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Study of Biosurfactant “Cocktails” with Enhanced Properties

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ABSTRACT

Biosurfactants are molecules produced by a variety of microorganisms that exhibit pronounced surface and emulsifying activities. A wide range of chemical structures can be found among these compounds. Hence, it is reasonable to expect diverse properties and physiological functions for different groups of biosurfactants. Thus, the aim of this thesis was to prepare mixtures of biosurfactants produced by different microorganisms, in order to evaluate their main characteristics (surface tension, emulsification ability, antimicrobial and anti-adhesive activities) for several applications, namely in the oil recovery, health care and biomedical fields.

Initial screening revealed that biosurfactants from *Lactobacillus agilis* CCUG31450, *Bacillus subtilis* PX573 and *Pseudomonas aeruginosa* PX112 possessed the most notable activity. Therefore, strategies to improve biosurfactants yield and reduce production costs were conducted. The *B. subtilis* PX573 and *P. aeruginosa* PX112 biosurfactants yields increased 2.8 and 2.5 times, respectively; due to the optimization of the agitation and use of low-cost substrates. Moreover, the development of an efficient downstream process led to an additionally increase of the amount of *P. aeruginosa* PX112 biosurfactants recovered. The evaluation of biosurfactants stability at extreme environmental conditions was performed. The results showed that biosurfactants were very stable, suggesting their application in bioremediation and in the oil industry. The antimicrobial, anti-adhesive and oil recovery activity of the produced biosurfactants were evaluated, first individually and then within different mixtures. In the antimicrobial assays, individual biosurfactants from *B. subtilis* PX573 and *P. aeruginosa* PX112 showed the most interesting results, mainly against Gram-positive bacteria. However, it was with the mixture of biosurfactants from lactic acid bacteria *Lactobacillus paracasei* A20 and *Lactobacillus animalis* ATCC35046 that the most remarkable synergetic effect was observed. In the case of the anti-adhesive assays, the probiotic biosurfactants from *L. agilis* CCUG31450 were the most effective and the mixture *P. aeruginosa* PX112 (CSLM) and *L. agilis* CCUG31450 (MRS) biosurfactants showed the most positive interaction. At last, from the oil recovery assays it was found that biosurfactants from *B. subtilis* PX573 and *P. aeruginosa* PX112 were equally or even a more effective than the chemical surfactants tested.

In conclusion, each mixture tested in the different assays showed a unique activity that was dependent on the type of biosurfactant, the proportion of the mixture and the pathogenic strain studied. In some cases, an interesting synergetic effect between different biosurfactants was achieved that consequently enhanced their activity in the diverse applications tested.

SUMÁRIO

Os biosurfactantes são moléculas produzidas por uma variedade de microrganismos que exibem uma interessante atividade de superfície e emulsionante. Estes compostos podem apresentar uma grande variedade de estruturas químicas. Assim, é aceitável esperar que os diferentes grupos de biosurfactantes possam apresentar diversas propriedades e funções fisiológicas. Deste modo, esta tese teve como objetivo a preparação de misturas de biosurfactantes produzidos por diferentes microrganismos, de forma a avaliar as suas principais características (tensão superficial, capacidade de emulsificação, atividades antimicrobiana e anti-adesiva) e testar a sua aplicação nas áreas de recuperação do petróleo, saúde e biomédica.

Uma análise inicial revelou que os biosurfactantes produzidos por *Lactobacillus agilis* CCUG31450, *Bacillus subtilis* PX573 e *Pseudomonas aeruginosa* PX112 apresentavam uma atividade mais notável. Assim, foram aplicadas estratégias para promover o aumento da produção de biosurfactantes e a redução dos respetivos custos. O rendimento dos biosurfactantes de *B. subtilis* PX573 e *P. aeruginosa* PX112 aumentou 2.8 e 2.5 vezes respetivamente, devido a otimização da agitação e do uso de substratos de baixo custo. Além disso, o desenvolvimento de um processo eficiente de recuperação levou a um aumento adicional dos biosurfactantes de *P. aeruginosa* PX112 recuperados. Foi realizada a avaliação da estabilidade dos biosurfactants em condições ambientais extremas. Os resultados obtidos mostraram que os biosurfactantes eram bastante estáveis, sugerindo a sua aplicação na biorremediação e na indústria petrolífera. Foi, de igual modo, avaliada a atividade antimicrobiana, anti-adesiva e de recuperação de petróleo, primeiro individualmente e depois com diferentes misturas de biosurfactantes. Nos testes antimicrobianos os biosurfactantes individuais produzidos por *B. subtilis* PX573 e *P. aeruginosa* PX112 revelaram os resultados mais interessantes, principalmente contras as bactérias Gram-positivas. Contudo, foi na mistura preparada com biosurfactantes das bactérias lácticas *Lactobacillus paracasei* A20 e *Lactobacillus animalis* ATCC35046 que se observou o efeito sinergético mais notável. No caso dos ensaios anti-adesivos, os biosurfactantes probióticos de *L. agilis* CCUG31450 foram os mais efetivos e a mistura com biosurfactantes de *P. aeruginosa* PX112 (CSLM) e *L. agilis* CCUG31450 (MRS) revelou a interação entre biosurfactantes mais positiva. Por fim, nos ensaios de recuperação de petróleo verificou-se que os biosurfactantes de *B. subtilis* PX573 e *P. aeruginosa* PX112 eram igualmente ou até mesmo mais efetivos do que os surfactantes químicos testados.

Em conclusão, cada mistura testada nos deferentes ensaios revelou uma atividade única que era dependente do tipo de biosurfactante, da sua proporção na mistura e da estirpe patogénica testada. Em alguns casos, foi observado um efeito sinergético entre os biosurfactantes misturados, levando consequentemente um aumento da sua atividade nas diversas aplicações testadas.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CMC	Critical Micelle Concentration
CSL	Corn Steep Liquor
CSLM	Corn Steep Liquor and Molasses
CTAB	Cetyltrimethylammonium bromide
CWM	Cheese Whey Medium
E_{24}	Emulsification index
E_{24}^{-1}	Emulsification index of a sample 10 times diluted
E_{24}^{-2}	Emulsification index of a sample 100 times diluted
FTIR	Fourier Transform Infrared Spectroscopy
HLB	Hydrophilic-Lipophilic Balance
LB medium	Medium introduced by Luria-Bertani
LPS	Lipopolysaccharide
M17 medium	Medium for improved growth of lactic streptococci
MEL	Mannosylerythritol lipids
MEOR	Microbial Enhanced Oil Recovery
MIC	Minimum Inhibitory Concentration
MRS medium	Medium introduced by De Man, Rogosa and Sharpe for cultivation of *Lactobacillus* species
OOME	Olive oil mill effluent
PBS	Phosphate-buffered saline
Rf	Retention factor
RSM	Response surface methodology
SD	Standard deviation
ST^{-1}	Surface tension of a sample 10 times diluted
ST^{-2}	Surface tension of a sample 100 times diluted
TLC	Thin-layer chromatography

CONTEXT AND MOTIVATION

The interest in microbial surfactants has been progressively increasing in the recent years due to their diversity, environmental friendly nature, performance under extreme conditions and their biological activities (antimicrobial, anti-adhesive, antifungal, antiviral). These remarkable characteristics have led biosurfactants to be an asset in a wide range of potential applications, such as therapeutics, biomedical, enhanced oil recovery and food processing. In spite of the immense potential of biosurfactants, their use and production at an industrial scale still remains limited, mainly due to their high production and extraction costs, low production yields and lack of information on their toxicity towards human systems.

