace their chemical counterparts, given their functional properties that allow them to have a wide range of applications. However, the biosurfactant production can be in some cases more expensive than the chemical ones. The use of low cost raw materials and the discovery of strains with high production yields are key factors in overcoming these economic obstacles.

In this context, the main objective of this work was to validate a preliminary screening test of biosurfactants producing microorganisms, so-called foaming test. In parallel, it was also evaluated the influence of the culture medium and cellular growth in the BS production.

The combination of several screening methods, including emulsification index (E_{24}), oil spreading test and Du-Nouy-Ring assay was the approach adopted to validate the foaming test. Several culture media were optimized for the BS production by the strains under study (several "carotenoids-producing" strains, *Starmerella bombicola* Rosa & Lachance, *Rhodococcus erythropolis* DCL14), including the production under nitrogen-limiting conditions or the addition of lipophilic precursors to the culture medium. The evaluation of the BS production was based on the cell growth and properties of the biosurfactants (ability to form foam and emulsion, disperse oil and reduce the surface tension).

Four "carotenoids-producing" strains and *S. bombicola* Rosa & Lachance and *R. erythropolis* DCL14 were found to produce biosurfactants.

Bio16EMS5Ng03, a "carotenoids-producing" strain, can be produce extracellularly, intracellularly or membrane-associated BS under nitrogen-limiting conditions, which can be responsible for forming 3.7 mL foam from an liquid volume of 0.8 mL culture sample; E_{24}= 50%; a diameter in the oil spreading test of 35 mm; and a surface tension of 36.0 mN/m ± 0.05. However, cell membrane components could have been released to the culture medium in the cellular decline phase, which can have contributed to the effects observed in the screening tests.

S. bombicola Lachance & Rose grown in a higher glucose concentration (100 g/L) was able to produce biosurfactants with better properties, namely 2.2 mL foam (in 0.8 mL culture sample); E_{24}= 50 % (unstable); a diameter in the oil spreading test 30 mm and a surface tension of 35.5 mN/m ± 0.25. The addition of oleic acid combined with glucose, after the exponential growth of the *S. bombicola* strain in solely hydrophilic medium, also led to the biosurfactant production (brown oily precipitate) with E_{24}= 30 % and a diameter in the oil spreading test of 40 mm.

R. erythropolis DCL14 produced, eventually, membrane-associated biosurfactants, during the exponential and stationary phases, both in hydrophilic medium or medium combined with hydrophilic and hydrophobic substrates. The biosurfactants produced in hydrophilic medium were able to form 3.2 mL foam (in 0.8 mL culture sample); a light emulsion and oil dispersion.

The biosurfactants produced in the second culture medium were able to emulsify the hydrophobic substrates.

Finally, the validation of the foaming test as a preliminary screening assay for the selection of biosurfactants-producing organisms was successfully achieved, given the similarity of the results obtained in this test and in the other complementary screening methods as well as its characteristics such as precision, specificity, fasteness, simplicity and ability to analyze a great number of potential candidates in microplates.

Resumo

Os biosurfactantes (BS) podem substituir os seus homólogos químicos, dadas as suas propriedades funcionais que lhes permitem ter uma variedade de aplicações. No entanto, a produção de biosurfactantes pode ser, em alguns casos, mais cara que a dos químicos. O uso de matérias-primas de baixo custo e a descoberta de estirpes com elevados rendimentos de produção são fatores-chave para superar estes obstáculos económicos.

Neste contexto, o principal objetivo deste trabalho foi validar um teste preliminar de rastreio de microorganismos produtores de biosurfactantes, designado "teste de espuma". Em paralelo, foi também avaliada a influência do meio de cultura e do crescimento celular na produção de BS.

A combinação dos vários métodos de rastreio, incluindo o índice de emulsificação (E_{24}), o teste de espalhamento de óleo e o teste do anel (*Du-Nouy-Ring*) foi a abordagem adotada para validar o "teste de espuma". Diversos meios de cultura foram otimizados para a produção de BS pelas estirpes em estudo (várias estirpes " produtores de carotenóides", *Starmerella bombicola* Rosa & Lachance, *Rhodococcus erythropolis* DCL14), incluindo a produção em condições limitantes de azoto ou a adição de precursores lipofílicos ao meio de cultura. A avaliação da produção de BS foi baseada no crescimento celular e nas propriedades dos biosurfactantes (capacidade para formar espuma e emulsão, dispersar o óleo e reduzir a tensão superficial).

Quatro estirpes "produtoras de carotenóides", *S. bombicola* Rosa & Lachance e *R. erythropolis* DCL14 foram encontradas estar a produzir biosurfactantes.

Bio16EMS5Ng03, uma estirpe "produtoras de carotenóides", pode estar a produzir BS extracelularmente ou intracelularmente/ associados à membrana em condições de azoto limitante, os quais podem ser responsáveis por formar 3.7 mL de espuma a partir de um volume de líquido de 0.8 mL de amostra de cultura; E_{24}= 50 %; um diâmetro no teste de dispersão de óleo de 35 mm; e uma tensão superficial de 36.0 mN/m ± 0.05. Contudo, componentes da membrana celular podem ter sido libertados para o meio de cultura na fase de declínio celular, os quais podem ter contribuido para os efeitos observados nos testes de screening.

S. bombicola Lachance & Rose crescida numa concentração de glucose mais elevada (100 g/L) foi capaz de produzir biosurfactantes com melhores propriedades, nomeadamente 2 mL de espuma (em 0.8 mL de amostra de cultura); E_{24}= 50% (instável); um diâmetro no teste de dispersão de óleo de 30 mm; e uma tensão superficial de 35.5 mN/m ± 0.25. A adição de ácido oleico combinado com glucose, após a fase exponencial de crescimento da estirpe *S. bombicola* em meio somente hidrofílico, também levou à produção de biosurfactantes (precipitado óleoso castanho) com E_{24}= 30 % e um diâmetro no teste de espallhamento de óleo de 40 mm.

R. erythropolis DCL14 produziu, eventualmente, biosurfactantes associados à membrana, durante a fase exponencial e estacionária, tanto em meio hidrofílico como em meio combinado com substratos hidrofílicos e hidrofóbicos. Os biosurfactantes produzidos em meio hidrofílico

foram capazes de formar 3.2 mL de espuma (em 0.8 mL de amostra de cultura), uma ligeira emulsão e dispersão de óleo.

Os biosurfactantes produzidos no segundo meio de cultura foram capazes de emulsionar os substratos hidrofóbicos.

Por fim, a validação do "teste de espuma" como um ensaio de rastreio preliminar para a seleção de organismos produtores de biosurfactantes foi alcançada com sucesso, dada a similaridade dos resultados obtidos neste teste e nos outros métodos de screening complementares, assim como as suas características tais como, precisão, especificidade, simplicidade, rapidez e capacidade de analisar um grande número de potenciais candidatos em microplacas.

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GENERAL INTRODUCTION

MOTIVATION AND AIMS

Motivation

Biosurfactants are compounds with tensioactive properties synthesized by living organisms, from plants (e.g. saponins), humans (e.g. pulmonary surfactant) or microorganisms [1]. These natural surfactants can be considered alternative to the synthetic surfactants, due to their unique properties, such as biodegradability, low toxicity, effectiveness at extreme environmental conditions and ability to be produced from renewable raw-materials [9, 16, 18]. These molecules have gained an increased interest in several applications, such as environmental, food-processing, biomedical and cosmetic [3, 13, 16].

The microbial biosurfactant production can present higher costs compared to their synthetic analogous, mainly due to the extraction and purification steps, but also to the low production yields, thus limiting the commercial biosurfactants availability [3, 19, 27]. Several studies have focused on the development of new strategies to improve this biotechnological process at an indutrial scale.

Biosurfactants industrial producers focus their efforts in the use of renewable raw materials, such as agro-industrial residues, as an economic sustainable strategy, which could contribute to a decrease of 10-30% in the final process [18, 20]. Moreover, the adoption of such strategy can be viewed as an environmental sustainability measure, since it enables the establishment of an end-use for the wastes, which are considered pollutants [18, 20]. In parallel, in the last years, a broad range of different screening methods for the discovery of new biosurfactants and producer strains have been proposed. The search for new biosurfactants, as well as super producing strains is indeed a key factor to overcome the economic limitations that currently exist in the biosurfactants commercialization [31].

Research aims

The main goal of this work was to validate a preliminary screening technique for the discovery of biosurfactant-producing microrganisms, the so-called foaming test. Additionally, the influence of the culture medium was evaluated, including the use of renewable substrates, in the biosurfactants production as well as in their physicochemical properties.

The current study was developed at the biporocess development company Biotrend - Inovação e Engenharia em Biotecnologia, S.A., under the scope of the European Project "O4S (Organic for Surfactants)", which aims at developing fermentation technologies for the sustainable production of organically certified biosurfactants for application in cosmetic products. The main focus areas of the thesis were:

- validation of the foaming test for the screening of biosurfactant-producing microorganisms, evaluating its selection performance by comparison with other methods described in the literature;
- selection of potential biosurfactant-producing microorganisms;
- optimization of the fermentation processes, assessing the influence of the substrates in the biosurfactant production. Evaluation of the potential use of renewable raw materials (olive oil) in those fermentation processes;
- evaluation of the influence of growth kinetics in the biosurfactant production.

CHAPTER 1

STATE OF THE ART

1.1 Market Study

1.1.1 Global biosurfactants market

Global biosurfactants market was evaluated in 2011 with a value of USD 1,735.5 million and it is expected an overall average annual growth of 3.5% up to 2018, reaching therein a worth USD 2,210.5 million and a market volume of 476,512.2 tons [2]. Asia, Africa and Latin America will be responsible for 21% of the consumption of this biosurfactants volume. Currently, Europe leads the global biosurfactants market, in terms of volume and revenue (53.3%), followed by North America [2].

Europe gathers great biosurfactant manufacturers, such as: Fraunhofer IGB (Germany) and BASF (Germany) that produce glycolipids, cellobiose lipids and mannosylerythritol lipids (MEL), as well as the Ecover (Belgium) and Groupe Soliance (France) that manufacture sophorolipids. Recently, Cognis (now BASF) announced a biosurfactant from vegetable oil or starch designated green surfactant alkyl polyglucoside (APG®) [3]. In the United States, Jeneil Biosurfactants is responsible for selling ZONIX (a biosurfactant used in cleaning and oil recovery), Paradigm Biomedical Inc. and AGAE Technologies Ltd. are involved in the rhamnolipids production [3]. In Asia, Saraya (Japan) and MG Intobio (South Korea) are sophorolipids producing companies, while Urumqi Unite (China) focuses on rhamnolipids production [3, 4]. Kanebo, a Japanese cosmetic industry, lately announced the production of MEL-B [5].

1.1.2 Global surfactants/biosurfactants market for cosmetic applications

The first surfactants marketed in the cosmetic field were intended for the soap production [6]. Currently, surfactants represent 40 % of the cosmetics ingredients used in personal care products in the European Union [7].

Biosurfactants have been shown to be superior to their chemical counterparts, including in the cosmetic field, since they are less irritating to the skin due to their low toxicity [6]. According to a report published by Transparency Market Research [2], the domestic detergents along with the personal care products sector will be the focus of attention in the coming years, contributing to more than 56% of the global biosurfactants market in 2018.

1.2 Biosurfactant: structure and mode of action

Biosurfactants can be produced by a diversity of microorganisms (bacteria, yeast or fungi), either extracellularly, associated to the cell membrane or intracellullarly [3, 10, 36].

These microbial surfactants are amphiphilic molecules, comprising hydrophilic (polar head and soluble in water) and hydrophobic moieties (nonpolar tail and soluble in oil/air), as illustrated in Figure 1. The hydrophilic group can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or an alcohol, while the hydrophobic group usually consists of long-chain fatty acids or derivatives of fatty acids [3, 13-16].

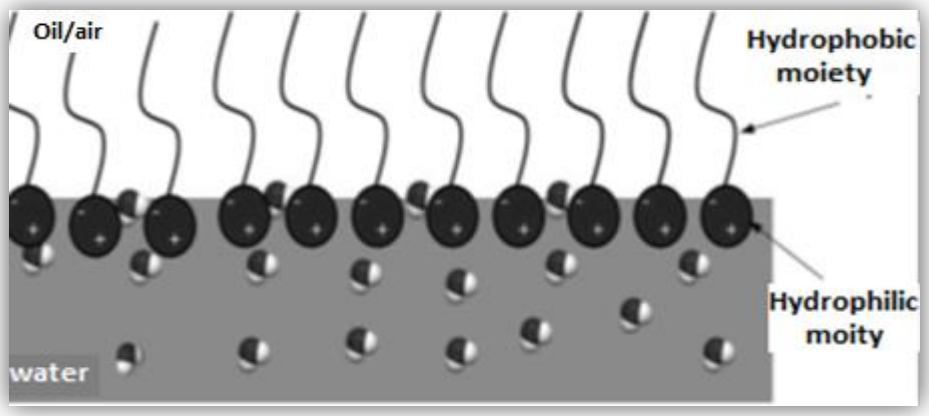

Figure 1 – Accumulation of biosurfactants at the interface between liquid and oil/air (taken from [13]).

This molecular structure confers the biosurfactants the ability to accumulate at the interface of heterogeneous systems such as air/liquid, solid/liquid or oil/liquid, reducing the surface tension between a liquid and a solid or a liquid and a gas (air) and the interfacial tension between two immiscible fluids, thereby aiding the mixture of two different phases [10, 12-14].

The biosurfactant molecules can associate to form micelles, usually occurring at a given concentration at which no further reduction of the superficial tension can be observed. This concentration at which micelles begin to form (micellization) is designated as the critical micelle concentration (CMC). The CMC and surface/ interface tensions are two basic properties that are commonly used to determine the efficiency and effectivity of the biosurfactants [10, 13, 14]. The low CMC and surface/interface tensions make them excellent detergency/foaming agents, dispersants, wetting, emulsifiers, among other, with practical use in several industrial fields [13, 14, 16].

1.3 Biosurfactants Natural Functions

Although most biosurfactants are considered secondary metabolites, they may have an essential role in the survival of their producing strains due to their natural functions [12].

Mechanism of transport and assimilation of hydrophobic substrates: Water-insoluble substrates can only be degraded by cells if they are somehow first solubilized. Some microorganisms produce biosurfactants that remain associated to their cell walls, which changes the structure of the cell wall, increasing thus the bioavailability of these substrates for the cell. Other microorganisms secrete the biosurfactants to the culture medium during their growth on hydrocarbon susbtrates, emulsifying them in order to enable their transport and assimilation by the cell [12, 31, 33].

Heavy metal binding: Some biosurfactants have the ability to capture heavy metals from the culture medium. Since these elements are important cofactors for the metabolism of their producer organisms, the production of such biosurfactants has been reported as a survival strategy [12, 30, 31, 33].

Bacterial pathogenesis: Biosurfactants have the capacity to solubilize the major components of the microbial cell membranes (proteins, phospholipids, lipids). Therefore, biosurfactant producing microrganisms acquire a greater chance of survival in the competition for nutrients with other microbes [12, 31, 33].

Attachment/detachment of microorganisms to/from surfaces: Biosurfactants attached to the cell wall can change the cell surface properties (hydrophobic or hydrophilic) depending upon the orientation of the molecule, allowing their producing microrganisms to adhere or detach from a particular surface, according to their needs to find favorable environments or to get rid of unfavorable environments [12, 31, 33].

1.4 Biosurfactants Classification

Biosurfactants can be classified according to their chemical composition, molecular weight, physicochemical properties and microbial origin [14, 16, 17].

Rosenberg and Ron [22] suggested the division of biosurfactants into two main classes: low-molecular-weight compounds, which efficiently lower surface/interfacial tension; and high-molecular-weight compounds, designated also as bioemulsifiers (BE) since they are more effective as emulsion stabilizing agents than as tensioactive agents. The low-molecular weight biosurfactants group includes the glycolipids, lipopeptides and lipoproteins, phospholipids, neutral lipids and fatty acids, while the high–molecular-weight biosurfactants group (BE) includes the polymeric and particulate biosurfactants.

1.5 Advantages and Limitations of the Biosurfactant Production

Biosurfactants (BS) have become attractive molecules in several industrial and biotechnological applications owing to their functional properties and advantages (Table 1) over synthetic substances [30].

However, the biosurfactants production processes present higher costs compared to their synthetic analogous, mainly due to their high extraction and purification costs, thus rendering the large scale biosurfactants production very limited [3, 19, 27, 30]. The biosurfactants purification, essential for pharmaceutical, food and cosmetic applications, requires the downstream processing with multiple consecutive steps [19, 27]. Besides, to recover biosurfactants from diluted broths it is necessary that high yields can be obtained, however for many biosurfactants production processes these yields are quite low [19, 30]. During the past few years, the scientific and industrial communities have been conducting several efforts to overcome this issue, namely through the use of low cost raw materials, as well as the search and design of novel biosurfactant-producers with improved production yields [3, 13, 19, 31].

Table 1 – Properties and advantages of biosurfactants (BS) as compared to chemical surfactants [9, 12, 16, 18, 19]

Property	Description
Superficial/ interfacial activity	BS are considered good surfactants if they can lower the surface tension (ST) of water from 72 to ≤ 35 mN/m and the interfacial tension (IT) of water/hexadecane from 40 to 1 mN/m. (e.g. Surfactin from *Bacillus subtilis* reduces ST and IT to 25 mN/m and <1 mN/m, respectively). Generally, less BS is necessary to get a maximal decrease on ST, since their CMC is about 10–40 times lower compared to chemical surfactants, thus BS can be considered more effective and efficient.
Biodegradability	BS are easily degraded in the water and soil as opposed to the synthetics surfactants, allowing their large use in environmental applications, such as bioremediation and dispersion of oil spills.
Low toxicity	BS are considered generally low/non-toxic products, thus with added value for pharmaceutical, cosmetic and food applications. (e.g. BS from *Pseudomonas aeruginosa* was considered slightly non-toxic and non-mutagenic comparing with Marlon A-350 (synthetic surfactant).
Tolerance to extreme conditions	Several BS are effective at extreme conditions of temperature, pH and ionic strength. (e.g Lichenysin produced by *Bacillus licheniformis* JF-2 was not affected by temperatures up to 50^{o}C, pH values between 4.5 and 9.0, and NaCl concentrations up to 50 g/L.)
Availability of raw material	BS can be produced from cheap raw materials that are available in large amounts, including oil wastes, starchy substances, cheese whey, glycerol, among others.
Specificity	BS are complex organic molecules of great chemical diversity and specific functional groups, which enables their use in specific applications, such as the detoxification of specific pollutants or in the cosmetic, pharmaceutical and food industry.
Sustainable process	Besides their biodegradability, BS can be produced from agro-industrial wastes, which also has a positive impact in the environment since some of these substrates are considered pollutants.
Antimicrobial/Anti-adhesive activities	Some BS are good antimicrobial and antifungal agents, thus can be potentially used for biomedical applications as alternatives to antibiotics. Additionally, their anti-adhesive features can be further explored for the development of biomedical materials.