In order to overcome this problem, making biosurfactant production economically feasible, a number of strategies have been well explored, for instance the use of cheaper substrates, optimal growth and production conditions coupled with novel and efficient multistep downstream processing methods, and the use of recombinant and mutant hyper producing microbial strains. Another alternative, not yet explored, is the use of biosurfactant "cocktails" with different microbial origins to enhance a given feature for a particular application. Since biosurfactants can show a wide range of chemical structures, it is predicted that their positive interaction could enhance their properties.

In summary, the combination of these strategies will help to overcome the economic barriers of biosurfactants production and at the same time to improve their properties and make them more attractive for industrial applications.

REASERCH AND AIMS:

The main goal of this work was the preparation of mixtures of biosurfactants produced by different microorganisms to study their main characteristics towards different applications. In this sense, the secondary aims were the following:

- Evaluation of biosurfactants production by different microorganisms, as well as their production profile;

- Optimization of biosurfactants yields and reduction of production costs using low-cost substrates;

- Characterization of biosurfactants, namely their physicochemical and biological properties;

- Preparation of mixtures of biosurfactants synthetized by different bacteria;

- Validation of biosurfactants mixtures activities in diverse applications, namely as anti-adhesion and antimicrobial agents, and their capacity to recover oil envisaging their potential application in MEOR.

1. INTRODUCTION

Over the years biosurfactants have been studied and characterized in order to assess their activity and understand their biological role and value.

1.1. Microbial surface-active compounds

Several microorganisms produce molecules with surface activity, designated biosurfactants [1], which are mostly either anionic or non-ionic [2]. These molecules can be cell-bound or excreted by the microorganisms. Structurally, biosurfactants are amphipihilic molecules, i.e. consist of a hydrophilic (polar) moiety that can be an amino acid, carbohydrate, cyclic peptide or phosphate; and a hydrophobic (nonpolar) moiety composed of unsaturated or saturated hydrocarbon chains or fatty acids. Based on their structure the biosurfactants are able to accumulate between fluid phases such as oil/water or air/water, lower the surface and interfacial tension of liquids, and form micelles and microemulsions between two different phases [1–3]. The biosurfactants have unique properties, such as biodegradability, low toxicity and stability in adverse conditions, that confers to these molecules several advantages as compared to the chemical surfactants [2]. Additionally, due to their characteristics and natural roles these molecules have gained importance in diverse applications. Depending on their chemical composition and properties, biosurfactants could be more suitable for one or other application.

1.1.1. Classification of biosurfactants

Generally, biosurfactants are classified according to their chemical composition or microbial origin [3]. Rosenberg and Ron [4] proposed that microbial surface active compounds can be divided into low-molecular-weight molecules, the so-called biosurfactants that reduce surface and interfacial tension more efficiently; and high-molecular-weight polymers, named as bioemulsifiers that possess powerful emulsion-stabilizing activity.

The most common biosurfactants include glycolipids, lipopeptides and phospholipids, while the bioemulsifiers include polymeric and particulate compounds [5]. Table 1 summarizes the different classes of biosurfactants and their producers.

Table 1. Types of biosurfactants and their producers. (Adapted from [2–4, 6]).

Biosurfactant class	Producing microorganism
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas chororaphis*
Sophorolipids	*Candida bombicola, Candida apicola, Candida antarctica, Torulopis petrophilum*
Mannosylerythritol lipids (MEL)	*C. antarctica, Pseudozyma aphidis, Pseudozyma rugulosa*
Trehalose lipids	*Norcadia erithropolis, Rhodococcus erythropolis, Arthobacter sp.*
Lipopeptides	
Surfactin	*Bacillus subtilis*
Iturin/fengycin	*B. subtilis*
Lichenysin	*Bacillus licheniformis*
Phospolipids	*Acinetobacter sp., Corynebacterium lepus*
Fatty acids/ neutral lipids	*Corynebacterium insidibasseosum, Candida ingens, Rhodotorula glutinis*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Alasan	*A. calcoaceticus*
Particulate biosurfactants	*A. calcoaceticus,* Cyanobacteria
Surlactin	*Lactobacillus*

1.1.2. Biosurfactants properties

Biosurfactants have aroused interest to several applications due to their properties such as surface, interfacial and emulsion activity, as well as stability to extreme environmental conditions (temperature, pH and salinity). As previously mentioned, biosurfactants show remarkable properties as biological molecules that are advantageous comparing to their chemical counterparts, namely biodegradability and low toxicity [2, 5, 7]. These properties are described in detail in the following sections.

i. Surface and interfacial tension

Mulligan [8] stated that a good biosurfactant can lower surface tension of water from 75 to 35 mN/m, and the interfacial tension of water/hexadecane from 40 to 1 mN/m. Several biosurfactants showed good surface and interfacial tensions. For instance, the rhamnolipids

produced by *P. aeruginosa* strains decreased the surface tension of water from 72 mN/m to 30 mN/m and the interfacial tension of water/hexadecane to values lower than 1 mN/m [9]. The biosurfactants produced by *B. subtilis* strains, namely surfactin, can reduce the surface tension of water to 25 mN/m and interfacial tension of water/hexadencane to values below 1 mN/m [10]. Moreover, a reduction of surface tension to 33 mN/m and interfacial tension to 5 mN/m were reported by Cooper and Paddock [11] for sophorolipids from *Torulopsis bombicola*.