1.6 Biosurfactant-Producing Microorganisms and Applications

The increasing interest in biosurfactants by the scientific community is mainly due to the great diversity of producer microorganisms, as well as their unique properties that allow them to be used in a wide range of applications in the environmental, food, biomedical and cosmetic industries [3, 13, 16]. The potential applications of biosurfactants in the cosmetic field will be described in more detail in this thesis.

Environmental applications: Biosurfactants can accelerate different environmental treatment processes since they can be used to: promote the formation of emulsions, thus facilitating the removal hydrocarbons or heavy metals in bioremediation and biosorption; act as dispersants to prevent that the particles sediment/agglutinate in soil leaching processes; and removal/recovery of crude oils (MEOR - Microbial enhanced oil recovery), through their capacity of decreasing the surface tension of oil-rock interface, reducing the capillary forces preventing the movement of the oil through the pores of the rock [3, 9, 19]. Furthermore, biosurfactants are easily degraded in the water and soil comparing to their chemical counterparts, as mentioned previously, thus reducing the environmental impact [3, 9, 19].

Food applications: Biosurfactants can be incorporated like food-formulation ingredients to: facilitate the formation and stabilization of emulsions of fat-based products (butter cream); improve the consistency and texture of food products; slow staling and to confer flavour to confectionery products and ice-cream [10, 16]. Apart from these functions, biosurfactants can be used as anti-adhesive agents, preventing the surface microbial contaminations [23].

Biomedical and therapeutics applications: Some biosurfactants may replace antibiotics due to their antimicrobial or antifungal activities [9, 10, 12]. Additionally, biosurfactants can exhibit anti-adhesive activity, inhibiting microbial adhesion to solid surfaces or infection sites, and therefore these can be used as coating agents in several biomedical related surfaces, including prostheses and catheters [9, 12]. Also, noteworthy are other interesting properties for potential therapeutic applications, such as the anticancer activity of some glycolipids (differentiation-inducing activities against human leukemia cells) and the immune action of lipopeptides [9, 12].

Cosmetic applications: Biosurfactants can be used as multifunctional ingredients in the formulation of cosmetics [6]. Due to their physicochemical properties, biocompatibility and biodegradability, the biosurfactants widely used in the cosmetic industry are glycolipids, including sophorolipids, rhamnolipids and mannosylerythritol lipids (MEL) [6, 8, 15]. Sophorolipids (figure 2A) from *Candida bombicola* and *C. apicola* exhibit antibacterial and antioxidant properties, emulsifying and moisturizing activities, as well as wetting and foaming effects. These properties allow their application in several cosmetic products, such as lotions, liquid soaps, hair products, deodorants and creams for acne treatment, skin smoothing and anti-wrinkle [6, 15]. Rhamnolipids (figure 2B) and MEL (figure 2C) produced by *P. aeruginosa* and *C. antartica*, respectively, possess antimicrobial and emulsification properties, thus showing potential for application in anti-wrinkle face and anti-aging creams, toothpastes, among other [6, 15].

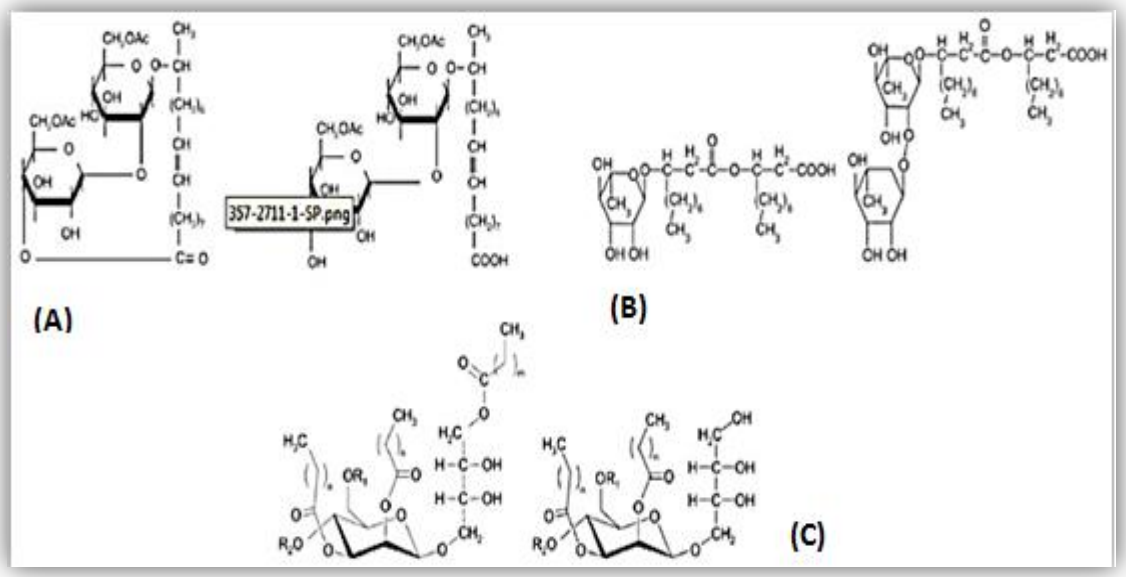

Figure 2 – Chemical structures of common glycolipids types: (A) sophorolipids in lactonic form (left) and free acid (right); (B) monorhamnolipid (left) and dirhamnolipid (right); (C) triacylated (left) and diacylated (right) mannosylerythritol lipids (adapted from [6]).

A summary of the different biosurfactants, their producer microorganisms and their common applications is provided in the following tables: glycolipids (Table 2), lipopeptides and lipoproteins (Table 3), phospholipids, fatty acids, neutral lipids (Table 4), polymeric biosurfactants (Table 5) and particulate biosurfactants (Table 6).

Table 2 – Glycolipids biosurfactants produced by different microorganisms and their applications

Biosurfactant type	Microrganism (s)	Application (s)	Ref.
Rhamnolipids	*Pseudomonas aeruginosa*, *P. alcaligenes*, *P. chlororaphis*, *P. fluorescens*, *Serratia rubidea*, *Thermus thermophilus* HB8, *Burkholderia plantari* DSM 9509, *Pseudoxanthomonas* spp PNK-04	• Bioremediation • Improves the texture/consistency of the food products • Anti-aging cosmetic creams • Antimicrobial and anti-adhesive properties	[10-16, 18-20, 23, 25]
Trehalose lipids	*Rhodococcus erythropolis*, *Arthrobacter* sp., *Corynebacterium* sp., *Micrococcus luteus* BN56, *Nocardia erythropolis*, *N. rhodochrous*, *Mycobacterium tuberculosis*	• Bioremediation and MEOR • Antiviral activity against HSV and influenza virus • Anti-adhesive activity against several bacterial and yeast strains isolated from voice prostheses	[10-14, 18-20, 25]
Sophorolipids	*Candida bombicola*, *C. antartica*, *C. botistae*, *C. apicola*, *C. stellata*, *C. riodocensis*, *C. lipolytica*, *C. bogoriensis*, *Torulopsis petrophilum*	• Bioremediation and MEOR • Cosmetic products: lotions, hair products, creams, etc • Antimicrobial and antiviral properties	[9, 10, 12-15 18-20]
MEL (mannosylerythritol lipids)	*C. antartica*, *Pseudozyma siamensis*, *P. graminicola* CBS 10092, *P. antarctica* JCM 10317T, *Calyptogena soyoae*, *Kurtzmanomyces* sp.	• Antimicrobial/immunological/anticancer properties • Anti-wrinkle cosmetic creams • Bioremediation	[9, 11-13, 15, 18, 20]
Glycolipids	*Streptococcus thermophilus*, *Alcanivorax borkumensis*, *Pseudozyma hubeiensis*, *Rhodococcus wratislaviensis* BN38	• Anti-adhesive properties (surface coatings) • Bioremediation	[11, 12, 20, 34]
Surlactin	*Lactobacillus*	• Anti-adhesive properties	[12, 24]
Diglycosyl diglycerides	*Lactobacillus fermentum*	• Bioremediation	[13, 19]
Cellobiolipids	*Ustilago zeae*, *U. maydis*	• Antifungal proprieties	[10,13, 19]

Table 3 – Lipopeptides and lipoproteins biosurfactants produced by different microorganisms and their applications

Biosurfactant type	Microrganism (s)	Application (s)	Ref. (s)
Surfactin	*Bacillus subtilis*, *B. amyloliquefaciens*, *B. licheniformis* F2.2	• Bioremediation • Antimicrobial and immunological properties	[10, 11-14 18, 19]
Iturin	*Bacillus subtilis* *B. amyloliquefaciens*	• Antimicrobial and antifungal properties • Non-toxic and non-pyrogenic immunological adjuvant	[9, 11-13]
Fengicin	*Bacillus subtilis*	• Antimicrobial properties	[13, 28]
Lichenysin	*Bacillus licheniformis*, *B. subtilis*	• MEOR • Antibacterial Activity	[11-14, 18 19-20]
Flavolipid	*Flavobacterium* sp. MTN11	• Emulsifying activity	[16]
Viscosin	*Pseudomonas fluorescens*, *Leuconostoc mesenteriods*	• Antimicrobial and antibiotic properties	[10-13, 17-19]
Viscosinamide	*Pseudomonas fluorescens*	• Antifungal proprieties	[12]
Serrawettin	*Serratia marcescens*	• Bioremediation	[10, 11, 13,17]
Peptide-lipid	*Bacillus licheniformis*	• Antimicrobial properties	[10, 13]
Subtilisin	*B. subtilis*	• Antimicrobial properties	[10, 13]
Ornithine lipids	*Pseudomonas* sp., *Myroides* sp. SM1 *Thiobacillus thiooxidans*, *Agrobacterium* sp.	• Emulsifying activity	[13, 26]
Gramicidins, Polymyxins and Antibiotic TA	*Brevibacterium brevis*, *B. Polymyxa*, *Myxococcus xanthus*	• Antibiotic properties	[9-11, 18]

Table 4 – Phospholipids, fatty acids and neutral lipids biosurfactants produced by different microorganisms and their applications

Biosurfactant type	Microrganism (s)	Application (s)	Ref. (s)
Phospholipids, fatty acids and neutral lipids	*Acinetobacter* sp., *Corynebacterium lepus*, *Thiobacillus thiooxidans, Nocardia erythropolis*	• Bioremediation • Emulsifying activity	[10, 11, 13, 18]
Spiculisporic acid	*Penicillium spiculisporum*	• Bioremediation	[14]
Corynomycolic acid	*Corynebacterium insidibasseosum, C. lepus*	• MEOR	[14]
Bile acids	*Myroides*	• Good surface active agent	[26]

Table 5 – Polymeric biosurfactants (bioemulsifiers) produced by different microorganisms and their applications

Biosurfactant type	Microrganism (s)	Application (s)	Ref. (s)
Emulsan	*Acinetobacter calcoaceticus, A.venetianus* RAG-1	• MEOR	[10- 14, 18]
Alasan	*Acinetobacter radioresistens*	• Bioremediation	[13, 14, 18, 19, 21]
Liposan	*Acinetobacter tropicalis, Candida(Yarrowia) lipolytica*	• Emulsifying activity	[10-14, 18]
Biodispersan	*Acinetobacter calcoaceticus* A2	• Dispersing agent	[10, 14]
Carbohydrate-protein-lipid	*Yarrowia lipolytica*, *Pseudomonas fluorescens P. náutica, Debaryomyces polymorphis*	• Emulsifying activity	[10, 13, 26]
Mannan-lipid-protein	*Saccharomyces cerevisiae, Candida tropicalis*	• Emulsifying activity	[9-11, 14, 18]

Table 6 – Particulate biosurfactants (bioemulsifiers) produced by different microorganisms and their applications

Biosurfactant type	Microrganism (s)	Application (s)	Ref. (s)
Vesicles & fimbriae	*Acinetobacter calcoaceticus, P. marginilis, P. Maltophila*	• Bioremediation	[10, 13, 18]
Whole cells	*Cyanobacteria,* variety of bacteria	• Emulsifying activity	[10, 13, 18, 26]

1.7 Screening Biosurfactant-Producing Microrganisms

The main goal of screening for new biosurfactants producing strains is to find new structures/molecules with improved physicochemical properties, as well as to search for strains with high production yields that can render the production of biosurfactants economically competitive [31].

The screening methods can provide quantitative or qualitative results, being that for a first screening the qualitative methods are generally sufficient [31].

The choice of a screening method depends on both the goal of the screening as well as on the advantages and the disadvantages of each method. Some tests are limited to a given group of biosurfactants, such as the colorimetric assay with cetyl trimethylammonium bromide (CTAB) and the haemolytic activity assay that are specific for extracellular glycolipids/other anionic surfactants or biosurfactants capable of lysing blood cells, respectively [31]. The screening techniques must be simple, require unsophisticated equipment/material and small amounts of sample, fast and specific (i.e. detect with accuracy the metabolite of interest in the presence of interferents [31]). Several studies have been focused on the development of High Throughput Screening methods (HTS), allowing a fast and reliable screening in microplates of organisms from thousands of potential candidates [31]. However, it should be noted that the combination of these methods is suitable and in most cases preferred for a successful screening [31]. Table 7 summarizes some of the screening methods that have been used for selecting biosurfactants producing microorganisms.

Table 7 – Screening methods for the selection of biosurfactants producers [31, 32, 35]

Method	Principle/ Description	Features
Hemolysis Activity	Cultures inoculated on sheep blood agar (48h at 25˚C) are considered biosurfactant producers if a colorless zone around the colonies is formed – blood cells lysis. This test can be performed with purified biosurfactants (BS).	Qualitative Assay; slow (days); non-specific (lytic enzymes can also lead to clear zones and some BS do not show any hemolytic activity); limited
Bacterial Adhesion to Hydrocarbons Assay (BATH)	Aqueous suspension of washed culture cells is mixed with an equal volume of a hydrophobic solvent (hexadecane/octane) and mixed for 2 min. After separation of the two phases, the turbidity (O.D) of the aqueous phase is measured at 600nm. The turbidity decrease correlates to the cells hydrophobicity (cells adherence to hydrocarbons): $Adhesion_{\%} = O.D_{\text{initial cell suspension}} - O.D_{\text{aqueous phase}} / O.D_{\text{initial bacterial suspension.}}$	Qualitative assay; very fast (min); easy; indirect
Drop Collapse Assay	In a glass surface is placed a drop of cell suspension or culture supernatant culture. If BS are present the drop will collapse, contrarily to the negative control (distilled water) in which the drop remains stable (round) – the destabilization of liquid droplets is correlated with surface/interfacial tension.	Qualitative assay; very fast (min); easy; HTS
Oil Spreading Assay	In a petri dish containing distilled water covered by an oil surface, a drop of culture sample is placed on the centre of the oil layer. BS producing organisms will form a clearing zone on the oil surface – oil displacement capacity. For a pure BS, a linear correlation between surfactant amount and clearing zone diameter can be established.	Qualitative assay very fast (min); easy; reliable

Table 7 (Cont.) – Screening methods for the selection of biosurfactant producers [31, 32, 35]

Method	Principle/ Description	Features
Emulsification Assay – E_{24}	A mixture (1:1, v/v) of an hydrophobic/hydrocarbon solvent and culture sample is vortexed for 2 min. After 24 h, the emulsion index height , E_{24}, is determined: $E_{24} = h_{emulsion} / h_{liquid\ total}$	Qualitative assay; slow (24h); easy
Du-Nouy-Ring Method	The force required to elevate the platinum ring of a tensiometer from the sample surface/interface is proportional to the superfacial/interfacial tension (ST/IT). A culture is considered a BS producer if it is able to reduce the ST of water from 72 to ≤ 35 mN/m and the IT of water/hexadecane from 40 to 1 mN/m.	Qualitative/quantitate assay; very fast (min); accurate; large sample volumes (mL); one sample measured at the time
CTAB Agar Assay	Cultures are inoculated in a medium with CTAB and methylene blue dye. In the presence of anionic surfactants (or extracellular glycolipids) dark blue halos are formed around of colonies due their capacity to react with cationic indicators.	Qualitative assay; slow (days); limited; CTAB toxicity inhibits the growth of some microbes
Optical distortion assay	The culture samples are placed in a microplate. The plate is viewed using a graph paper in order to check for the occurrence of optical distortion. In the presence of BS the fluid surface becomes concave and takes the form of a diverging lens. In contrast, the water surface is flat (negative assay) – the optical distortion is correlated with surface/interfacial tension.	Qualitative assay; very fast (min); easy; dependent on the visual acuity of the observer; HTS
Penetration Assay	The culture samples colored with a red stain are placed in a microplate containing a hydrophobic paste (oil + silica gel) covered by an oil surface. After 15 min, the wells are inspected for color changes. If BS are present the color will change from clear red to cloudy white (negative assay: the sample will turn cloudy but will stay red) – the color change occurs due to contacting of the two insoluble phases (aqueous sample and hydrophobic paste).	Qualitative assay; very fast (min); easy; HTS

1.8 Biosurfactants Production

Biosurfactants can be produced as extracellular, intracellular or cell-bound metabolites by bacteria, yeast or fungi. The biosurfactants production depends on the type of biosurfactant being produced, the producing microorganisms as well as the fermentative process kinetics and substrates and the conditions used [3, 10, 36].