In general, these studies showed that biosurfactants are more effective and efficient than chemical surfactants, with critical micelle concentrations (CMC) about 10 to 40 times lower, i.e. lower amounts of biosurfactants are required to achieve a maximal decrease of the surface tension [5].

ii. Emulsifying activity

An emulsion is defined as an heterogeneous system, comprising at least one immiscible liquid dispersed in another in the form of droplets [5]. These types of systems possess a minimal stability, which may be enhanced by the addiction of surface active compounds. In general, the bioemulsifiers are better emulsifiers than the biosurfactants. As an example, it was found that sophorolipids from *T. bombicola* show a good surface and interfacial tension, however did not revealed good emulsifying activity [2]. On the contrary, the bioemulsifier liposan from *C. lipolytica* could not reduce the surface tension but showed a good emulsifying activity, being successfully used to emulsify edible oils [2]. The emulsifying activity of these microbial surface active molecules is especially interesting in the food industry, but also for environmental applications [12].

iii. Stability to different environmental conditions

The stability of biosurfactants to extreme conditions of pH, temperature and salinity make them desirable molecules for applications where these conditions prevail. Several studies showed that many biosurfactants are not affected by extreme environmental conditions. The lichenysin produced by *B. licheniformis* JF-2 is an example of a biosurfactant with good stability, not being affected by temperature up to 50°C, pH 4.5 – 9.0, and by NaCl concentrations up to 50 g/l [13]. A thermostable (30 - 100°C) and pH (2.0 – 12.0) stable biosurfactant produced by *Arthrobacter protophormiae* was described by Pruthi and Cameotra [14]. Nitschke and Pastore [15], studied

the stability of a lipopeptide produced by *B. subtilis* LB5a. The surface activity did not change within the pH range from 4 to 11, and NaCl concentrations up to 20 %. The lipopeptide was stable after autoclaving (121°C, 20 min), and even at low temperatures (-18°C) the biosurfactant maintained its activity.

Some industrial processes require the use of new microbial compounds that can resist to these extreme conditions. As such, biosurfactants could be considered for such applications [5].

iv. Biodegradability

One of the most attractive properties of the biosurfactants compared with the chemical ones is their biodegradability [16], which make them useful for environmental applications such as bioremediation [8, 17]. Lima et al. [18] evaluated the biosurfactants biodegradability as compared to sodium dodecyl sulfate. The authors concluded that the biosurfactants tested were more suitable for bioremediation applications due to their biodegradability and impact in the environment.

The increasing environmental concerns, as well as the regulatory obligations imposed by governments, make the biosurfactants interesting alternatives to replace chemical surfactants [5].

v. Low toxicity

Although the toxicity of biosurfactants has not been widely explored, in general they are classified as low or non- toxic molecules, thus being considered appropriate for pharmaceutical, food and cosmetic uses [2]. For instance, the synthetic anionic surfactant, Corexit was found to have a ten times lower LC_{50} (lethal concentration to 50 % of test species) against *Photobacterium phosphoreum* than the rhamnolipids tested, proving the higher toxicity of the chemical surfactant. Similarly, Marlon A-350, a chemical surfactant commonly used in industry, showed higher toxicity and mutagenic activity when compared with biosurfactants produced by *P. aeruginosa* [19].

1.1.3. Natural roles

A wide range of microorganisms have been shown to produce surface active agents as a strategy to survive in the environment. These agents can increase the bioavailability of water-insoluble substrates, provide the uptake of key metal ions necessary as co-factors for enzymes, exhibit antimicrobial activity in order to inhibit the growth of other microorganisms, and are involved in the interaction of microorganisms with surfaces.

Biosurfactants are responsible for increasing the bioavailability of hydrophobic water-insoluble substrates. For instance, microorganisms living in environments rich in hydrophobic substrates with low solubility in water, such as hydrocarbons, have some limitations to obtain the essential nutrients for their metabolism. Due to the hydrophobic character of these substrates, they adsorb to surfaces, thus restricting their bioavailability. Therefore, these microorganisms produce biosurfactants that can either be excreted to the surrounding environment or remain attached to the cell membrane. The biosurfactants are then predominantly produced during growth on water immiscible substrates, in order to desorb the substrate that is adsorbed on the surfaces or to increase their apparent solubility [7]. Specifically, biosurfactants due to their ability to reduce the interfacial tension are particularly effective in making the bound substrates available for biodegradation. Moreover, the ones with low CMC are capable of incorporating the hydrocarbons in the hydrophobic cavity of their micelles, increasing therefore the substrate solubility in water (Figure 1). As an example, the rhamnolipids produced by *P. aeruginosa* strains, have been involved in the degradation of polymeric hydrocarbons. Other authors, showed that rhamnolipids besides emulsifying alkanes, also stimulate the growth of *P. aeruginosa* strains in hexadecane [3]. Some authors isolated two mutants of *P. aeruginosa*, PU-1 and PU-2, that were unable to produce rhamnolipids and also could not grow adequately on alkanes, proving therefore the importance of biosurfactants in the cell growth on hydrophobic substrates [3]. In the same way, biosurfactant-negative mutants of *P. aeruginosa* KY-4025 and *P. aeruginosa* PG-201 [20] showed poor growth on n-paraffin and hexadecane, respectively, as compared to wild type strains. This study clearly demonstrates that the addiction of rhamnolipids enables restoring the microbial growth on the hydrophobic substrates [3].

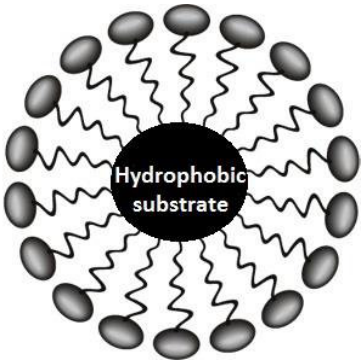

Figure 1. Incorporation of hydrophobic substrates in the hydrophobic cavity of biosurfactants micelles.

The uptake of metal ions, required for the metabolism of microorganisms, can be stimulated by biosurfactants. Some metal ions are important co-factors in the microbial metabolism, therefore microorganisms produce biosurfactants that form a complex with the metal ion and interact with the cell surface prompting their uptake. Herman et al. [21] found that rhamnolipids are capable of removing cadmium, lead and zinc from soil. Moreover, bioemulsifiers have also been described to interact with metal ions by binding them, it is the case of emulsan produced by *A. calcoaceticus* that binds to uranium [22].

The potential of biosurfactants as antibiotics confers the producing-microorganisms advantages to compete with other microorganisms present in the same environment. For example, biosurfactants have been described to show antimicrobial activity against bacteria, fungi and viruses [5].

The microorganism's ability to place themselves in an ecological niche, in which they can multiply, is one of their survival strategies. Biosurfactants play a crucial role in this strategy, since they are responsible for the interaction of the microorganism with a specific surface. In the case of biosurfactants that are excreted, they can form a conditioning film on the interface, thereby stimulating certain microorganisms to attach to a surface while inhibiting the attachment of others. On the other hand, cell-bound biosurfactants lead to the modification of the cell surface hydrophobicity [4]. For instance, *Acinetobacter* strains that produce cell-bound biosurfactants showed a reduction of their hydrophobicity due to biosurfactants [23]. In sum, biosurfactants have been shown to be involved in cell adherence, which imparts a great stability under hostile environmental conditions and virulence; and in cell desorption when organisms need to find new habitats for survival [7].

Some biosurfactants have been reported as being produced by microorganisms in association with virulence factors. For instance, Van Delden and Iglewski [24] suggested that rhamnolipids can be associated to virulence factors in *P. aeruginosa* strains that are involved in opportunistic infections. Olvera et al. [25] found that the synthesis of rhamnolipids in *P. aeruginosa* strains is mediated by the same proteins that are associated to other virulence-factors, such as alginate and lipopolysaccharides (LPS). For that reason, the evaluation of rhamnolipids for biomedical and therapeutic applications should take into account this aspect.

1.2. Analytical methods to study biosurfactants

The advances in the discovery of biosurfactants were largely attributed to the development of rapid and reliable methods for the screening and selection of biosurfactants producing-microorganisms, as well as for the evaluation of the biosurfactants properties. Table 2 describes the main analytic methods used to study biosurfactants.

Table 2. Analytic methods used to screen biosurfactant producers and to evaluate biosurfactants activity.

Method	Description	Ref.
Blood agar screening method	Based on the hemolytic activity of biosurfactants. This method is widely used for screening biosurfactants producing-strains. Briefly, each strain is streaked onto blood agar plates and incubated during 24h at 37°C. The visualization of clear zones around the colonies indicates the presence of biosurfactants. The diameter of the clear zones is a qualitative method used as an indicator of biosurfactant production. This assay does not detect specific types of biosurfactants.	[26, 27,28]
Drop-collapsing test	Evaluates the presence of biosurfactant in the broth supernatant, based on their surface activity. A drop of cell suspension is placed on an oil-coated-surface; if the drop collapses it indicates the presence of surfactant (a positive result); if the drop remains stable, indicates the absence of surfactant (a negative response). The assay is fast, easy to perform, reproducible and does not require specialized equipment.	[3, 29]
Tensiometric measurements	The surface tension of a solution containing biosurfactants is measured by a ring-tensiometer. Willumsen and Karlson defined a good biosurfactant producer as the one being able to reduce the surface tension of the growth medium by $\geq$20 mN/m as compared to distilled water.	[30]

Table 2. Analytic methods for a quick detection of biosurfactants producing-strains and evaluation of biosurfactants activity (continuation).

Method	Description	Ref.
Emulsification index value (E_{24})	The estimation of emulsification index value is performed by vigorously shaking the culture broth samples with an equal volume of kerosene or *n*-hexadecane. After 24h, the value of E_{24} is given by the equation: $$E_{24}(\%) = \frac{Height\ of\ the\ emulsion\ layer}{Total\ height} \times 100$$ This method is suitable to determine the activity of emulsifying biosurfactants.	[31, 32, 33]
Critical micelle concentration (CMC)	The CMC is defined as the concentration above which micelles are formed. Briefly, the surface tension of different biosurfactant concentrations is measured. Once the CMC value is reached, the surface tension will remain constant. The determination of CMC is commonly used to measure the efficiency of the biosurfactants.	[3, 34,35]
Hydrophilic-lipophilic balance (HLB)	The HLB value indicates the biosurfactant emulsifying activity. The HLB value indicates whether a surfactant will promote water-in-oil or oil-in-water emulsion. The HLB scale can be constructed by assigning a value of 1 to oleic acid, and a value of 20 to sodium oleate. Emulsifiers with HLB values less than 6 will favor the stabilization of water-in-oil emulsions, whereas emulsifiers with HLB values between 10 and 18 have the opposite effect and will favor oil-in-water emulsions. These values can be calculated by measuring the contact angles.	[3, 28]
Colorimetric methods-CTAB (cetyltrimethylammonium bromide)	This simple test allows the determination, in bacterial culture broth, of the presence of anionic biosurfactants. Briefly, anionic biosurfactants can form a colored complex with the cationic surfactant CTAB and the basic dye methylene blue. This complex can be quantified by spectrophotometry.	[27,36, 37]

In general, the methods above described are easy to perform and provide fast results on the biosurfactants activity. However, more than one method should always be performed, since most of these methods are complementary.

To establish the biochemical composition of biosurfactants in terms of carbohydrate, protein and lipid contents several techniques can be used. For instance, the carbohydrate content of biosurfactants can be determined by the phenol–sulfuric acid method using D-glucose as a standard [38]. Regarding the protein content, this can be determined using the method described by Lowry et al. [39], using bovine serum albumin as a standard, or alternatively by the Coomassie

blue method [40, 41]. Additionally, to determine the lipid content it is first necessary to conduct an extraction with chloroform:methanol. The organic phase is then evaporated under vacuum and the lipid content can be determined by gravimetric estimation [42, 43].

For a better understanding of the relationship among the chemical structures of biosurfactants, the composition of mixtures and a structural detailed analysis is necessary. This can be achieved by mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance spectroscopy [27]. Moreover, Fourier transform infrared spectroscopy (FTIR) is most useful for identifying types of chemical bonds (functional groups), therefore it can be used to elucidate some unknown components of unknown given mixture [28]. Thin-layer chromatography (TLC) has also been extensively used for determining the composition of biosurfactants [27].

1.3. Biosurfactants production

The improvement of biosurfactants production, in order to make their adoption feasible as components of large-volume commercial products, is a constant demand by industry [44]. Therefore, since the biosurfactants production has distinct characteristics, depending on the type of biosurfactant and its producer, it is first mandatory to understand the process before optimization can be envisaged. The following sections describe the kinetics of biosurfactants production, as well as the influence of the carbon and nitrogen sources, environmental factors and trace elements on the production.

1.3.1. Kinetics of fermentative production

The kinetic of biosurfactants production can be classified in three different groups depending on the different production conditions: growth–associated production, production under growth-limiting conditions, and production by resting cells (Figure 2).