1.8.1 Kinetics of biosurfactant production

The kinetics of biosurfactants production can be characterized according to different production types (Figure 4): growth-associated production, production under growth-limiting conditions, production by resting or immobilized cells, and production with precursor supplementation [3, 10, 37].

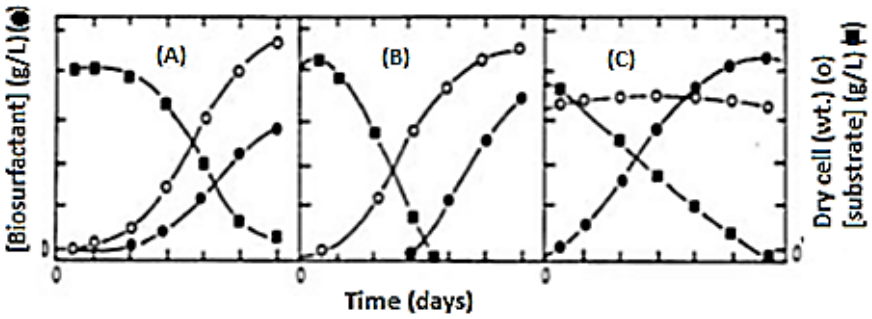

Figure 3 – Different types of fermentation kinetics for biosurfactants production: (A) Growth-associated production (*Acinetobacter calcoaceticus*), (B) Production under growth-limiting (*Pseudomonas* spp.), (C) Production by resting or immobilized cells where cells (*Torulopsis* spp.) (Taken from [10]).

- **Growth-associated production:** In this type of production process there is a parallel relationship between microbial growth, substrate consumption and biosurfactants production (Figure 3A) [10]. Examples of biosurfactants production associated with growth include the rhamnolipids by *Pseudomonas* spp.; glycoprotein AP-6 by *P. fluorescens* 378; a biosurfactant by *Bacillus cereus* IAF 346; and a biodispersant by *Bacillus sp*. strain IAF 343 [3, 10, 37].

- **Production under growth-limiting conditions:** Some microorganisms present an increased biosurfactant production under growth-limiting conditions, i.e. when the exhaustion of one or more nutrients from the culture medium occurs (Figure 3B) [10]. The limitation of nitrogen and iron in the culture medium was found to stimulate the biosurfactants production by *Pseudomonas* spp., *Candida tropicalis* IIP-4 and *Nocardia sp.* strain SFC-D [3, 10, 37].

- **Production by resting or immobilized cells:** the biosurfactant production is characterized by the use of cells that are in the stationary growth phase (Figure 3C) [10]. The carbon source in the culture medium is used in a continuous way by the cells solely to produce biosurfactants [10]. Rhamnolipids by *P. aeruginosa* CFTR-6; sophorolipids by *Torulopsis bombicola*; cellobiose lipids by *Ustilago maydis;* and trehalose lipids by *Rhodococcus erythropolis* are included in this type of production [3, 10, 38].

- **Production with precursor supplementation:** The addition of precursors to the culture medium promotes quantitative/qualitative changes in the biosurfactants production. Lipophilic precursor led to an increased biosurfactants production by *Torulopsis magnoliae, T. bombicola* and *T. apicola*, with yields ranging from 120 g/L-150 g/L [10].

1.8.2 Factors affecting biosurfactant production

The characteristics and amount of biosurfactants produced depend not only on the producing microorganism, but also on the composition of the culture medium (carbon, and nitrogen source, micronutrients) and environmental factors [9, 10, 13, 37].

- **Carbon:** Depending on the carbon source it is possible to obtain different biosurfactant structures and therefore different properties. Several carbon sources can be used for the biosurfactants production: hydrophilic (water-soluble) as glucose, glycerol, sucrose, ethanol, sodium acetate, molasses; or hydrophobic (water-insoluble) such as n-alkanes, vegetable oils, olive oil, diesel, crude oil [9, 10, 13, 37].

- **Nitrogen:** It is a component of proteins which are essential for microbial growth and enzyme production. Besides, nitigen also contributes to control the pH [9, 13, 37]. The yeast extract is the most commonly used nitrogen source for the biosurfactant production but there are other such as urea, peptone, ammonium sulfate, ammonium nitrate, sodium nitrate, and extract of meat and malt [9, 13, 37].

- **C:N Ratio:** The quantitative ratio between the carbon and nitrogen sources (C/N) is the an important parameter in the biosurfactant production [10, 37]. The biosurfactants production very often occurs when the nitrogen source is depleted during the stationary growth phase [13]. Guerra-Santos *et al.* [39] evidenced a maximum production of rhamnolipids for values of C:N between 16:1 and 18:1.

- **Micronutrients:** The presence of iron, magnesium, manganese, phosphorus and sulfur in low concentrations can influence the amount, composition, and consequently the characteristics of the biosurfactant [10]. Iron and manganese were found to increase the biosurfactants production by *Bacillus subtilis* [13, 37].

- **Environmental factors:** pH, temperature, aeration rate and agitation are factors extremely important because they affect the microbial growth or biosurfactant production [9, 10, 13]. The influence of the initial pH on the production of a biosurfactant by *Yarrowia lipolytica* (marine strain) was studied by Zinjarde and Pant [40], that reported the best biosurfactant production occurring at pH 8.0 (seawater natural pH). The majority of the biosurfactants are produced at temperatures between 25˚C and 30˚C [9, 13]. The aeration and agitation are important factors in the biosurfactants production since they facilitate the oxygen transfer from the gas to the aqueous phase. The oxygen transfer is one of the key parameters in the surfactin production by *B. subtilis* [13]. Metal ions in the culture medium have a crucial role for the production of some biosurfactants. Overproduction of surfactin occurs in the presence of Fe^{2+} in mineral salt medium and its properties are modified in the presence of inorganic cations [13].

1.8.3 Fermentative process

The fermentation processes for the biosurfactants production can be classified according to the nutrients feeding regime (batch, fed-batch and continuous) [43, 44].

Batch processes are characterized by the addition of all nutrients to the culture medium at the begining of the fermentation and the removal of products only at the end of fermentation [45]. In the fed-batch processes, the nutrients supply occurs during the fermentation and products recovery is performed only at end of fermentation [45]. Some studies on the sophorolipids production by *C. bombicola* have shown that these biosurfactants can be obtained either in batch mode or in semi-batch mode of fementation [20]. Continuous fermentation is characterized by a continuous feed of nutrients and removal of products along the fermentation [45]. Noah *et al.* [46] showed that surfactin production by *B. subtilis* can be conducted through a continuous fermentation in an airlift reactor and from potato process effluent.

A fermentative process can be carried out in solid-state or submerged. The submerged fermentation is characterized by the microbial growth in the presence of water in the culture medium. In the solid-state fermentation, the microorganisms grow on solid moisture that contains the substrates [45]. The solid state fermentation presents a number of advantages as compared to the submerged fermentation, including the diversty of products that can be obtained, the stability of the products in a concentrated form, the use of less energy, and the inexistence of oxygen supply requirements [45]. In the case of biosurfactants, Ohno *et al.* [47] reported the surfactin production by *B. subtilis* RB14 and recombinant *B. subtilis* MI113 specie in solid state (okara, a soybean residue) showed higher productivity than in the submerged state.

1.8.4 Raw-materials of low cost

Every year, about 18 million tons of surfactants are produced from petroleum-based chemical sources. A quarter of this value is now produced by microorganisms from renewable raw-materials [48].

Low cost raw materials from different sources, especially from agro-industrial wastes, have been evaluated for the production of biosufactants. The use of these substrates can lead to a reduction of the raw materials costs, thus contributing to a 10–30% decrease of the final biosurfactant production cost [18, 20]. Several alternative substrates have been widely explored including vegetable oils and waste frying oils, waste animal fat, whey and molasses, starch and lignocellulosic residues [13, 18, 20]. Table 8 gathers some information from the literature on different renewable raw materials, biosurfactant types, producer microorganisms, kinetic parameters obtained with those alternative substrates ($Y_{P/S}$ – product/substrate yield and t – optimum fermentation time) and the biosurfactants properties (ST – surface tension; E_{24} – emulsion index correspondent to the ratio of the height of the emulsion layer and the total height of liquid; among others).

Table 8 – Biosurfactant production from renewable substrates. Unidentified biosurfactant is given by "NI" and no information about kinetic parameters/ properties by "-"

Renewable substrates	Biosurfactants	Microorganisms	Kinetic parameters/properties	Ref. (s)
Sunflower oil	Rhamnolipids	*Pseudomonas aeruginosa* 47T2	$Y_{P/S}$ (g/L) = 2.98	[13, 20]
	Lipopeptides	*Serratia marcescens*	$Y_{P/S}$ (g/L) = 2.98	[18]
	Glycolipids	*Tsukamurella* sp. DSM 44370	$Y_{P/S}$ (g/L) = 30	[20]
	Sophorolipids	*Candida bombicola*	$Y_{P/S}$ (g/L) = 120; t (d) = 8	[20]
Palm oil	Rhamnolipids	*Pseudomonas aeruginosa* A41	good surface activity and oil displacement	[13, 20]
	N.I	*P. alcaligenes*	$Y_{P/S}$ (g/L) = 2,3; E_{24} =70%	[20]
	N.I	*Bacillus subtilis* PT2	t (d) = 2; good surface activity	[20]
Rapeseed oil	Rhamnolipids	*Pseudomonas* sp. DSM 2874	$Y_{P/S}$ (g/L) = 45	[13, 18]
	Sophorolipids	*Candida bombicola* ATCC 22214	$Y_{P/S}$ (g/L) > 300	[20]
Soybean oil	Mannnosylerythritol lipid	*Candida* sp. SY16	$Y_{P/S}$ (g/L) = 95	[18]
	Sophorolipids	*Pichia anomala* PY1	t (d) = 7; TS (mN/m) = 28	[20]
	Rhamnolipids	*P. aeruginosa* DS10-129	$Y_{P/S}$ (g/L) = 4.31	[20]
Turkish corn oil and honey	Sophorolipids	*Candida bombicola* ATCC 22214	$Y_{P/S}$ (g/L) = 400	[18, 20]
Olive oil	Glycolipids	*Penicillium citrinum*	$Y_{P/S}$ (g/L) = 0.54; t (h) = 60	[20]
Babassu oil	Sophorolipids	*Candida lipolytica* IA 1055	$Y_{P/S}$ (g/L) = 11.72	[18]
Peanut oil	Lipopeptides	*Candida lipolytica*	$Y_{P/S}$ (g/L) = 4.5	[20]
Animal fat	Sophorolipids	*Candida bombicola*	$Y_{P/S}$ (g/L) = 120	[13,20,41]

Table 8 (Cont.) – Biosurfactant production from renewable substrates. Unidentified biosurfactant is given by "NI" and no information about kinetic parameters/ properties by "-"

Renewable substrates	Biosurfactants	Microorganisms	Kinetic parameters/properties	Ref. (s)
Potato process effluent	Surfactin	*Bacillus subtilis ATCC 21332*	$Y_{P/S}$ (g/L) = 0.6	[13, 18, 41]
	Lipopeptides	*B. subtilis*	$Y_{P/S}$ (g/L) = 2.7	[18]
Cassava flour wastewater	Surfactin	*Bacillus subtilis* ATCC 21332 and *B. subtilis* LB5a	$Y_{P/S}$ (g/L) = 2.2 TS (mN/m) = 25.9	[13, 20, 41]
Soap stock	Rhamnolipids	*Pseudomonas aeruginosa* LBI	$Y_{P/S}$ (g/L) = 15.9	[13, 20, 41]
	Emulsan	*Acinetobacter calcoaceticus* RAG-1	$Y_{P/S}$ (g/L) = 25 e t (h) = 50	[13, 41]
	Biodispersan	*A. calcoaceticus* A2	$Y_{P/S}$ (g/L) = 12 e t (h) = 45 h	[13, 41]
Olive oil mill effluent (OOME)	Rhamnolipids	*Pseudomonas aeruginosa* 47T2	$Y_{P/S}$ (g/L) = 8.1	[13] [20]
	Rhamnolipids	*Pseudomonas* sp. JAMM	$Y_{P/S}$ (g/kg) = 14 e t (h) = 150	[41]
Restaurant oil waste	Sophorolipids	*Candida bombicola* ATCC 22214	$Y_{P/S}$ (g/L) = 34	[20]
	Sophorolipids	*Pseudomonas aeruginosa* zju.u1 M.	$Y_{P/S}$ (g/L) = 20	[20]
	Rhamnolipids	*P. aeruginosa* 47T2	$Y_{P/S}$ (g/L) = 2.7	[13, 20]
	Rhamnolipids	*P. aeruginosa* PACL	$Y_{P/S}$ (g/L) = 3.3; E_{24} (%) = 100 TS (mN/m) = 26.0	[20]
Cheese whey	Sophorolipids	*Candida bombicola*	$Y_{P/S}$ (g/L) = 34	[20]
		Cryptococcus curvatus ATCC20509 and *C. curvatus* ATCC 22214	$Y_{P/S}$ (g/L) = 422	[13, 20]

Table 8 (Cont.) – Biosurfactant production from renewable substrates. Unidentified biosurfactant is given by "NI" and no information about kinetic parameters/ properties by "-"

Renewable substrates	Biosurfactants	Microorganisms	Kinetic parameters/properties	Ref. (s)
Molasses	Rhamnolipids	*Pseudomona aeruginosa* GS3	$Y_{P/S}$ (g/L) = 0.25; t (h) = 96	[13, 20, 41]
	Surfactin	*Bacillus subtilis* BS5	$Y_{P/S}$ (g/L) = 1.45; t (h) = 96	[20]
	N.I	*B. subtilis* MTCC 2423 and *B. subtilis* MTCC1427	$Y_{P/S}$ (g/L) = 1.12	[20, 41]
Mixture of molasses and soybean oil	Sophorolipids	*Candida bombicola*	$Y_{P/S}$ (g/L) = 60	[20]
Glycerol	Rhamnolipids	*Pseudomonas aeruginosa*	$Y_{P/S}$ (g/L) = 15.4	[20]
	Mannnosylerythritol lipid	*Pseudozyma antarctica* JCM 10317	$Y_{P/S}$ (g/L) = 16.3	[20]
	N.I	*Pseudomonas aeruginosa* UCP0992	$Y_{P/S}$ (g/L) = 8.0 ; t (h) = 96; TS (mN/m) = 27.4	[20]
Clarified cashew apple juice	Surfactin	*Bacillus subtilis* LAMI008	$Y_{P/S}$ (mg/L) = 3.5	[20]
	Surfactin	*B. subtilis* LAMI005	$Y_{P/S}$ (mg/L) = 123	[20]
Fish oil	Rhamnolipids	*Pseudomonas aeruginosa* BYK-2 KCTC 18012P	$Y_{P/S}$ (g/L) = 17	[13, 20]
Okara	Surfactin/iturin	*Bacillus subtilis* NB22	-	[20]
Agricultural residues (hemicellulose sugars)	N.I	*Lactobacillus* sp.	$Y_{P/S}$ (mg/g) = 71	[20]
Grape pomace (hemicellulose sugars)	N.I	*Lactobacillus pentosus*	$Y_{P/S}$ (mg/g) = 0.60	[20]

1.9 Recovery, Purification and Chemical Characterization

Downstream processes refer to the recovery and purification of biosynthetic products, as well as to the recycling of salvageable components and to the proper treatment and disposal of wastes [49]. In many biotechnological processes, the downstream processing can represent up to 60% of the total production cost [10]. Generally, the combination of several methods allows the removal, isolation and required purification degree of the product [49].

The selection of the most suitable recovery method depends on the characteristics of the biosurfactants, namely their ionic charge (chromatography), solubility (water/organic solvents) and location (intracellular, extracellular, cell bound) [10, 13]. The techniques most commonly used to recover and purify microbial surfactants are listed in Table 9.

The chemical characterization of biosurfactants is based on several spectroscopic techniques (NMR: Nuclear magnetic resonance; IR: Infrared Spectroscopy; MS: Mass spectrometry; FTIR: Fourier Transform Infrared Spectrometer; GC−MS: Gas chromatograph − mass spectrometry; FAB−MS: Fast atom bombardment − mass spectrometry) and chromatography (TLC: Thin Layer Chromatography, HPLC: High-performance liquid chromatography), among others [19, 34]. Some of the analytical methods that are used to characterize biosurfactants are listed in Table 10.