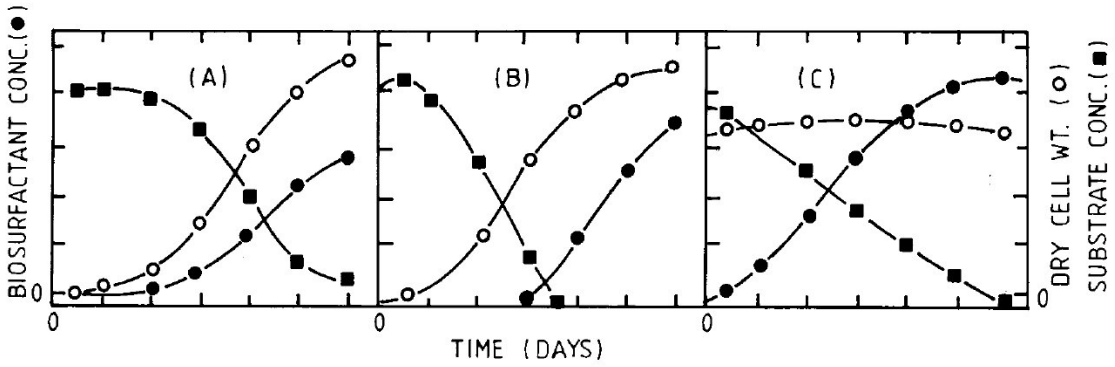

Figure 2. Different biosurfactant production kinetics. **(A)** Growth-associated production; **(B)** Production under growth-limited conditions and **(C)** Production of biosurfactants by resting cells. (Taken from [3]).

The first case, growth–associated production, describes a parallel relation between biosurfactants production, substrate consumption and cell growth (Figure 2A) [3]. Several researchers have reported biosurfactants being produced in association to cell growth, e.g. rhamnolipids production by *Pseudomonas* spp. [3]; biodispersan by *Bacillus* sp. strain IAF- 343 [31]; a surface-active agent by *B. cereus* IAF-346 [31], and the glycoprotein AP-6 produced by *Pseudomonas fluorescens* 378 [45].

However, there are some biosurfactants for which the production is favored by the limitation of one or more medium components, as for example nitrogen limitations (Figure 2B) [3]. Examples include the production of a glycolipid by *Norcadia* sp. strain SFC-D [3]; bioemulsifier by *Candida tropicalis* IIP-4 [46]; and a water-soluble biosurfactant by *Torulopsis apicola* [47].

For the cases in which no cell growth occurs but the cells continue using the carbon source from the medium, biosurfactants production is conducted by resting cells (Figure 2C) [3]. Examples of these type of biosurfactant production include the rhamnolipids produced by *P. aeruginosa* CFTR-6 [48]; MELs produced by *C. antarctica* [49]; and sophorolipids produced by *T. bombicola* [3].

1.3.2. Factors affecting biosurfactant production

The composition and properties (e.g. surface and emulsifying activity) of a biosurfactant not only depends on the producer strain, but also on the culture conditions. In other words, the nature of the carbon and nitrogen sources, as well as the nutritional limitations, trace elements, and

operational parameters such as temperature, aeration and pH will greatly influence the amount and type of biosurfactant being produced.

i. Carbon Source

Several microorganisms can use different types of carbon sources to produce biosurfactants. In general, glucose, sucrose, glycerol, diesel and crude oil, have been reported as good sources of carbon for biosurfactant production. However, the use of different substrates influences the biosurfactant structures, and consequently their properties. These changes are welcome if they induce improvements in the biosurfactants proprieties making them more suitable for some applications [7]. Robert et al. [50] found that *P. aeruginosa* 4431 could grow on different carbon sources producing different rhamnolipids species, thus illustrating the influence of the carbon source. Moreover, the chain length of the carbon substrate also affects the biosurfactants production. For instance, Kitamoto and co-workers [51] demonstrated the influence of different *n*-alkanes on the production of MEL by *C. antarctica*. Their results showed that the chain length of *n*-alkanes affected significantly the production of MEL, the highest productivity being observed with *n*-octadecane. On the contrary, for sophorolipids produced by *C. bombicola* ATCC22214 it was found an increase in the yield with a *n*-alkane chain length from C12 to C15 [52].

ii. Nitrogen Source

Nitrogen is an important constituent on the culture medium for biosurfactants production, since it is an essential component of the proteins that play a role in the growth of microorganisms, and therefore in the production of enzymes required for the fermentative process. In the literature, several sources of nitrogen have been reported for the production of biosurfactants, e.g. urea, peptone, ammonium sulfate, ammonium nitrate, sodium nitrate, meat extract and malt extract. Yeast extract has been widely used for the production of biosurfactant. However, its concentration greatly depends on the nature of the producing-microorganism and the specific culture medium used. Johnson and co-workers [53] showed that potassium nitrate yield a greater amount of the biosurfactants produced by *R. glutinis* as compared with other nitrogen sources, namely urea or ammonium sulfate [54]. The influence of different nitrogen sources has also been evaluated for

the rhamnolipids production, and the presence of NO_3^-, glutamate, and aspartate has been suggested to promote the production of rhamnolipids, whereas NH_4^+, glutamine, asparagine, and arginine to inhibit the rhamnolipids production [55]. Nitrogen limitation has been described to increase the biosurfactant production by *P. aeruginosa* [56] and *C. tropicalis* IIP-4 [46]. According to Desai et al. [3], nitrogen limitation not only causes an increase in the biosurfactants production, but also changes their composition. An important parameter studied by several researchers is the quantitative ratio between carbon and nitrogen sources (C:N) that is required for biosurfactants production. Guerra-Santos et al. [57, 58] found a maximum rhamnolipids production under nitrogen limitation at a C:N ratio of 16:1 to 18:1. On the contrary, no biosurfactant production was reported for C:N ratios below 11:1 (no nitrogen limitation). Singh et al. [46] tested different C:N ratios and hydrocarbons for the production of biosurfactants by *C. tropicalis* and also reported an increase in the production under nitrogen limitation.

iii. Environmental Factors

Environmental factors and growth conditions such as temperature, pH, agitation, and oxygen availability also affect the biosurfactant production through their effects on cellular activity and growth.

The biosurfactants produced by *Pseudomonas* sp. strain DSM-2874 [3] and *Arthrobacter paraffineus* [59] were found to present different compositions as a result of temperature changes.

Although most of the biosurfactants are produced at temperatures ranging from 25°C to 30°C [6], thermophilic bacteria such as *Bacillus* sp. have also been reported to grow and produce biosurfactants at temperatures above 40°C [60].

Casas and Ochoa [61] studied the production of sophorolipids from *C. bombicola* at different temperatures, specifically 25°C and 30°C, and verified that temperature only slightly affects sophorolipid production when resting-cells were used. Desphande and Daniels [62] also found the maximum growth of *C. bombicola* at 30°C, but the highest biosurfactants production was observed at 27°C.

As mentioned above, the pH of the culture medium also plays an important role in the biosurfactants production. For instance, Zinjarde and Pant [63] reported that the best biosurfactant production by a marine yeast, *Yarrowia lipolytica* NCIM3589, occurred when the pH value was 8.0,

which corresponds to the natural sea water pH. Desai and Banat [3] described that the best pH for rhamnolipids production by *Pseudomonas* spp. is in the range between 6.0 and 6.5. The pH role in the glycolipids synthesis by *C. antarctica* and *C. apicola* was evaluated [54]. When the pH value is maintained at 5.5, the biosurfactants production reaches the maximum. However, without pH control the synthesis of glycolipids decrease, thus confirming the relevance of controlling the pH during the fermentation process.

Aeration and agitation are also relevant in the production of biosurfactants, since both facilitate the oxygen transfer from the gas phase to the aqueous phase. Sheppard and Cooper [64] showed that oxygen transfer is a main parameter for the optimization and scale-up of surfactin production by *B. subtilis.* The influence of aeration in the production of biosurfactants by *C. antarctica* was evaluated, an air flow rate of 1 vvm and dissolved oxygen concentration of 50 % was observed to provide the highest yield. However, the increase of the air flow rate to 2 vvm caused foam formation, thus leading to a decrease in the biosurfactants production up to 84 % [54].

iv. Trace elements

Metal ions can act as co-factors of many enzymes that are involved in the biosurfactants production, therefore the concentration of these species in the culture media play an important role in production of same biosurfactants [54]. Thimon et al. [65] showed that the presence of Fe^{2+} in mineral salt medium increased drastically the production of surfactin *B. subtilis.* Furthermore, the supplementation of 0.01 mM Mn^{2+}, which affected the nitrogen utilization, to a defined glucose medium led to an increase from 0.33 g/l to 2.6 g/lof surfactin, produced by *B. subtilis* [66].

1.3.3. Cost reduction strategies for biosurfactants production

As previously mentioned, biosurfactants exhibit several advantages over their chemical counterparts. However, the biosurfactants production processes are still not economically interesting, thus limiting their commercialization. Indeed, the low production yields, expensive substrates and expensive recovery are the main causes for their low competitiveness [1, 67]. As such, in order to compete with chemical surfactants, the biosurfactants must present interesting functionalities/activities and better production yields [68]. For some applications, such as for

medicines, the higher production costs can be acceptable, since in this case biosurfactants represent high value products and the required amounts are low. Nevertheless, for the most common biosurfactants applications, namely environmental ones, high volumes of biosurfactants are required and the costs of production are unbearable [69].

To make biosurfactants production a cheaper process and commercially attractive, it is necessary to boost their production and recovery at larger scales. Therefore, several strategies have been proposed to make biosurfactants cost-competitive, including the development of more efficient bioprocesses, i.e. (a) optimization of fermentative conditions and cost-effective downstream recovery processes; (b) replacement of synthetic media by cheaper and waste substrates; and (c) development of overproducing mutant or recombinant strains (Figure 3) [1, 70].

The first two strategies have been widely explored and reported in several studies that showed considerably improvements in the biosurfactants production.

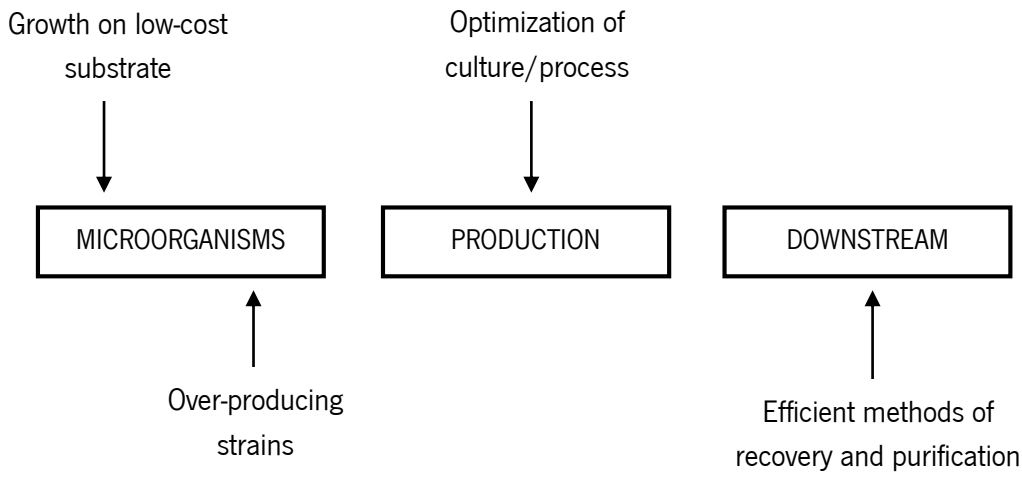

Figure 3. Different cost-reduction strategies for biosurfactants production. (Adapted from [1]).

i. Growth on low-cost substrates as promising alternatives

The amount and type of raw substrate used for the production of any bioproduct can contribute considerably to the production cost. Specifically, it is estimated that raw substrates account for 10-30 % of the total production costs. Therefore, to reduce this cost it has been

suggested the use of low-cost raw substrates. For example, the use of agro-based raw materials or wastes (e.g. potato, cassava and soybean) have been extensively explored, since these contain high levels of carbohydrates or lipids to support biomass growth and biosurfactants synthesis. Moreover, plant-derived oils, oil wastes, starchy substrates, distillery wastes and lactic whey have also been reported as cheap alternative raw materials for biosurfactants production [5, 69, 70]. Table 3 compiles the main low-cost substrates used for biosurfactants synthesis, as well as the yield and type of biosurfactant produced.

Table 3. Biosurfactants producing microorganisms growing in different low-cost substrates. (Adapted from [2, 5, 67, 70]).