Table 9 – Methods to recover and purify biosurfactants (BS)

Method	Principle	Biosurfactants	Ref.
Acid precipitation	BS become insoluble at low pH values and precipitates	Surfactin, glycolipids, lipopeptides	[10, 13, 18, 34]
Ammonium sulphate precipitation	High-molecular-weight BE (protein rich compounds) are precipited - *salting-out*	Emulsan, biodispersan	[10,13,18,34]
Solvent extraction	BS are soluble in organic solvents due to their hydrophobic moieties. Solvents as methanol, chloroform and acetone have been replaced by less toxic solvents (MTBE - methyl-tert-butyl)	Trehalose lipids, rhamnolipids liposan, sophorolipids	[10,13,18,34]
Centrifugation	Insoluble BS precipitate due to centrifugal force	Glycolipids	[10,13,18, 34]
Foam fractionation	BS due to their surface activity partition to the foam phase	Surfactin	[10,13,18]
Ultrafiltration	BS form micelles above their CMC, thus being held by a polymer membrane	Rhamnolipids, surfactin	[7,10,13,18]
Adsorption / Desorption	BS are adsorbed in polymer resins or activated charcoal and subsequently can be desorbed using organic solvents	Rhamnolipids lipopeptides	[10,13,18,34]
Ion Chromatography	BS bind to ion exchange resins and can be eluted with an appropriate buffer	Rhamnolipids	[7,13,18]
Crystallization	BS precipitate in the form crystals. The addition of a non-polar solvent (hexane) combined with the reduction of temperature causes the product crystallization	Cellobiolipids, glycolipids rhamnolipids	[7,10,13,18,34]
Dialysis and lyophilization	BS are separated from the solution due to their different concentrations by a semipermeable membrane and are further preserved by lyophilisation	Bioemulsifiers sophorolipids	[2, 34]

Table 10 – Chemical characterization of biosurfactants using analytical method (Taken from [19])

Biosurfactant/microrganisms	Method	Chemicals/solvents
Rhamnolipids		
Pseudomonas aeruginosa	TLC	$CHCl_3/CH_3OH/CH_3COOH$
	HPLC	$CHCl_3/CH_3OH$
	HPLC	CH_3CN
		2-Propanol-NH_4OH-H_2O
	Western blot	
	TLC	Carbenicillin, Tetracycline
	HPLC	CH_3CN-H_2O
	HPLC	Tetrahy drofuran-H_2O
	HPLC	CH_3CN/Phosphate burffer pH 6
	TLC	CH_3OH/H_2O
	FTIR	
	TLC	Solv. A: $CHCl_3/CH_3OH/CH_3COOH$
		Solv. B: 2-Propanol-NH_4OH-H_2O
Pseudomonas aeruginosa LBI	HPLC	CH_3CN/H_2O
	TLC	$CHCl_3/CH_3OH/H_2O$
Pseudomonas aeruginosa 57RP	HPLC-MS	CH_3CN/H_20
	TLC	$CHCl_3/CH_3OH/CH_3COOH$
Pseudomonas aeruginosa UG2	HPLC	CH_3CN-H_3PO_4
	ESI	N2
	HPLC-UV	CH_3CN-H_3PO_4
Pseudomonas aeruginosa 47T2	HPLC	CH_3CN/CH_3COOH
	TLC	$CHCl_3/CH_3OH/CH_3COOH$
Pseudomonas fluorescens	TLC	CH_3CN/H_2O
Sophorolipids - *Candida bombicola*	HPLC + ELSD	
and Torulopsis sp.	HPLC-UV	CH_3CN/H_2O
	FTIR	
Trehalose lipids – *Rhodococcus* sp. P32C1	HPLC	CH_3CN
Lipopeptide - *Bacillus licheniformis*	FTIR	
	HPLC-MS	CH_3CN/TFA
Phospholipids - *Acinetobacter* sp.	GC-MS	$CHCl_3/CH_3OH$ (extraction method)
Surfactin – *B. subtilis* ATCC 21332	HPLC	CH_3CN/TFA

CHAPTER 2

MATERIALS AND METHODS

2.1 Strains

In the current work several microbial strains were tested for their ability to produce biosurfactants, namely twenty three "carotenoids-producing" strains (bacteria) obtained from the culture collection of Biotrend SA, one yeast *Starmerella bombicola* Rosa & Lachance (nº 29811, Canada) and one gram positive bacterium *Rhodococcus erythropolis* DCL14 kindly provided by Dr. Carla Carvalho from Instituto Superior Técnico (Lisboa, Portugal).

S. bombicola (also called *Candida bombicola* ATCC 22214 or Torulopsis *bombicola*) and *R. erythropolis* are described in the literature as biosurfactant producers. The "carotenoids-producing" strains used in this study have never been tested for biosurfactant production before, although they are known to be able to produce sphingolipids and related molecules.

All strains were cultured from stock cryotubes, containing 15% (v/v) glycerol solution and stored at - 80 ° C.

2.2 Chemicals and Reagents

Most medium components were purchased from VWR BDH Prolabo, (Paris, France), with the exception of the Urea (Merck, Darmstadt, Germany), Yeast extract (Organo Technie, La Courneuve, France), Peptone (Himedia, India). Glycerol and oleic acid were used with a purity of 97% and 70%, respectively. The olive oil used as carbon source in some media was the "Azeite tradicional Serrata - refinado e virgem" (Lote 56412180, Portugal).

The commercial surfactant "PLANTACARE® 2000 UP (decyl glucoside)" used in screening tests as a positive control was from Cognis (Monheim, Germany). The concentration of this surfactant solution was standardized at 1 g/L.

Querosene (Parafina Líquida, Lacrilar, Ramalhal, Portugal) and lipid soluble dye Sudan black B (Panreac, Barcelona, Spain) were used for conducting the emulsification test and car engine oil was used in the oil spreading test.

2.3 Media and Culture Conditions

All microorganisms under study were initially cultured in the media previously established by Biotrend SA, containing basic nutrients for growth of each organism. These media were designed "Initial culture medium".

Subsequently, other experiments were performed to optimize the biosurfactants production and referred as "Optimal biosurfactantproduction". The media used for this optimization differed among the microorganisms studied.

Shake flask experiments (25 mL) were carried out using submerged culture and in duplicate (except for the strains from the "carotenoids" group grown in "Initial culture medium" due to the high amount of samples). The material and cultures media were sterilized in an autoclave for 20 min at 121 ° C and 1 bar. Different tables are shown below for each microorganism study, including the conditions and composition of the culture media used.

Table 11 – Media and culture conditions for the "carotenoids-producing" strains: initial culture medium and optimal biosurfactant (BS) production

Components/ conditions	**Concentrations**	
	Init. cult. medium	**Optimal BS production** Nitrogen limitation
Phosphate buffer - pH 6. 5 (1) (mL/L)	10	10
Oligo elements (2) (mL/L)	10	10
Yeast extract (g/L)	7.5	3.5
Glycerol (g/L)	10	10
Glucose (g/L)	10	10
Temperature (˚C)	28	28
Time (h)	72	168
Shaker speed (rpm)	150	150
pH	-	6.5 (3)
Fed–batch: addition of glucose (g/L)	-	10 (4)

(1) Phosphate buffer - pH 6. 5: 176 g/L $NaH_2PO_4.2H_2O$, 86 g/L $K_2HPO_4.3H_2O$.

(2) Oligo elements: 1.00 Na_2EDTA, 0.20 g/L $ZnSO_4.7H_2O$, 0.10 g/L $CaCl_2.2H_2O$, 0.50 g/L $FeSO_4.7H_2O$, 0.02 g/L $Na_2MoO_4.5H_2O$, 0.02 g/L $CuSO_4.5H_2O$, 0.07 g/L $Co(NO_3)_2.6H_2O$, 0.13 g/L $MnSO_4.H_2O$, 9.10 g/L $MgSO_4.7H_2O$.

(3) The pH of the flask cultures was adjusted to 6.5 with 1N NaOH whenever necessary along fermentation.

(4) The fermentation was carried out in fed-batch mode, through the addition of 10 g/L glucose whenever the pH value was adjusted.

Table 12 – Media and culture conditions for *S. bombicola* Rosa & Lachance: initial culture medium and optimal biosurfactant (BS) production

Components/ conditions	Concentrations		
	Init. cult. medium	Optimal BS production	
		High concentration hydrophic substrate [62]	Hydrophic and hydrophobic substrates (1)
Yeast extract (g/L)	10	10	10
Peptone/urea*(g/L)	20 (pept.)	1 (urea)	20 (pept.)
Glucose (g/L)	20	100	20
			Substrates solutions (2)
Temperature (˚C)	28	28	28
Shaker speed (rpm)	150	150	150
Time (h)	72	168	48 h + 96 h
pH	-	3.5 (3)	-

(1) After 48 h of fermentation in medium containing yeast extract (10 g/L) peptone (20 g/L) glucose (20g/L), the cells were washed twice (under sterile conditions) and resuspended in new shake flask (25 mL) of four different solutions of carbon sources (2): 100 g/L oleic acid; 100 g/L olive oil; 1 g/L glucose + 100 g/L oleic acid; 1 g/L glucose + 100 g/L olive oil. The cultures were incubated for 96 h more under the same initial conditions.

(3) The pH of the cultures was adjusted to 3.5 with 1N NaOH whenever necessary along the fermentation.

Table 13 – Media and culture conditions for *R. erythropolis* DCL14: Initial culture medium and optimal biosurfactant (BS) production

Components/ conditions	Concentrations	
	Init. cult. medium	Optimal BS production: Hydrophic and hydrophobic substrates
Phosphate buffer - pH 6. 5 (mL/L)	10	10
Oligo elements (mL/L)	10	10
Yeast extract (g/L)	7.5	7.5
Glycerol (g/L)	10	10
Glucose (g/L)	10	10
Oleic acid or olive oil (g/L)	-	100
Temperature (˚C)	28	28
Time (h)	72	72
Shaker speed (rpm)	150	150

2.4 Cellular Growth

Microbial growth was followed by measuring the optical density at 600 nm using a spectrophotometer (UV-1700 PharmaSpec, Shimadzu) at different time points. The characteristic growth curve for each microorganism under study was established as absorbance values versus time.

2.5 Screening Methods

Biosurfactant-producing microorganisms were screened through different methods: foaming test, emulsification index, oil spreading and the Du Nouy Ring assay. These screening methods were performed mainly using culture supernatants, obtained by centrifugation (Sigma 2-16KC, Sartorius) at 9000 rpm during 10 min. The culture broth from *R. erythropolis* DCL14 and the cellular suspension of *S. bombicola* Rosa & Lachance were also used.

Several controls were included in these screening methods, namely a control prepared with culture medium, a negative control prepared with distilled water and a positive control with a commercial surfactant (1 g/L).

2.5.1 Preliminary test - Foaming Test

The foaming test allows quantifying the formation of liquid foams resulting from gas bubbing in a small amount of liquid containing surfactants [50, 54]. The procedure of this test was based on the FoamScan procedure [50]. A peristaltic pump (120U pump, Watson Marlow) was used to pump air into in Falcon tubes containing 0.8 mL culture sample at a flow rate of 1 mL/min during 3 min. The height of foam generated (foamability) after this time was measured. The foam stability (decrease of the foam volume) was evaluated 5 min later. The foam height was measured in millimeters (mL) and may be related with the presence of biosurfactants. These measurements were done in duplicate and performed on samples taken throughout the fermentation, (except for the strains from the "carotenoids" group grown in "Initial culture medium"). A rotation speed of 8 rpm, at which the pump was set, was determined using the equation of the calibration curve of the peristaltic pump (annex A).

2.5.2 Emulsification Index

This screening method, developed by Cooper and Goldenberg [51], allows evaluating the stability of the emulsions formed by biosurfactants in a hydrophobic medium, e.g querosene. Equal volumes of culture samples and querosene colored with 0.1 g/L Sudan Black dye were vigorously mixed in a test tube using a vortex for 2 min. The mixture was allowed to setle for 24 h. Afterwards, the emulsion stability was evaluated after 24, 48 and 72 h, by measuring the height of the stable emulsion layer (E_{24}) through of ratio of the height of the emulsion layer and the liquid total height:

$$\boldsymbol{E_{24}}(\%) = \frac{\boldsymbol{h_{emulsion}}}{\boldsymbol{h_{\,total\ liquid}}} \times \mathbf{100} \quad \textbf{(Equation 1)}$$

These measurements were done in duplicate (except for the strains from the "carotenoids" group grown in "Initial culture medium").

2.5.3 Oil Spreading Test

The oil spreading test was developed by Morikawa *et al.* [52], and consists in the evaluation of the oil displacement in the presence of biosurfactants. Car engine oil was added to the surface of distilled water (10 mL) placed in a petri dish (5.5 cm of diameter). Subsequently, a drop of culture sample was gently placed on the centre of the oil layer. After 30 sec, it was possible to detect a clear zone on the oil surface in the cases in which biosurfactants were present. The diameter of the clearing zone was measured and could be correlated with the surfactant activity, also called oil displacement activity. This test was performed at room temperature and in duplicate (except for the strains from the "carotenoids" group grown in "Initial culture medium").

2.5.4 Du-Nouy-Ring Method

The Du-Nouy-Ring method, first proposed by Pierre Lecomte du Noüy [53], is a method based on the direct measurement of the biosurfactants surface activity. A KRUSS Tensiometer equipped with a 1.9 cm De Nouy platinum ring was used at room temperature. The samples were placed in a small watch glass and in contact with the ring. The force to uplift the ring from the sample surface corresponds to the surface tension. These measurements were done in duplicate.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Screening of Biosurfactant-Producing Microorganisms

The results of the first screening, which was based on the physical effects of the biosurfactants, allowed the selection of the biosurfactant-producing strains. For this first screening, all strains were grown in media designed as "Initial culture medium".

3.1.1 Preliminary Screening – Foaming Test

Foaming is an important feature of the surfactants because it is closely related to surface tension [50]. The foam is created when the surface tension between an aqueous solution and the air is reduced due to the presence of surfactant, thus causing the mixture of the two different phases and hence bubbles formation [50, 54]. Therefore, foaming can be used as a preliminary screening test of biosurfactant-producing microorganisms. As previously mentioned, this test was based on the determination of the foam height formed (foamability) by the culture sample after 3 min of bubbling. The stability of the foam formed was observed after 5 min. The results of the foaming test obtained for all the strains studied along with their culture media (control – basal activity), distilled water (negative assay) and commercial surfactant at 1 g/L (positive assay) are shown in table 14.

Table 14 – Foam height (mL) for the "carotenoids-producing" strains, *S. bombicola* Rose & Lachance, *R. erythropolis* DCL14 and controls. Non-foaming is given by "-"; total collapse by "T.C" and no collapse by "N.C"

Strains		Height (mL)	
		Foamability	Stability
"carotenoids-producing"	Bio16Org	-	-
	Bio16EM55Ng03	3.2	N.C
	Bio16EM55Ng22	-	-
	Bio16EM55Ng22Y	-	-
	Bio16Ng22O	-	-
	16UVM12	0.7	T.C
	M63	-	-
	M63Y	0.7	T.C
	M64	0.7	T.C
	M18	3.2	N.C
	M21	-	-
	M67	3.2	N.C
	M7UV	-	-
	M9UV	-	-
	M50UV	0.7	T.C
	M66UV	0.7	T.C
	M138UV	0.7	T.C
	M141UV	0.7	T.C
	P57-H8	-	-
	P97_C9	3.2	N.C
	R5	-	-
	R5Y	-	-
	Bio 16	-	-
***S. bombicola* Rosa & Lachance**		1.2	T.C
***R. erythropolis* DCL14**	supernatant	-	-
	broth	3.2	N.C
Controls	Distilled water	-	-
	Commercial surfactant (1 g/L)	4.0	N.C
	"Carotenoids-producing" strains initial medium	-	-
	S.bombicola Rosa & Lachance initial medium	-	-
	R. erythropolis DCL14 initial medium	-	-

Regarding the twenty-three "carotenoids-producing" strains evaluated, it could be seen that twelve strains were negative (non-foaming) and four were positive strains (stable foaming). The positive strains were *Bio16EM55Ng03*, *M18*, *M67* and *P97_C9*. The remaining strains of this group produced small foam amounts (much less than 3.2 mL) that totally collapsed, and therefore were also considered as negative strains.

S. bombicola and *R. erythropolis* have been described as biosurfactant-producing microorganisms. In the current study, *S. bombicola* Rose & Lachance and *R. erythropolis* DCL14 were used as positive control strains. The first strain presented a small and unstable amount of foam (1.2 mL) that collapsed totally. For the second strain, the foaming test was conducted with the supernatant and the culture broth (with cells) since *R. erythropolis* strains can produce biosurfactant both extracellularly as associated with the cell membrane [30, 67]. Stable foaming was only observed for the culture broth (3.2 mL), thus indicating that the strain being used mainly produced cell membrane-associated biosurfactants.

All culture media (negative assay) studied were found to be negative in this test, meaning that no foam was formed, thus discarding the possibility that the formed foam could result from some medium component with surface activity. On the other hand, the commercial surfactant at 1g/L (positive assay) showed the formation of 4 mL of foam. As such, the foam obtained in the culture samples may be an indicative of the presence of biosurfactants. However, to verify the accuracy of the test, namely the relation between foaming and biosurfactant activity, other screening methods for biosurfactants described in the literature were also performed. The combination of all the screening methods was the approach adopted to validate the foaming test as a preliminary screening method of biosurfactant-producing microorganisms, which will be discussed in section 3.3.

3.1.2 Screening Methods

The screening methods for biosurfactant-producing microorganisms can provide qualitative and/or quantitative results, being the qualitative methods generally enough for a first screening [31]. Oil spreading, emulsification index (E_{24}) and Du-Nouy-Ring-method were the screening methods performed in this work. Table 15 gathers the results obtained in these assays for all the strains studied along with their controls. The results were scored as follows: "-" no oil spreading/ no emulsion, "+" slight oil spreading/ slight change (emulsion) in the hydrocarbon phase, "++" oil spreading, "TS" total oil spreading. The surface tension values (ST) are the average of duplicate measurements along with the standard deviation. Some "carotenoids-producing" strains were not used in the Du-Nouy-Ring-method (classified as "NR" not result) since the other tests were sufficient to demonstrate that they were not biosurfactant producers.

Table 15 – Results obtained in the screening tests, oil spreading, emulsification index (E_{24}) and Du-Nouy-Ring test for the "carotenoids-producing" strains, *S. bombicola* Rose & Lachance, *R. erythropolis* DCL14 and controls

Strains		Clearing zone	E_{24}	ST (mN /m)
"carotenoids-producing"	Bio16Org	-	-	NR
	Bio16EM55Ng03	++	+	61.4 ± 0.60
	Bio16EM55Ng22	-	-	NR
	Bio16EM55Ng22Y	-	-	NR
	Bio16Ng22O	-	-	NR
	16UVM12	-	-	NR
	M63	-	-	NR
	M63Y	-	-	NR
	M64	-	-	NR
	M18	++	+	60.2 ± 0.70
	M21	-	-	NR
	M67	++	+	59.1 ± 0.15
	M7UV	-	-	NR
	M9UV	-	-	NR
	M50UV	-	-	NR
	M66UV	-	-	NR
	M138UV	-	-	NR
	M141UV	-	-	NR
	P57-H8	-	-	NR
	P97_C9	++	+	58.9 ± 0.05
	R5	-	-	NR
	R5Y	-	-	NR
	Bio 16	-	-	NR
***S. bombicola* Rose & Lachance**		++	+	36.7 ± 0.05
R. erythropolis DCL14	supernatant	-	-	59.0 ± 0.90
	broth	+	+	56.9 ± 0.15
Controls	Distilled water	-	-	71.1 ± 0.15
	Commercial surfactant (1 g/L)	TS	0.50	30.9 ± 0.05
	Carotenoids-producing strains initial medium	-	-	58.8 ± 0.20
	S. bombicola Rose Lachance initial medium	-	-	57.2 ± 0.20
	R. erythropolis DCL14 initial medium	-	-	58.8 ± 0.20

The results obtained for the "carotenoid-producing" strains are in good agreement with the previous results obtained in the foaming test, i.e. only four strains showed the expected activities in these tests, with the exception of the Du-Nouy-Ring-method. *Bio16EM55Ng03*, *M18*, *M67*, *P97_C9* similarly demonstrated oil dispersion and a small change in the hydrocarbon phase which could indicate the presence of a slight emulsion. None of these four strains demonstrated ability to reduce surface tension, presenting surface tension values greater than their own culture medium.