Low-cost substrate	Biosurfactant	Producing strain	Yield (g/l)
Vegetable oils and oil wastes			
Sunflower and soybean oil	Rhamnolipids	*P. aeruginosa* DS10-129	4.31
Rapeseed oil	Rhamnolipids	*Pseudomonas* sp. DSM 2874	45
Sunflower oil	Lipopeptide	*Serratia marcescens*	2.98
Oil refinery waste	Glycolipids	*C. antarctica*; *C. apicola*	10.5
Groundnut oil refinery residue	Lipopeptide	*C. lipolytica*	4.5
Palm oil	Rhamnolipids	*Pseudomonas alcaligenes*	2.3
Starchy substrates			
Potato process effluents	Lipopeptide	*B. subtilis*	2.7
Cassava flour wastewater	Lipopeptide	*B. subtilis* ATCC 21332, *B. subtilis* LB5a	2.2
Sugar industry wastes			
Molasses	Lipopeptide	*B. subtilis* (MTCC 2423 and MTCC1427)	-
	Rhamnolipids	*P. aeruginosa GS3*	0.25
Dairy industry whey wastes			
Curd whey and distillery	Rhamnolipids	*P. aeruginosa* BS2	0.92

Molasses are a co-product of the sugar cane and sugar beet industry, being widespread used as substrate due to its low price, compared to other sources of sugar, and the presence of several other compounds and vitamins. Specifically, molasses consist of sugars (sucrose 48-56 %), non-sugar organic matter (9-12 %), proteins, inorganic components and vitamins [67]. Onbasli and Aslim [71] reported the production of rhamnolipids by two strains, *Pseudomonas luteola* B17 and *Pseudomonas putida* B12, on 5 % (w/v) of sugar beet molasses. There are also reports on the use of molasses in combination with other cheap substrates. Patel and Desai [72] reported the

production of rhamnolipids by *P. aeruginosa* GS3 combining molasses and corn steep liquor (CSL) as carbon and nitrogen source, respectively.

Lactic whey is an effluent from dairy industry that has been reported as a good and cheap substrate for microbial growth and biosurfactants production. Lactose is the main component of lactic whey corresponding to 75 % of dry matter, being also composed by 12-14 % of protein, vitamins and organic acids [68]. To use the lactose effectively, the chosen organism must be capable of consuming the lactose and the breakdown products, galactose and glucose [69]. Koch and co-workers [73] developed a strain of *P. aeruginosa* able to produce rhamnolipids from whey. Daniel et al. [74] reported the production of sophorolipids, obtained by a two-stage process starting from deproteinized whey concentrate, using *Cryptococcus curvatus* ATCC20509 and *C. bombicola* ATCC22214. The *B. licheniformis* strain M104 was also described to grow in whey producing biosurfactants [75], as well as the *B. subtilis* 20B strain, to produce surfactin [76]. Rodrigues et al. [77] performed a screening of *Lactobacillus* strains able to produce biosurfactants. The lactic acid bacteria *Lactobacillus casei*,*Lactobacillus rhamnosus*, *Lactobacillus pentosus* and *Lactobacillus coryniformis torquens* were select as biosurfactants-producing strains, with *L. pentosus* being considered the most promising strain and whey as a potential alternative substrate.

The combination of cheese whey and molasses was evaluated by Rodrigues and co-workers [78], as substrates for the biosurfactants production by *Streptococcus thermophilus* A and *Lactococcus lactis* 53 strains. The authors reported an increase of 1.2-1.5 times in the mass of biosurfactants produced per gram of cell dry weight, with 75 % cost reduction. Therefore, the supplemented molasses and cheese whey media was suggested as a relatively inexpensive and economical alternative to synthetic media, for biosurfactants production by these probiotic bacteria.

ii. Optimization of culture parameters

As already mentioned, the final amount, quality and type of produced biosurfactants depend on the culture conditions. Environmental factors are exceptionally significant in the yield and characteristics of the biosurfactants produced. The optimization of process conditions, in order to increase the amounts of biosurfactants, is a required step for any profit-making biotechnology industry [54]. Several elements, media composition and precursors, affect the biosurfactant production process. Different elements, such as nitrogen, iron and manganese have been reported

to affect the biosurfactants yield. For example, the addition of iron and manganese to the culture medium was reported to increase the production of biosurfactants by *B. subtilis* [79]. The interaction of different components in the medium also affects biosurfactant production, therefore the ratios of different elements, C:N, C:P, C:Fe or C:Mg, should also be studied and optimized to enhance the production yields [70].

To maximize the biosurfactants production and reduce the costs it is necessary the use of process-optimization strategies that involve multiple factors. The classical method of medium optimization involves changing one variable at time, while keeping the others at fixed levels. Nevertheless, this methodology is laborious, time consuming and does not guarantee the determination of the optimal conditions for biosurfactants production. To overcome some of the disadvantages of traditional methodologies, a statistical optimization strategy based on response surface methodology (RSM), which explores the relationships between several variables, has been used by several researchers [70]. For instance, this methodology was used to determine the optimum media and environmental conditions for enhanced surfactin production by *B. subtilis* [80–82]. RSM has also been used to optimized culture media for rhamnolipids production by *P. aeruginosa* strains [83]. Rodrigues et al. [84] described the application of RSM for the optimization of medium components for biosurfactants production, by probiotic bacteria. An increase in productivity was observed in all the cases, showing that optimization methods would help to design the best media containing cheaper substrates, and to use the most favorable environmental conditions, for the improved production of biosurfactants.

iii. Recovery and purification of biosurfactants

For many biotechnological products, the downstream processing costs account for most of the total production cost, approximately 60 %. Therefore, optimization of biosurfactants production using optimal media and culture conditions is not enough to reduce the total costs, i.e. the production process is still incomplete without an efficient and economical recovery process. Several recovery methods for biosurfactants have been reported. Conventional methods include acid precipitation, solvent extraction, centrifugation and ammonium sulfate precipitation. In recent years, a few unconventional recovery methods have also been described, such as foam fractionation, ultrafiltration and ion exchanged chromatography [2, 70] (Table 4).

Extraction with solvents, namely chloroform, acetone and methanol, has shown some disadvantages, since they are toxic and harmful to the environment. Due to these disadvantages, cheap and less toxic solvents, such as methyl tertiary-butyl ether, have been successfully used to recovery biosurfactants produced by *Rhodococcus* species [41].

Often, a single downstream processing technique is not enough for biosurfactants recovery and purification. In these cases, it is applied a multi-step downstream strategy [2]. For instance, extraction of low-molecular-weight biosurfactants normally involves an initial precipitation step, followed by extraction with different organic solvents according to the hydrophobicity and hydrophilic-lipophilic balance value of the compounds. As an example, rhamnolipids are usually precipitated by acidification; followed by solvent extraction, e.g. with ethyl acetate. In the case of high-molecular-weight biosurfactants, normally they are extracted by ammonium sulfate precipitation and then purified by dialysis [1].

In sum, these recovery techniques are already well established for lab-scale applications, however some of them could not yet be applied to an industrial scale due to their operational costs. Therefore, several efforts have been conducted towards the development of low-cost extraction and purification processes, avoiding the use of hazardous and costly organic solvents [1].

Table 4. Properties-based biosurfactants recovery strategies and their relative advantages. (Adapted from [2 , 3]).