The surface tension of the culture medium (58.8 ± 0.20 mN/m) was lower than the surface tension of distilled water (71.05 ± 0.15 mN/m) indicating that it contained compounds capable of reducing the surface tension (e.g yeast extract). With the consumption of these compounds during the cell growth, the value of the surface tension increased, thus possibly accounting for the slightly higher value in the culture sample at the end of the fermentation. Furthermore, these strains can be producing bioemulsifiers, which are high-molecular-weight biosurfactants that are known to not exhibit ability to reduce surface tension [22].

S. bombicola Rose & Lachance showed biosurfactant activity through the presence of a slight emulsion, capacity of oil spreading and surface tension reduction of the medium, from 57.2 to 36.7 mN /m. This surface tension value differs only slightly from the value described by Cooper and Cavalero [55] who demonstrated that sophorolipids (SLs) *S. bombicola* are capable of reducing the surface tension of culture medium (containing hydrophobic substrates) to 33 mN /m. This small difference is related to the fact that the culture sample containing SLs is not purified.

Regarding *R. erythropolis* DCL14, using the culture supernatant no biosurfactant activity could be observed. On the other hand, using the culture broth it was possible to visualize a small emulsion and slight oil spreading, suggesting that biosurfactant production can be associated to the cell membrane. This strain promoted only a small decrease in the surface tension of the medium containing hydrophilic substrates. Gogotov and Khodakov [56] showed that a *R. erythropolis* strain, grown in different hydrophilic substrates, presented values of emulsification index and surface tension ranging from 60 to 61 mN/m, and 75 to 85%, respectively. The surface tension value obtained can be related with the sample type used, culture broth, that may interfere with the measure of the surface tension, giving negative false results (indicating incorrectly the biosurfactant absence in the sample). A further extraction and purification procedure to obtain a purer biosurfactant could lead to better surface activity parameters.

The results of this first screening for the strains under study were obtained in a very small scale (compared with the commercial surfactant 1 g/L), which may be indicative that small amounts of biosurfactants are being produced. As previously mentioned, the amount and type of biosurfactant produced largely depends on the culture medium, fermentation process and cell growth [29]. Therefore, new culture media were used for growing the four "carotenoids-producing" strains selected, *S. bombicola* Rose Lachance and *R. erythropolis* DCL14 in order to optimize the biosurfactant production.

3.2 Biosurfactant Production

The microbial growth and the foam formation capability throughout the fermentations, as well as the influence of the culture medium in the biosurfactant production are presented and discussed for each microorganism under study. Different culture media were used to optimize the biosurfactant production and called "Optimal biosurfactant production".

3.2.1 "Carotenoids-Producing" Strains

3.2.1.1 Cellular Growth and Biosurfactant Production

- Initial culture medium

Cell growth is measured as optical density values (OD) and foam height is measured in mL. The evaluation of the biosurfactant production for four "carotenoids-producing" strains (*Bio16EM55Ng03*, *M18*, *M67* and *P97_C9*) in medium containing glucose and glycerol, both at 10 g/L, during 72 h, is given by the relation between cell growth and foam formation, as illustrated in figure 4. In this first experiment, the points taken over time to perform both curves (cell growth and foam height) correspond to only a single measurement, , as already mentioned.

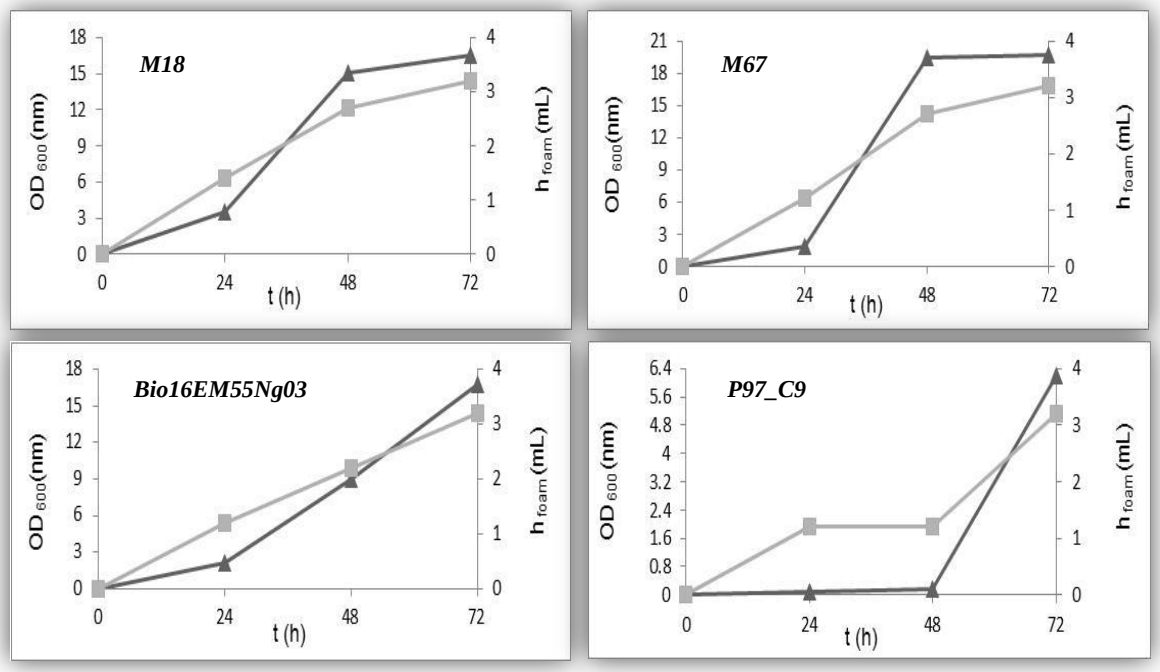

Figure 4 – Cell growth (O.D600nm) (triangle) and foam height (mL) (square) along time (h) for four "carotenoids-producing" strains grown in shake flask (25mL) containing 10 g/L glucose and 10 g/L glycerol.

The growth curves of *M18* and *M67* show an adaptation (0-24 h), exponential (24-48 h) and stationary phase (48-72 h), whereas *Bio16EM55Ng03* shows the adaptation (0-24 h) and exponential phase (24-72 h). *P97_C9* strain was found to grow more slowly; being visible a longer adaptation (0-48 h) and the start of the exponential phase (48-72 h).

Regarding the foam formation curve, it could be observed an increase in the amount of foam being formed along the fermentation time for all the strains. *P97-C9* revealed initially lower values of foam height, probably due to its slower growth as compared to the other strains. However, all strains showed the same amount of foam formed (3.2 mL) at the end of the fermentation, corresponding to the stationary phase in the case of the *M18* and *M67*; or to the exponential phase in the case of the *Bio16EM55Ng03* and *P97-C9* strains. For the last two strains more samples should have been taken after 72 h in order to evaluate the foaming parameter also in the stationary phase and to verify, possibly, a greater foam production at this stage as was observed for the other strains.

The increase of the foam amount along the fermentation, with maximum height values obtained in the stationary growth phase, suggest that this foam is due to the production of metabolites with ability to form foam, as for example the biotensoactives. Ron and Rosenberg [33] indicated that the majority of the biosurfactants are generally produced when the cultures reach the stationary phase. Furthermore, some studies about the surfactin recovery by foam fractionation have demonstrated that larger foam amounts are obtained in the stationary phase, again correlating foam formation with the biosurfactants production in this growth stage [23, 44].

- Optimal biosurfactant production

Nitrogen limitation

The strains were grown under stress conditions, such as low nitrogen concentrations (3 g/L yeast extract) in order to increase the biosurfactant production. The fermentation occurred in "fed-batch" mode, through the addition of 10 g/L glucose whenever the pH value was adjusted. Figure 5 shows the microbial growth of the "carotenoids-producing" strains in such conditions during 168 h of fermentation.

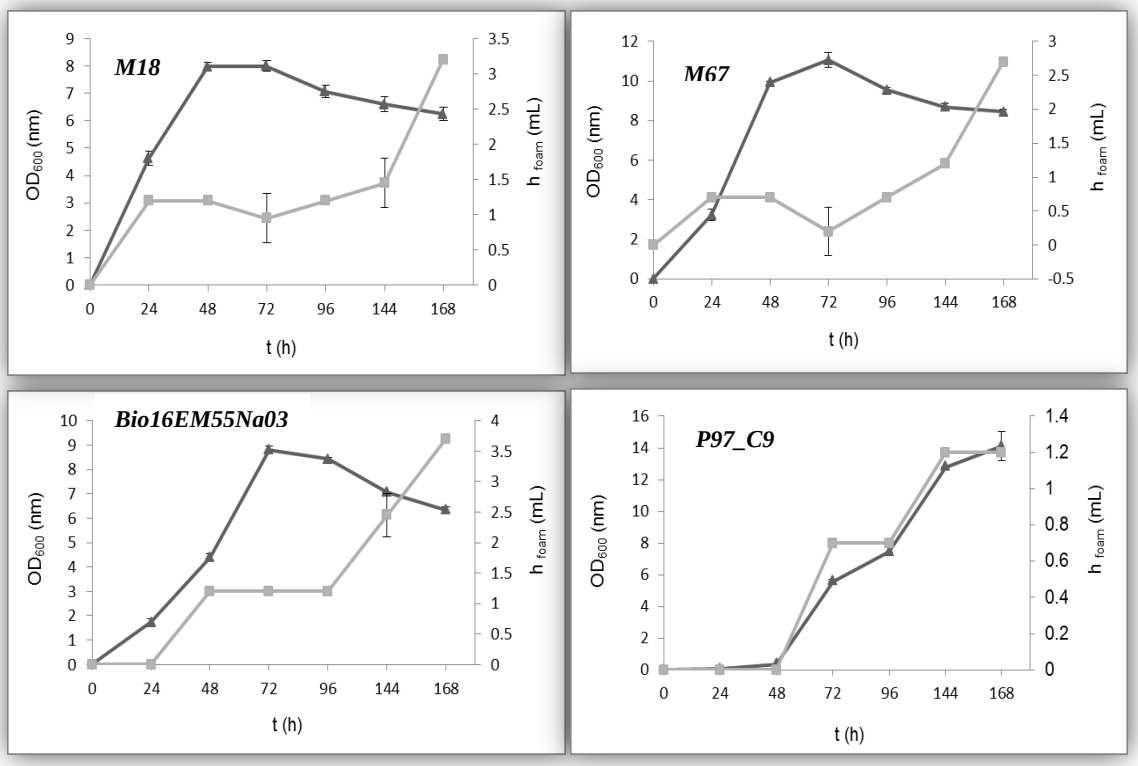

Figure 5 – Cell growth (OD600nm) (triangle) and foam height (mL) (square) along time (h) for four "carotenoids-producing" strains grown in shake flask (25mL) under nitrogen-limiting conditions

Figure 5 shows that generally the growth of the strains was very small, since that the strains grew under nitrogen-limiting conditions. The nitrogen source is a constituent element of the proteins and nucleic acids, being the second component used in large quantity in growth medium of the microorganisms. A lower nitrogen concentration may lead to a slower adaptation / growth of cells to the medium or even limit the cell growth. The decrease of OD values was aso visible for most strains, except for the *P97_C9*.

The foaming curve of *M18* presented a constant behavior during the exponential growth (0-48 h), with 1.2 mL of foam height. In the stationary phase (48-72 h), it was registered a decrease in the foam height, mainly due to the differences obtained for the foam height in the duplicate experiments for this time point (see standard deviation error). If this value is omitted, it can be seen that the strain continued to produce 1.2 mL of foam. Even with the decrease of the OD values (decrease of cell growth), the foam height increased, reaching the higher foam height value of 3.2 mL at the end of the time.

Regarding the *M67* strain, it was possible to see low values of foam height (0.7 mL) in the first two days of fermentation, corresponding to the exponential growth phase. During the stationary phase (48-96 h), if the point corresponding to the foam height at 72 h is omitted, for the same reasons as the ones mentioned for the *M18* strain, it can be verified that the foam amount remained constant along the exponential and stationary

phases. One more time, the decrease of the OD values were acompained by an increase of the foam heigth, with 2.7 mL of foam being obtained at the end of the fermentation.

For the *Bio16EM55N03* strain, the foam formation only began to be visible on the second day of fermentation, which presented 1.2 mL height and remained constant during the exponential phase (24-72 h) and stationary phase (72-96 h). Again, the foam amount increased even with cells at the decline phase, reaching a maximum foam height of 3.7 mL in the last day of fermentation.

Regarding the *P97_C9* strain, no foaming could be observed in the first two days of fermentation since the strain almost didn't grow. The beginning of the exponential phase was reached at 72 h and the following fermentation times were defined by a slight increase of the OD values and foam heigths, having been determined a foam height of 1.2 mL at 144 h. After this time, it was observed stabilization on the cell density and amount of foam being formed due to the start of the stationary phase.

Foaming may be related with biosurfactant activity present in the culture samples studied, as already mentioned.The distinct growth phases demonstrated different foam heigths, what may indicate the influence of cell growth in the foam production and hence in the biosurfactant production. A maximum value of foam height was obtained in the decline/death phase by most of the strains. The release of cellular membrane components into the culture medium, due to cell lysis that occurs normally in the cellular death phase, may have been responsible for forming foam. Other hypotheses about the origin of the foam will be better analyzed in the following section (3.2.1.2).

The initial pH value (6.5) of the culture medium was adjusted at 72 h for the *M18, M67, Bio16EM55N03* and at 144 h for the *P97_C9*, representing both the times of fermentation to stationary growth phase of strains. The decrease in the pH value reflects the presence of products as lactic acid, acetic acid or butyric acid resulting from the fermentation process by consumption of the substrate. Therefore, 1 g/L of glucose was added to the culture medium at this moment, so that there was sufficient substrate available for the biosurfactants biosynthesis. However, the addition of glucose did not affect cell growth of *M18, M67, Bio16EM55N03* because the cell density was further reduced. In the *P97_C9* growth, probably, it also does not have a significant effect, as the cells began to enter the stationary phase. After addition of glucose, it was observed an increase of foam, except for the *P97_C9*. However, as mentioned above, this increase of foam amount is also accompanied by death of some cells which may have been responsible for the foam formed.

3.2.1.2 Influence of Culture Medium on the Biosurfactants Production

The culture conditions and the medium composition influence the quantity and quality of the biosurfactant produced [10, 29]. The biosurfactant production can also be evaluated based on screening tests (as previously described). The results of these tests for the "carotenoids-producing" strains grown in nitrogen-limiting conditions are presented below. Culture medium, used as control to set the basal activity of the samples, allowed ascertaining that the effects observed were not caused by medium constituents with surface active properties.

Foaming test

Foaming test allowed determining the foam height (mL) formed after 3 min of bubbling by the different "carotenoids-producing" cultures and analyzing its stability. Figure 6 illustrates the results obtained for the four strains and culture medium (control).

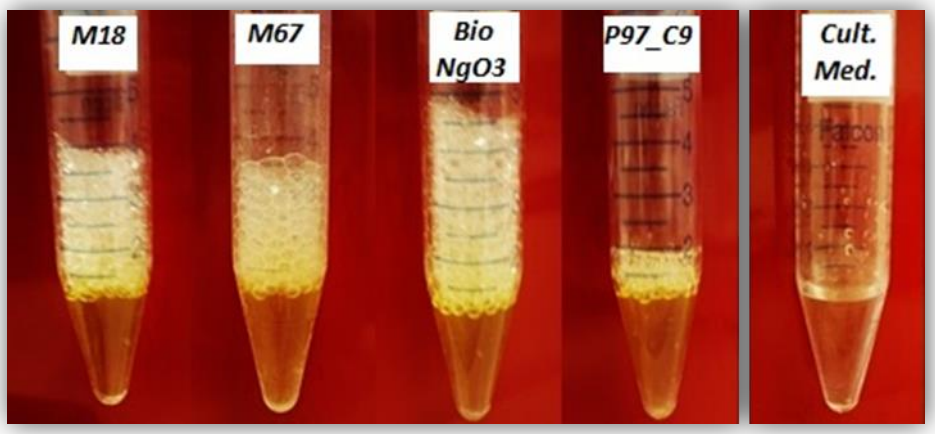

Figure 6 – Foam heights obtained for the supernatants of the four "carotenoids-producing" strains grown in nitrogen-limiting conditions. Culture medium showed no foam.

All strains were found to produce foam, being the highest value obtained for the *Bio16 EMS5Ng03* strain (3.7 mL), followed by *M18* (3.2 mL) and *M67* (1.7 mL), and finally by *P97_C9* (1.2 mL). In the foam stability analysis it was observed a small foam collapse, 1 mL for *M67* and *M18*, or 0.5 mL for *Bio16EMS5Ng03* and *P97_C9*.

Emulsification Index

Regarding the emulsification index assay, it was possible to visualize the formation of a third phase resulting from the mixture of two immiscible liquids after 24 h. Figure 7 shows the results obtained for the four "carotenoids-producing" strains along with the control (culture medium). The emulsion percentage was obtained as the index E_{24}.

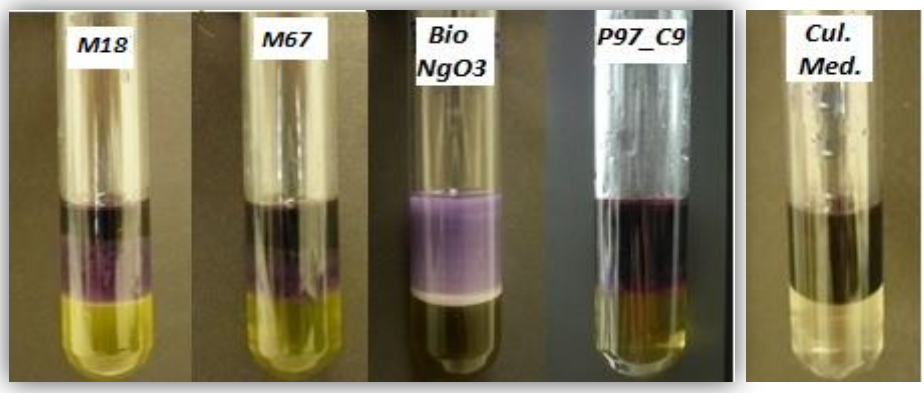

Figure 7 – Emulsification system kerosene/ culture supernatant for four "carotenoides-producing" strains grown in nitrogen-limiting conditions. Culture mediums showed no emulsion.

The emulsion formation was evident in the tube containing the *Bio16EMS5Ng03* strain, corresponding to 50% of the liquid total volume. The strains *M18* and *M67* showed a color change in the hydrocarbon phase, which corresponded to 37.5% and 25%, respectively. This effect was not observed for the *P97_C9* strain. After 72 h, all the emulsions remained, but according to Willumsen and Karlson [57] an emulsion is defined as stable if the emulsification index after 24 h was $E_{24} \geq 50\%$. Therefore only *Bio16EMS5Ng03* strain showed positive emulsification activity.

Oil Spreading test

The oil spreading test is based on the measurement of the diameter of the halo formed by the oil displacement in an aqueous surface, correlated to the surface activity of the biosurfactant. Figure 8 illustrates the halos formed in the surface after 30 sec of contact between the culture samples and the oil layer.

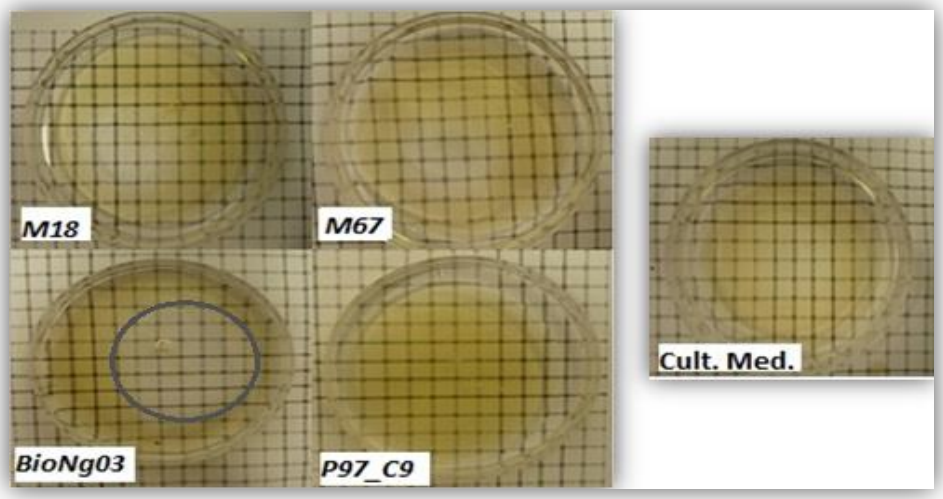

Figure 8 – Halos (diameters in mm) resulting from the oil dispersion when the supernatant of four "carotenoides-producing" strains grown in nitrogen-limiting conditions was placed onto the oil layer. Culture medium plates were negative for oil dispersion.

Clear zones could be observed for the strains *M18*, *M67* and *Bio16EMS5Ng03*, being the last strain the most potent dispersant with a halo of around 35 mm. Thavasi *et al* [32] also conducted a study to verify the presence of biosurfactants in 105 samples of culture through this screening method. Positive cultures were found when showed clear zones with diameters equal / greater than 5 mm. Within this group, 5 strains showed clear zones of 26 to 35 mm diameter and were considered strains with larger oil spreading activity. Therefore, the diameter obtained by *BioEMS516Ng03* under study suggests good activity oil displacement by this strain. The other strains showed small halos, which were not able to be quantified. In the plate corresponding to the *P97_C9* strain no oil displacement could be observed.

Du-Nouy-Ring Method

The surface tension values for the different strains and culture medium (control) were measured in duplicate using a tensiometer as previously described. The results shown in Table 16 correspond to the average of duplicate experiments.

Table 16 – Surface tension values for the four "carotenoids-producing" strains (supernatants) grown in nitrogen-limiting conditions. Culture medium was used as control

Strains	ST (mN/m)
M18	50.8 ± 0.75
M67	51.0 ± 0.50
Bio16EMS5Ng03	36.0 ± 0.05
P97_C9	58.8 ± 0.25
Culture medium	68.5 ± 0.50

As can be seen in the Table 16, only the *Bio16EMS5Ng03* strain presented biotensoactive activity, being able to reduce the surface tension of the culture medium to 36 mN/m. Cooper [58] suggested that the best surfactants can reduce the surface tension of water from 72 to less than or equal to 35 mN/m, while Willumsen and Karlson [59] defined a good surfactant has the one with the ability to reduce surface tension with greater differences than or equal to 20 mN/m, which is the case of the surface tension value herein obtained for the *Bio16EMS5Ng03* strain.

Often, the biosurfactant production occurs as a mechanism of adaptation from their producing microorganisms in conditions of stress, such as the low nitrogen concentrations used in these experiments [27, 30]. According to Albrecht *et al.* [60], the nitrogen limitation in the culture medium leads to a decline in the specific activities of NAD^+ and $NADP^+$ dependent isocitrate dehydrogenase, which is responsible for the oxidation of isocitrate α-ketoglutarate in the citric acid cycle. With the decline of the activity of this enzyme, a continuous accumulation of isocitrate occurs, and hence citrate accumulates in the mitochondria. Citrate is cleaved by citrate synthase yielding acetyl-CoA. This product is the precursor of the fatty acid synthesis, thus leading to an increase of the hydrophobic chain of the biosurfactants and consequently of their production.

Overall, the screening results demonstrated that *Bio16EMS5Ng03* presented biosurfactant activity, contrary to the *P97_C9* strain. *M18* and *M67* strains were only positive for some of the screening tests, foaming and emulsification index. All these tests were carried out using the culture supernatants collected at the end of the fermentation, thus corresponding to the decline stage in the case of *BioNgEMS51603*, *M67* and *M18*; and to the end of the exponential phase/early stationary phase for the *P97_C9* strain. However, the biosurfactant production, namely by *Bio16EMS5Ng03*, may have occurred before the decline phase. Therefore, it was evaluated a new growth for this strain, in order to better follow the exponential and stationary phases.

Several shake flasks experiments (under the same culture conditions) were performed, of which two were used to follow cell density along the fermentation time. The other flasks were used to perform the screening tests, namely foaming, emulsification index (E_{24}) and oil spreading.

One more time, the beginning of the foam formation occurred at the beginning of the exponential phase (annex B.1) being obtained a foam heigth of 1.2 mL. This foam height remained constant during the stationary phase. A slight emulsion and oil dispersing effect were observed along these two growth phases.

These results are not in accordance with the effects obtained for the decline phase in the previous growth experiments. Although, the several shake flasks have been subjected to the same conditions, in practice it was very difficult to maintain the same conditions between them. As the results were not reproducible, it is assumed that the biosurfactants production by *Bio16EMS5Ng03* is associated to the decline phase as mentioned previously. The biosurfactants production may have occurred when some cells started to die, by other cells in order to protect themselves from the adverse conditions, acquiring a greater chance of survival. Furthermore, several organisms have been reported to accumulate greater amounts biosurfactants on the cell membrane or inside the cell during the stationary growth phase, which are released to the culture medium when cell lysis occurs [10].

From another point of view, the cells membrane disintegration that is characteristic of decline stage in the cellular growth may have released components (phospholipids, lipids and proteins) that have the same a tensoactive structure (polar head and nonpolar tail) and may be responsible for the results obtained.

3.2.2 *Starmerella bombicola* Rose & Lachance

3.2.2.1 Cellular Growth and Biosurfactant Production

- Initial culture medium

For the cellular growth curve and foaming curve of *S. bombicola* Rose & Lachance (Figure 9), culture samples were taken at different time intervals up to 72 h of fermentation in a medium containing glucose (20 g/L) as carbon source.

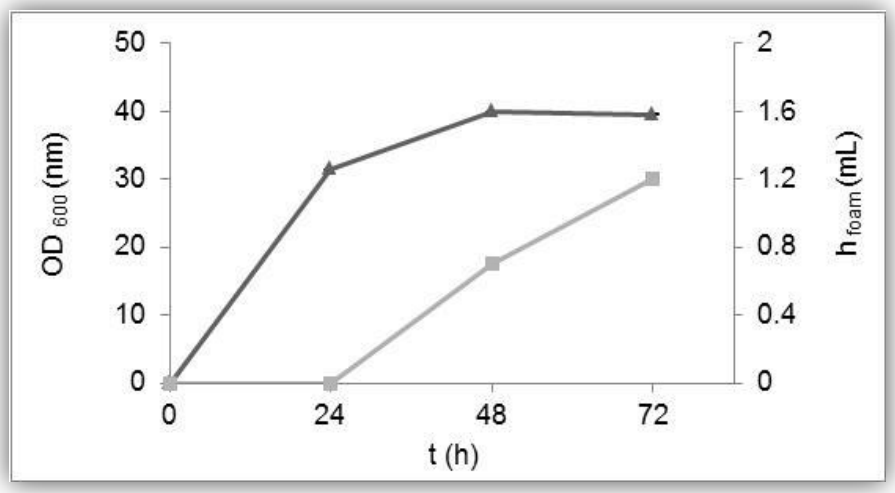

Figure 9 – Cell growth (OD600nm) (triangle) and foam height (mL) (square) along time (h) for *S. bombicola* Rose & Lachance grown in shake flask (25mL) containing 20 g/L glucose.

From Figure 9 it is possible to observe a progressive increase of the OD values in the first 24 hours (exponential growth) that after tend to stabilize until the 72 h (stationary phase). During the stationary phase, the foam is formed and at end of fermentation 1.2 mL foam were measured. The sophorolipids production by multiple species of the *Starmerella (Candida) bombicola* has been reported to occur when cells enter the stationary phase which is marked by nitrogen limitation [61, 65]. Therefore, these results suggest that the foaming occurring at the stationary phase may be associated with biosurfactant production. The stationary phase should have been extended after 72 h, i.e. more samples should have been taken at this stage, to determine if more foam amount would be produced.

- Optimal biosurfactant production

Media containing only hydrophilic substrate or combined with hydrophobic substrates were studied for optimizing the biosurfactant production by the *S. bombicola* Rose & Lachance.

High concentration of hydrophilic substrate

In the first experiment, *S. bombicola* Rose & Lachance was grown in a medium containing glucose as only carbon source, at a concentration of 100 g/L, during 168h. The growth and foaming curves are illustrated in Figure 10.

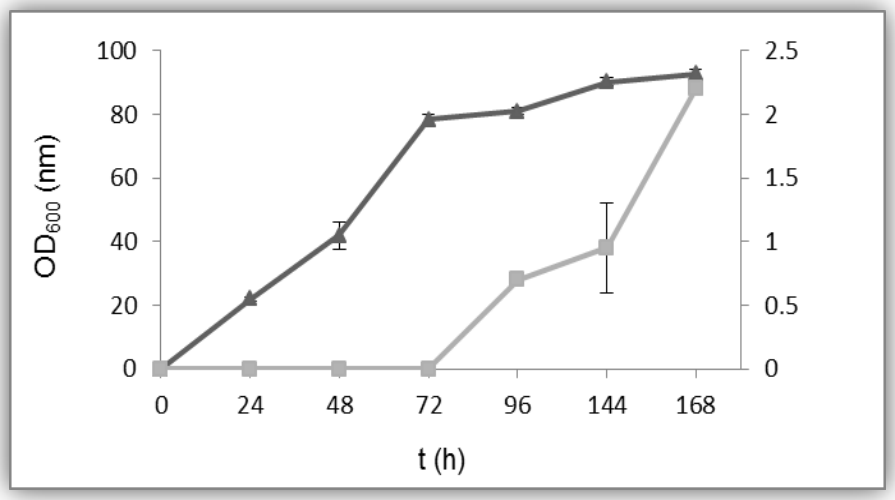

Figure 10 – Cell growth (O.D600nm) (triangle) and foam height (mL) (square) along time (h) for *S. bombicola* Rose & Lachance grown in shake flask (25mL) containing 100 g/L glucose.

The growth curve shows an exponential growth until 72 h, followed by the stationary phase until the end of the fermentation. The foaming began in the start of the stationary phase and increased until the end of the fermentation reaching 2.2 mL. Again, the foaming was evident at the stationary phase which is accordance with other studies reported on the biosurfactant production by several *S. bombicola* strains [61, 65].

During the exponentional growth phase (up to 72 h) in medium containing 100 g/L glucose, the pH was found to slightly drop. Therefore NaOH was added to the culture medium to maintain the pH at 3.5 which is the optimal value for sophorolipids production by *S. bombicola* strains [38]. This low pH and the antimicrobial effect of the sophorolipids allow the protection of the fermentation broth against contamination especially in processes that take more than 200 h [38].

Hydrophilic and hydrophobic substrates

S. bombicola Rose & Lachance was initially grown in a medium containing 20 g/L glucose. After the exponential growth phase, the culture was resuspended in different carbon sources (mainly hydrophobic) such as 100 g/L oleic acid; 100 g/L olive oil; 1g/L glucose combined with 100 g/L oleic; and 1 g/L glucose combined with 100 g/L olive oil. These lipophilic substrates acted as anti-foaming agents, thus preventing the evaluation of the foam formation along the fermentation. Consequently, Figure 11 only shows the cellular growth curves of the strain for the several carbon sources studied during 144 h of fermentation.

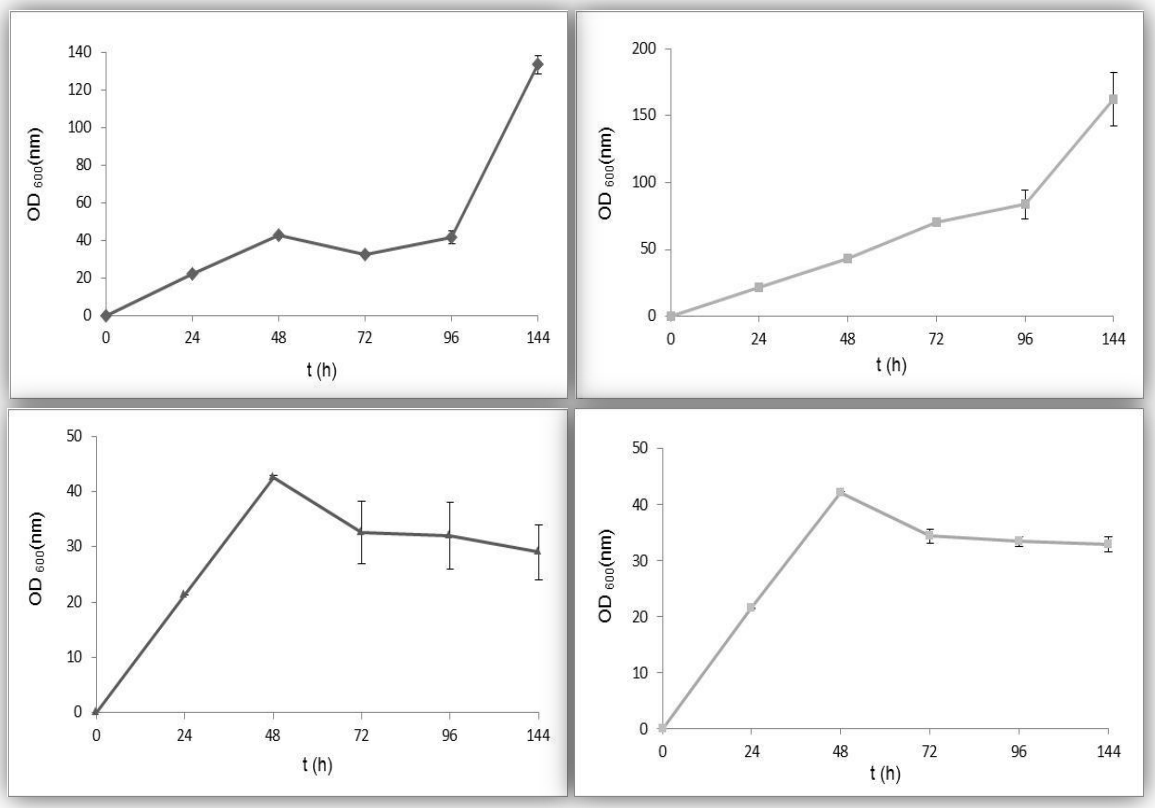

Figure 11 – Cell growth (OD600nm) along time (h) for *S. bombicola* Rose & Lachance grown in shake flask (25mL) containing initially 20 g/L glucose and resuspended in: 100 g/L oleic (the top left), 1 g/L glucose combined with 100 g/L oleic acid (the top right), 100 g/L olive oil (the bottom left) and 1g/L glucose combined with 100 g/L olive oil (the bottom right).

The strain, initially grown in glucose medium, reached the exponential phase after 48 h of fermentation. At this time point, the cells were resuspended in different substrate solutions and a different adaptation period was observed for each substrate studied.

From all curves, it is possible to see that the strain had a better adaption to the medium containing glucose and oleic acid, because OD values increased continuously along time.

In the other cases, it is observed a slight decrease of the cellular density at the time of the adaptation (72 h). This decrease could be related with the viscosity of the system due to the use of these lipophilic substrates and this increase in viscosity will lead to a decrease of mass transfer and aeration [64]. For this same reason, these substrates should be applied only after the cell growth stage. Besides, lipophilic elements are completely unnecessary for cell growth of *S. bombicola*, although could be of major relevance for the production of biosurfactants [63]. However, the following times (72-144 h) were marked by a new increase of the cell density in the medium with oleic acid; a slight decrease in the medium containing olive oil; whereas in the medium containing glucose and olive oil, the cell density reached a steady state. The media containing oleic acid showed higher OD values compared with other media evaluated, mainly due to the denser color of the sample (yellowish) resultant, eventually, from the reactions occurring within the cells due to the presence of oleic acid that resulted in biosurfactant production [68].

3.2.2.2 Influence of Culture Medium on the Biosurfactants Production

Several culture media were tested in order to study the best nutritional composition and conditions for the sophorolipids production by *S. bombicola* Rose & Lachance. The analysis of the biosurfactant production is initially presented for the cultures containing only a hydrophilic substrate, and then for the cultures containing both hydrophilic and hydrophobic subtrates.

- Hydrophilic substrate

Two media containing glucose (hydrophilic substrate) as the only carbon source at different concentrations, 20 g/L and 100 g/L, were used for the sophorolipids production by *S. bombicola* Rose & Lachance. The results obtained in the different screening tests for both culture media are shown below, in order to qualitatively evaluate the biosurfactant production in each culture condition tested. Culture medium was used as control to ascertain that the effects observed were not caused by any medium constituent with surface-active properties.

Foaming test

The foam heights obtained after 3 min of bubbling for *S. bombicola* Rose & Lachance grown in media containing 20 g/L and 100 g/L glucose are shown in Figure 12 together with their controls (culture media).

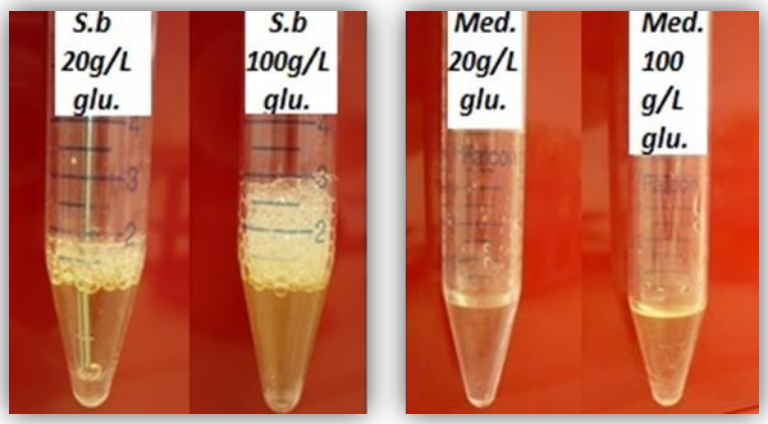

Figure 12 – Foam heights (mL) for the supernatants of *S. bombicola* Rose & Lachance grown in glucose, at 20g/L and 100g/L. Culture media (at the right) were negative for foam formation.

For both tubes containing culture supernatants (at the left) it was possible to visualize foaming, but for the second tube it was observed twice the foam amount as compared with the first tube. Thus, this *S. bombicola* strain was found to produce more foam in medium containing higher amounts of glucose (100 g/L). A decrease of 1mL in the foam volume was observed for both situations. In the case of culture grown in 20 g/L of glucose (first tube) it corresponded to the total foam collapse.

Emulsification Index

Emulsification capacity for *S. bombicola* Rose & Lachance grown in hydrophilic media are showed in Figure 13. Culture media were used as controls.

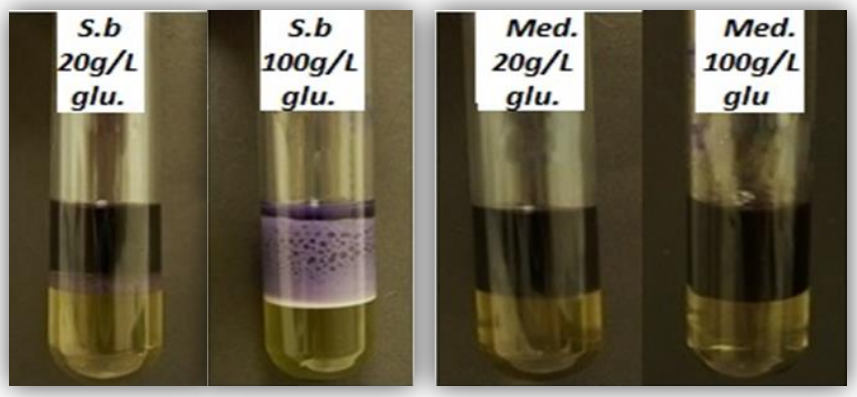

Figure 13 – Emulsification system kerosene/ supernatant of *S. bombicola* Rose & Lachance grown in glucose, at 20 g/L and 100 g/L. Culture media (at the right) were negative for emulsion formation.

In Figure 13 above it was possible to observe that the strain grown in 100 g/L glucose was able to form an emulsion with querosene. This emulsion corresponds to 50% of the original volume, but it didn't remain stable after 24 h, returning to its initial state. In the first tube (culture grown in 20 g/L glucose) it was possible to visualize the formation of a third phase (emulsion) that remained visible after 72 h but it could not be quantified. In both situations, it can consider that the strain was not able to form a stable emulsion (≥ 50% after 24 h). Some studies reported that sophorolipids from *S. bombicola* shown to reduce surface tension, but are not good emulsifiers [55].

Oil Spreading test

In the oil spreading test, the measurement of halos allows evaluating the dispersing power of the different samples, as can be seen in Figure 14.

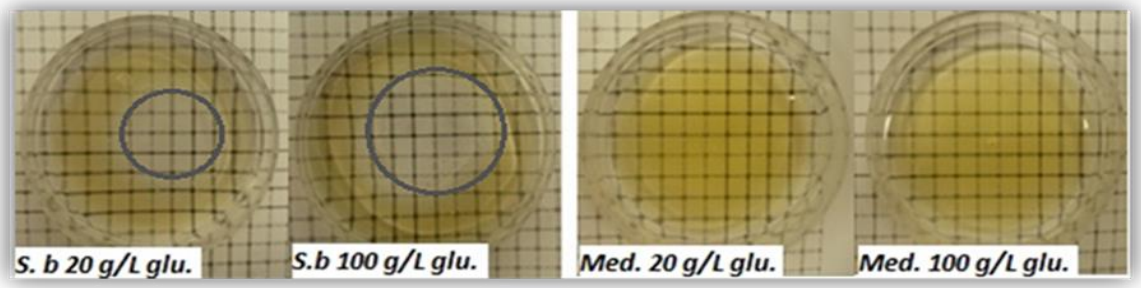

Figure 14 – Halos (diameter in mm) resulting from the oil dispersion when the supernatant of *S. bombicola* Rose & Lachance grown in medium containing 20 g/L and100 g/L was placed onto the oil layer. Culture medium plates were negative for oil dispersion.

The plates containing culture supernatant (at the left) showed oil dispersion comparing to their controls (at the right). The halos formed by the strain grown in medium with 20 g/L and 100g/L of glucose showed diameters of 20 mm and 30 mm, respectively, thus demonstrating that in the second case the dispersion power was higher. *S. bombicola* Rose & Lachance may be producing more biosurfactant in the second medium since the oil spreading power may be related with the biosurfactant concentration present in the medium [31]. However, both diameters of the halos obtained for *S. bombicola* Rose & Lachance showed good oil displacement activity compared to the diameters of the halos described in the literature for various strains as already referenced above [32].

Du Nouy Ring Method

Regarding the Du Nouy Ring method, the surface tensions of the culture grown in medium with 20 g/L and 100 g/L of glucose were measured, as well as of their controls. The values obtained correspond to the average of duplicated experiments along with their standard deviations (Table 17).

Table 17 – Surface tension values for *S. bombicola* Rosa Lachance (supernatant*)* grown in media with different concentrations of glucose, 20 g/L and 100 g/L. Culture media were used as control

Sample	ST (mN/m)
S. bombicola Rosa Lachance (20 g/L gluc.)	36.7 ± 0.05
S. bombicola Rosa Lachance (100 g/L gluc.)	35.5 ± 0.25
Culture medium (20 g/L gluc.)	57.2 ± 0.20
Culture medium (100 g/L gluc.)	59.8 ± 0.25

The results show that *S. bombicola* Rose & Lachance was able to reduce the surface tension in both media. The culture showed a surface tension reduction of 20.5 mN/m in the medium containing 20 g/L glucose and of 24.3 mN/m in the medium containing 100 g/L glucose. Although the surface tension values obtained are similar it is possible to infer that it is lower when the strain is grown in the medium with 100 g/L glucose. Both values obtained can be considered good results since they were not discrepant compared with the values referred in the bibliography for pure biosurfactants (as already mentioned).

The results of all secreening tests revealed that culture medium containing 100 g/L glucose was better for the production of biosurfactants by *S. bombicola Rose* & Lachance. Casas and Ochoa [64] demonstrated that a glucose concentration above or below of 100 g/L led to worst biosurfactant production yields, suggesting that this concentration is optimal for sophorolipids production. Most studies also reported that an initial glucose concentration of 100 g/L is optimal both for cell growth (maximum cell density could be reached in 30 h, although this has not been observed in the current work) and for sophorolipids production [63].

Besides changing the carbon source concentration, the peptone (20 g/L) used in the initial medium was replaced by urea in a lower concentration (1 g/L). Through this study can not conclude whether the urea also contributed to a better biosurfactant activity. For Rispoli *et al* [69], both the peptone as the urea were considered substrates with a negligible influence on sophorolipids production. Therefore, this change in the nitrogen source, possibly, had a little or no influences in the biosurfactant production.

- Hydrophilic and hydrophobic substrates

Several hydrophobic substrates (hexadecane, oleic acid and vegetable oils) have been studied for the sophorolipids production by *S. bombicola* strains [62, 65, 67]. Oleic acid or olive oil were used as secondary carbon sources and were added after the exponential cell growth of *S. bombicola* Rose & Lachance grown initially in 20 g/L glucose.

The evaluation of the biosurfactants production through screening tests was difficulted due to the interference of lipophilic substrates contained in the sample, as mentioned previously. The Du-Nouy-Ring test was not performed in these cases since the lipophilic substrates could damage the platinum ring of the tensiometer. This test can be used in a later step after the biosurfactants extraction, removing these lipophilic substrates with the hexadecane apolar solvent [62, 65].

The expected effects in the screening tests for the biosurfactants presence could only be detected when the culture was resuspended in the solution containing both carbon sources, hydrophilic (1 g/L glucose) and hydrophobic (100g/L oleic acid). In the foaming test it was observed some foam bubbles but it was not possible to quantify them because they totally collapsed. Figure 15 illustrates the results obtained in the emulsification index test and oil spreading test with the cells suspension and the controls (culture medium).

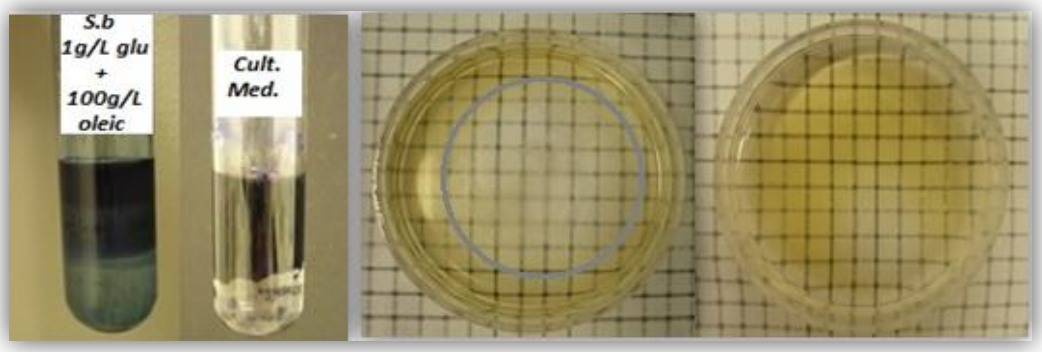

Figure 15 – Emulsification system kerosene/ cells re-suspension and halo (mm) resulting from the oil dispersion obtained with re-suspension of *S. bombicola* Rose & Lachance grown in 1 g/L glucose and 100g/L oleic acid was placed.

From Figure 15, it is possible to observe an emulsification index value (E_{24}) of 30 % and a diameter of the clearing zone 40 mm. Compared with the culture media containing only a hydrophilic substrate, the E_{24} obtained in this culture was higher than in the medium containing 20 g/L glucose and smaller than in medium containing 100 g/L glucose. However, in medium with 100 g/L glucose, the emulsion formed was not as stable as the one observed in this culture. Despite oleic acid also shows some oil dispersion (control plate), it is remarkable the greater dispersion power of the strain in this culture comparing with the other hydrophilic media.

After a centrifugation at 9000 rpm for 10 min, a brown and viscous oil precipited (crude-sophorolipid) was obtained at the bottom of the falcon containing glucose and oleic acid like carbon sources (Figure 16c). This compound could be the result of the reaction between the yeast biomass and the glucose and oleic acid [68]. For the last tube (d), it was possible to observe a slight brown precipitate, which indicates that a very small amount of biosurfactant may have been produced in the medium containing glucose combined with olive oil. In the other tubes, no precipitate could be observed.

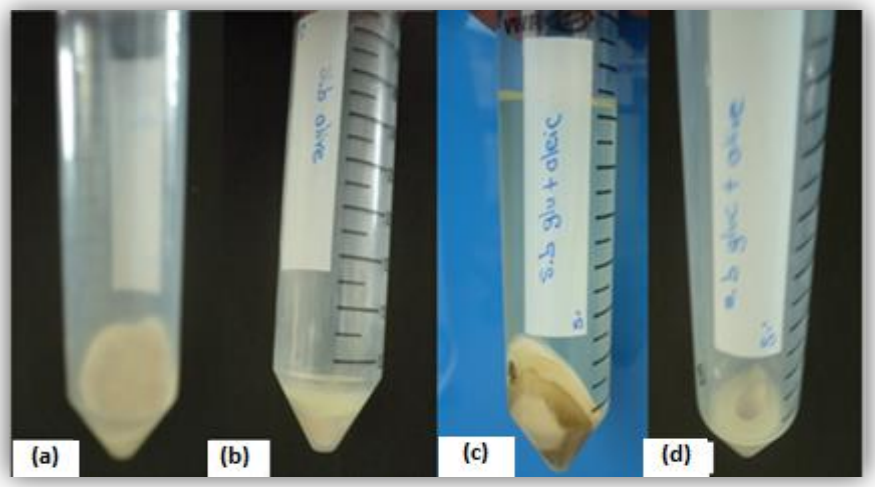

Figure 16 – Cell pellets and a brown and viscous liquid (crude-sophorolipid) obtained for *S. bombicola* Rose & Lachance resuspended in: (a) 100 g/L oleic acid, (b) 100 g/L olive oil, (c) 1 g/L glucose and 100 g/L oleic acid, and (d) 1g/L glucose and 100 g/L olive oil.

Of all these cultures, 1 g/L glucose and 100 g/L oleic acid was the only medium in which the presence of biosurfactants could be infered, confirmed by the brown oil precipitate and also the screening tests results. This result is consistent with the theory which states that a greater of biosurfatants production can be obtained when supplying a mixture of hydrophobic and hydrophilic substrates.The hydrophilic substrate is used primarily in cellular respiration and in the sophorose synthesis, while the lipophilic substrate is used exclusively for the lipidic portion production of the biosurfactants, thus leading to a highest sophorolipids production [63, 65].

In this study, oleic acid was the best lipophilic precursor for sophorolipids production. Solaiman *et al.* [66] also mentioned that among lipophilic substrates, the oleic acid presents the highest sophorolipids production yields. It may be related with the fact that the oleic acid molecule is more easily converted to free fatty acids and afterwards hydroxylated to sophorolipids than olive oil that is a mixture of glycerol and three fatty acids (oleic acid is predominant). However, it would be important to study the substrates consumption to confirm this hypothesis. The oleic acid cost is relatively high, thus in a future study should explore more inexpensive substrates, such as oil residues containing high oleic acid contents, e.g. rapeseed oil [63].

In order to verify that the effects observed in the screening tests are due to the presence of biosurfactants it would be necessary to extract, purify and characterize the product. The extraction could be carried with ethyl acetate and afterwards with hexadecane to remove the lipophilic subtrates in the cases where these were used [62, 65]. The chemical structure characterization could be determined using thin layer chromatography (TLC) or other chromatography techniques as HPLC (High-performance liquid chromatography) [18, 62, 65]. The morphology of the product (sophorolipids) is generally characterized by an oily brownish precipitate, or white crystals [62, 65]. Some studies reported that oily products are obtained when oleic acid or elevated glucose concentrations are used, as in this study [62, 65, 66]. These products are mixtures of several structural types of sophorolipids, thus being more complicated their recovery and purification [65]. Hence, this last step would be very time consuming and painful, besides being very costly.

3.2.3 *Rhodococcus erythropolis* DCL14

3.2.3.1 Cellular Growth and Biosurfactant Production

- Initial culture Medium

The relation between cellular growth and foaming for *R. erytropolis* DCL14 during 72 h of fermentation in a medium containing 10 g/L glucose and 10 g/L glycerol as carbon sources is shown in Figure 17. The foaming curve was performed both with supernatant as culture broth.

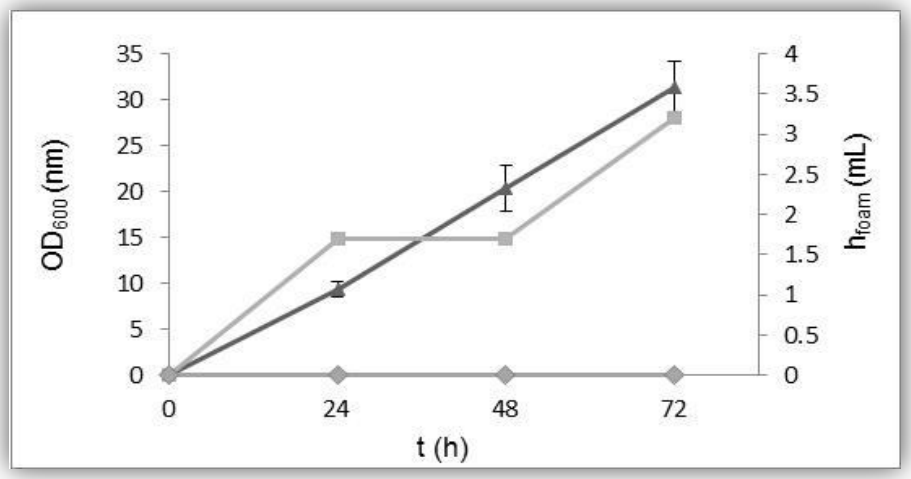

Figure 17 – Cell growth (OD600nm) (triangle) and foam height (mL) along time (h) for the broth (square) and supernatant (rhombuses) of *R. erythropolis* DCL14 grown in shake flask (25mL) containing 10 g/L glucose and 10 g/L glycerol.

Along fermentation time, an increase in the cell density was observed. However, in the last time it was not checked cellular multiplication, which may indicate the beginning of the stationary phase. The evolution of the foam height was only observed for the culture broth, thus indicating that *R. erythropolis* DCL14 can be producing biosurfactants associated to the cell wall. The foam height remained constant during the exponential growth phase with 1.7 mL and registered a maximum value of 3.2 mL at the end of the fermentation. Several studies have shown that the cell-bound biosurfactants production by *R. erythropolis* strains is cell-growth associated, but higher yields can be obtained in the stationary phase [10, 70]. However, it would be necessary to take more samples after the 72 h of fermentation to have a better assessement of the stationary phase.

- Optimal biosurfactant production

Hydrophilic and hydrophobic substrates

Media supplemented with 100 g/L oleic acid or 100 g/L olive oil were used to increase the biosurfactants production. Due to the lipophilic nature of these substrates, namely their anti-foaming ability, the foaming test could not be performed. Therefore, only the growth curves of *R. erythropolis* DCL14 for these lipophilic substrates are shown in Figure 18.

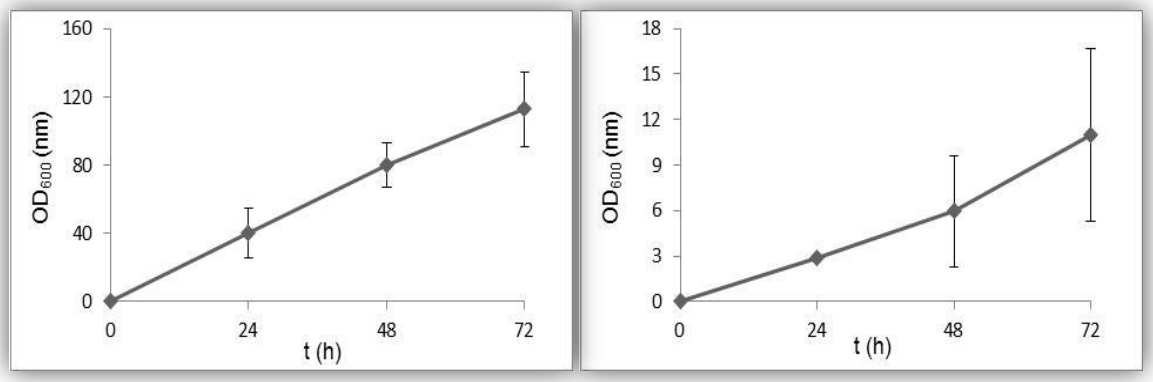

Figure 18 – Cell growth (OD600nm) along time (h) for *R. erythropolis* DCL14 grown in shake flask (25mL) containing hydrofilic medium suplemmented with 100 g/L oleic acid (at the letf) or 100 g/L olive oil (at the rigth).

In the left figure, corresponding to the strain grown in the medium containing oleic acid as a lipophilic precursor, it was observed an exponential increase of the cellular density up to 48 h. From this time point, a slowdown in cell growth was observed, thus reflecting the beginning of the stationary phase. The strain growth in the medium with olive oil occurred very slowly. However, it is important to notice that when recording the OD values the duplicates gave very different results, as can be seen through the standard deviations, due to the presence of several oil droplets in the sample.

Higher OD values were visible for the medium supplemented with oleic acid comparing with the medium with olive oil. It was due to the whitish color of the culture sample that became increasingly denser throughout the fermentation which can suggest the formation of a product or metabolite, as the biosurfactants. Therefore, the oleic acid appears to be degraded more easily than the olive oil, but this does not imply that the last substrate doesn't favor the biosufactant production. The biosurfactants production can occur as a strategy for the survival of this strain in the medium with olive oil, so that the strain is able to solubilize and assimilate the oil to cells [27, 30].

3.2.3.2 Influence of Culture Medium on Biosurfactant Production

Two different media were studied, the first contained only hydrophilic substrates (corresponding to the "initial culture medium") and the second contained both hydrophilic as hydrophobic subtrates (corresponding to the "optimal biosurfactant production"). The evaluation of the biosurfactants production by *R. erythropolis* DCL14 grown in the first medium was discussed previously, through the results screening presented in Tables 14 and 15. Due to the interference from oleic acid or olive oil present in the second medium, the screening tests could not be performed with success. However, it was possible to verify biosurfactant activity by the strain in these media as is demonstrated below.

- Hydrophobic and hydrophic substrates

At the end of the fermentation, after 72 h, different behaviors of the strain were observed when cultured in different culture media, supplemented with oleic acid or olive oil, by the analysis of the culture broths (Figure 19) and the respective cell pellets (Figure 20). Controls (media without cells) were also incubated for 72 h.

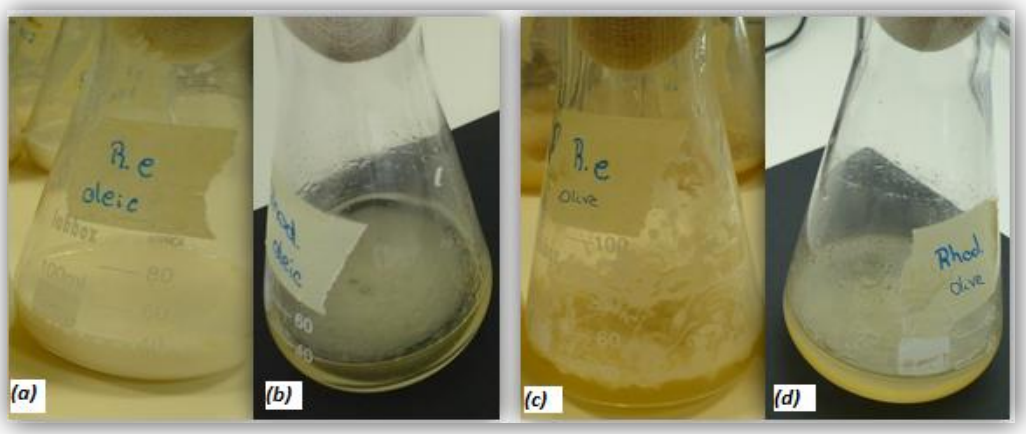

Figure 19 – Culture broth obtained at the end of the fermentation in medium containing lipophilic precursors: oleic acid (a) and its control (b), olive oil (c) and its control (d).

Figure 20 – Cell pellets: white fragments suspended and yellowish oily paste of *R. erythropolis* DCL14 grown in medium containing oleic acid and oil olive, respectively.

Both the culture broths presented a distinct appearance comparing to their controls in the end of the fermentation. It is further observed that none of these effects were verified for the culture in hydrophilic medium. A whitish homogeneous appearance was observed for the culture broth containing oleic acid, resultant of the emulsification between the hydrophobic phase (oleic acid) and the hydrophilic phase (aqueous phase). Regarding the culture broth containing olive oil it was observed a white oily upper phase (emulsion) and a bottom phase corresponding to the aqueous phase of the culture medium. These emulsions formed in both media, more visible in the oleic acid, may be due to the presence of biosurfactant. The biosurfactant production by *R. erythropolis* DCL14 may have occurred in lesser amount in the medium with olive oil, since also lesser emulsion amount was observed.

From another point of view, the different emulsions can be the result of the different biosurfactants production by the strain when cultured in distinct media, since the culture media are known to influence the properties and structures of the biosurfactants [10, 13].

After the centrifugation of the culture samples, cell pellets were not deposited at the bottom of the Falcon tubes as usually occurs. In the first Falcon tube white fragments were obtained suspended in the culture supernatant, corresponding to the oleic acid emulsified and pellet. The lipophilic material is lighter than the aqueous phase (supernatant), so it remained in the upper phase. In the second tube it was possible to observe a yellow paste (similar to a biofilm) on the tube walls.

Thus, in both cases the cells migrated to the hydrophobic phase. The structure of the cell membrane usually does not have affinity to hydrophobic materials (non-polar) due to its hydrophilic head (polar) that is exposed to the outside. However, the presence of a biosurfactant membrane-associated can function as a mediator allowing the contact between the polar exterior phase of the cellular membrane and the apolar lipophilic medium (oleic acid and olive oil), thus capturing these substrates to the cells [12, 31, 33].

In this study, *R. erythropolis* DCL14 grown both in hydrophilic medium or medium combined with hydrophilic and hydrophobic subtrates can be producing biosurfactants as part of the cell membrane. Trehalose lipids are biosurfactants membrane-associated that are commonly produced by *R. erythropolis* strains in the exponential or stationary phase, as it was also seen in the current work [30, 70]. These glycolipids type contain trehalose as the major carbohydrate along with (unsaturated and saturated) fatty acids and fatty alcohols [70].

Given the limitations associated with the extraction and purification of metabolites produced intracellularly or associated to the cell wall, Biotrend opted to not proceed with this study. However, some *R. erythropolis* strains have been reported as extracellular biosurfactants producers in other culture media, namely in the presence of heavy metals (Cu_2^+, Cd_2^+, and Pb_2^+) [30]. Therefore, the addition of heavy metals to the medium can be an alternative way to study biosurfactants production by the strain under study.

3.3 Foaming Test validation

There are features that make the screening methods more or less advantageous; being therefore determinants in the choice of the method for the selection of biosurfactants producing microrganisms (see 1.7).

All the experiments that were conducted using the foaming test demonstrated its usefulness for screening biosurfactant-producing microrganims. The selection power of this screening test was confirmed by the similarity of the results obtained in the other complementary screening methods that have been described in the literature and that were herein used.

Foaming is a simple and easy test (not requiring the use of very sophisticated materials), fast (min), and the sample amount required is quite small. The foaming test can be considered specific for the detection of biosurfactant-producing microrganisms since the controls (culture medium) were all negative, thus meaning that no medium components with surface activity (e.g. yeast extract) interfered with the test. The test specificity can become limited when components of the cell membrane disintegration are released to the culture medium, e.g phospholipids, which may also cause foaming.

Hydrophobic substrates cannot be included in this assay, since they act as anti-foaming agents, being this one of the major disadvantages of the foaming test.

The great interest of the Biotrend, in the implementation of this screening test in their experimental studies is due to the fact of the foaming test include the minimum requisites for a High Throughput Screening [32], such as: (1) ability to identify potential organisms; (2) ability to assess quantitatively how effective the surfactant is; (3) ability to analyze quickly a great number of potential candidates in microplates.

CHAPTER 4

CONCLUSION AND FUTURE PERPECTIVES

In order to fulfill the aim of this work, validation of a biosurfactants screening test, different strains and strategies were studied.

Four "carotenoids-producing" strains, *S. bombicola* Rosa & Lachance and *R. erythropolis* DCL14 were considered potential biosurfactant producers after a first screening.

The optimization of the biosurfactants production demonstrated that the nitrogen limitation in the growth of the "carotenoids producing" strains provided better results in the screening tests, except for *P97_C9*. The strain *Bio16EM55Ng03* led to 3.7 mL foam from an liquid volume of 0.8 mL culture sample; E_{24}= 50%; 35 mm oil dispersion; and demonstrated ability to reduce the surface tension of the culture medium to 36.0 mN/m ± 0.05. *M18* and *M67* only demonstrated foaming and emulsifying capacities. These properties obtained in the screening tests, namely, for *Bio16EM55Ng03* were observed in its decline growth phase. This strain may produce biosurfactants extracellularly or intracellularly/membrane-associated that can be released into the culture medium during cell membrane lysis. However, components of the cell membrane disintegration may also be responsible by the effects observed.

S. bombicola Lachance & Rose grown in 100 g/L glucose showed better results in the screening tests than in the 20 g/L glucose medium, with 2.2 mL foam (in 0.8 mL culture sample); E_{24}=50% (unstable); 30 mm oil dispersion; and a surface tension of 35.5 mN/m ± 0.25. It can be concluded that a high glucose concentration improves the biosurfactants production by this strain. The biosurfactants were found to be produced in the stationary phase.

The addition of hydrophobic substrates, after the exponential growth of *S. bombicola* strain in medium solely hydrophilic, demonstrated that the combination of glucose and oleic acid dually functioned as energy sources to the biosynthesis of a crude-biosurfactant (brown oily precipitate) with E_{24}= 30% and 40 mm oil dispersion. Despite of the oleic acid interference in the screening tests, it was considered a good substrate, which can be replaced by low cost raw materials as rapeseed oil.

R. erythropolis DCL14 grown in a hydrophilic medium was found to produce membrane-associated biosurfactants, leading to a foam height of 3.2 mL (in 0.8 mL

culture sample); a slight emulsion and oil dispersion. The biosurfactants were found to be produced from of exponential growth but better results were obtained in the stationary phase.

In culture media combining hydrophilic and hydrophobic substrates, *R. erythropolis* DCL14 was found to form emulsions. Oleic acid was the best lipophilic substrate emulsified. The affinity between the cell membrane and the lipophilic substrates may indicate that the biosurfactants production is associated with the cell membrane. During cell growth, the emulsification of oleic acid was observed from the exponential phase.

Finally, the validation of the foaming test as a preliminary screening assay for the selection of biosurfactant-producing organisms was successfully achieved, given its characteristics such as precision, specificity, fasteness, simplicity and ability to analyze a great amount of potential candidates in microplates.

In order to further confirm the results herein obtained and to improve the applicability of the foaming test, several studies should be conducte.

The biosurfactants production by strains under study should be confirmed through the implementation of extraction/purification steps and structural characterization of their molecules. Thereafter, it would be important to re-conduct the screening tests to characterize the biosurfactant regarding its physicochemical properties.

Although some satisfactory results were obtained, it is worth noting that there were some limitations at the experimental level. Therefore, it is suggested an accurate assessment of the optimization of biosurfactants production, namely the modification of one variable at the time (nutrient or culture condition) keeping the others fixed. Alternatively, more efficient statistical methods, including the fractional factorial design and response surface methodology, can be used to improve the production yields. Given that the biosurfactants production using non-conventional substrates was promising, it would advantageous to evaluate other alternative substrates.

For the validation of the foaming test, it would be important to follow a reference plan for analytical methods validation in order to assess whether the foam test gathers all the characteristics underlying in an analytical method.

In a more advanced phase of the study, it would be interesting to study the antimicrobial activity of the biosurfactants being produced, and also to perform a toxicity control test since these are essential characteristics and of utmost importance for the biosurfactants use in cosmetic products, which is within the interest area of the "O_4S" project.

CHAPTER 5

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CHAPTER 6

ANNEXES

Annex A – Determination of the rotation speed (rpm) of the peristaltic pump

The rotation speed (rpm) was determined replacing the value of the flow rate (1 mL/min) in the equation of the calibration curve of the pump supplied by Biotrend. The flow rate is given by "y" and the rotation speed by "x". Thus, applying equation 2 is obtained:

$$\boldsymbol{y = 0.1322\ x - 0.59} \quad \textbf{(Equation 2)}$$

$$1 = 0.1322\ x - 0.59$$

$$x = \frac{1}{0.1322} + 0.59 \approx 8\,rpm$$

Annex B - Cellular Growth and Biosurfactant Production for *Bio16EM55Ng03*

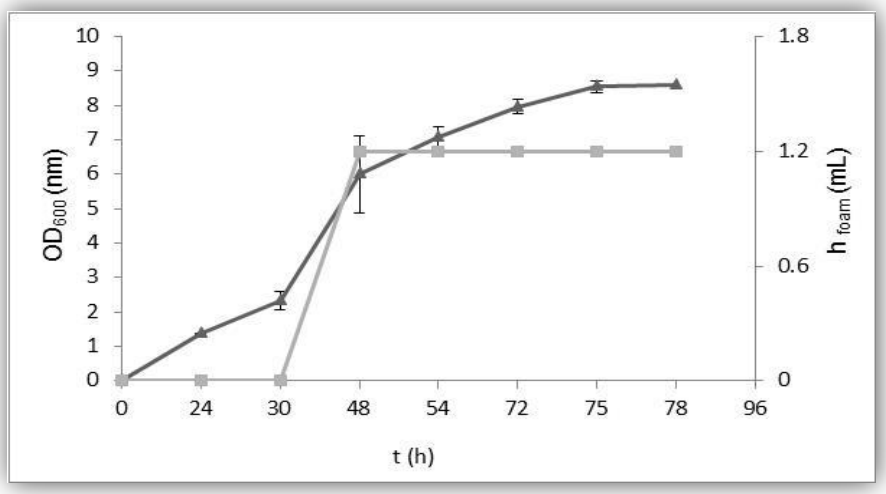

Figure B.1 – Cell growth (OD600nm) (triangle) and foam height (mL) (square) along time (h) for *Bio16EM55Ng03* grown in (25mL) under nitrogen-limiting conditions.