Recovery strategy	Description	Advantages	Biosurfactants
Acid precipitation	At low pH the biosurfactants become insoluble	Efficient in the recovery of crude biosurfactants, low cost	Surfactin, Glycolipids
Ammonium sulfate precipitation	Salting –out of polymeric or protein-rich biosurfactants	More suitable and efficient for the recovery of certain polymeric biosurfactants	Emulsan, Biodispersan
Organic solvent extraction	The presence of hydrophobic ends in biosurfactants turn them soluble in organic solvents	Solvents can be reused, efficient in the recovery of crude biosurfactants, partial purification	Trehalolipids, Sophorolipids, Liposan
Alternative organic solvent for extraction	The presence of hydrophobic ends in biosurfactants turn them soluble in organic solvents	Less toxic than conventional organic solvents, low cost, can be reused	-
Centrifugation	The centrifugation force leads to the precipitation of insoluble biosurfactants	Reusability, effective in crude biosurfactants recovery	-

Table 4. Properties-based biosurfactants recovery strategies and their relative advantages. (Adapted from [2 , 3]) (continuation).

Recovery strategy	Description	Advantages	Biosurfactants
Foam fractionation	Due to the surface activity, biosurfactants form and partition into foam	Suitable for bioreactors that facilitate the recovery of foam during the fermentation	Surfactin
Membrane ultrafiltration	The polymeric membranes can trap the micelles of biosurfactants formed above their CMC	Fast, highly purity	Glycolipids
Ion – exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	Reusability, high level of purity, fast recovery	-
Adsorption on wood – activated carbon	Biosurfactants are adsorbed on activated carbon and can be desorbed with organic solvents	Cheaper, reusability, highly purity	-
Adsorption on polystyrene resins	Biosurfactants are adsorbed on polystyrene resins and desorbed with organic solvents	Reusability, highly purity, fast recovery	-

1.4. Biosurfactants applications

In the last years, much attention has been direct towards biosurfactants due to their broad range of functional properties, as well as the diversity of producing microorganisms. Their unique properties allow their application in a wide variety of commercial areas and industrial processes, replacing the chemical surfactants. Some of the potential applications of biosurfactants are presented in Table 5, as well as their role in each industrial field.

Table 5. Biosurfactants applications in industry. (Adapted from [2]).

Application	Role of biosurfactant
Biomedical and therapeutic	Antibacterial, antifungal, antiviral agents, adhesive agents, immunomodulatory molecules, gene therapy
Microbial enhanced oil recovery (MEOR)	Improving oil draining into well bore, reduction of oil viscosity and oil pour point, lowering of interfacial tension and dissolving of oil
Bioremediation	Emulsification of hydrocarbons, lowering of interfacial tension, metal sequestration
Food Industry	Emulsifier, solubilizer, demulsifier, suspension, foaming, defoaming, thickener and lubricating agent
Biocontrol in agriculture	Facilitation the biocontrol mechanisms of microbes parasitism, competition, induced systemic resistance and hypo-virulence
Cosmetic	Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents

1.4.1. Biomedical and therapeutic applications

Several biosurfactants have been studied due to their potential as biological active compounds that could be useful in the medical field. Specifically, they constitute suitable alternatives to synthetic medicines and antimicrobial agents, and may be applied as safe and effective therapeutic agents [85].

i. Antimicrobial activity

Numerous biosurfactants have been shown to possess antimicrobial activity against bacteria, fungi, algae and viruses. For instance, the iturin lipopeptide produced by *B. subtilis* showed potential antifungal activity [86], as seen by the alterations in the morphology and structure of yeast cells [85]. Surfactin produced by *B. subtilis* was reported to inactivate the enveloped virus such as herpes and retrovirus [87].

Antimicrobial activity has been reported also for rhamnolipids that were shown to inhibit the growth of harmful bloom algae species, *Heterosigma akashivo* and *Protocentrum dentatum* at

concentrations ranging from 0.4 to 10.0 mg/l [2]. Abalos et al. [88] studied the activity of rhamnolipids produced by *P. aeruginosa* AT10. These biosurfactants showed a good inhibitory activity against several bacteria, *Micrococcus luteus*, *Escherichia coli*, *Alcaligenes faecalis* (32 mg/ml), *Mycobacterium phlei*, *Serratia marcescens* (16 mg/ml) and *Staphylococcus epidermidis* (8 mg/ml); it was also detected good antifungal properties against *Aspergillus niger* (16 mg/ml), *Chaetonium globosum*, *Aureobasidium pullulans*, *Enicillium crysogenum* (32 mg/ml) and the phytopathogenic *Botrytis cinerea* and *Rhizoctonia solani* (18 mg/ml).

Rodrigues and co-workers [89] reported the antimicrobial activity of two probiotic bacteria, *L. lactis* 53 and *S. thermophilus* A, against a variety of bacterial and yeast strains isolated from explanted voice prostheses. The authors found that the biosurfactants produced by both microorganisms have a high antimicrobial activity even at low concentrations, against *C. tropicalis* GB 9/9, one of the strains responsible for prostheses failure. Moreover, at the highest concentration tested, 100 mg/ml, the biosurfactants were effective against all the strains of bacteria and yeast tested. The activity of probiotic biosurfactants produced by lactobacilli was also discussed by Reid et al. [90, 91]. The authors suggested a possible probiotic role of these biosurfactants in the restoration and maintenance of healthy urogenital and intestinal tracts as an alternative treatment to antibiotics, conferring protection against pathogens. The biosurfactants produced by *Lactobacillus fermentum* RC-14 reduced, in a rat model, infections associated to surgical implants, which are mainly caused by *Staphylococcus aureus,* through inhibition of growth and reduction of the adherence to surgical implants [92].

ii. Anti-adhesive activity

The anti-adhesive activity of biosurfactants has also been extensively debated, since they are capable of inhibiting the adhesion of pathogenic organisms to solid surfaces or to infections sites. Moreover, it has been suggested that prior adhesion of biosurfactants to solid surfaces might constitute a new and effective means of combating colonization by pathogenic microorganisms [93]. For example, pre-coating vinyl urethral catheters by running a surfactin solution through them, before inoculation with media, resulted in a decrease in the amount of biofilm formed by *Salmonella enterica*, *Salmonella typhimurium*, *Proteus mirabilis* and *E. coli* [94]. These results showed the

great potential of biosurfactants to treat the infections caused by opportunist microorganisms, such as *Salmonella* species, including in urinary tract infections of AIDS (Acquired Immunodeficiency Syndrome) patients [93]. Efforts in the development of strategies to prevent the microbial colonization of silicone rubber voice prostheses was investigated by Rodrigues and co-workers [95, 96]. The ability of biosurfactants obtained from probiotic bacteria to inhibit adhesion of several bacterial and yeast strains, through the pre-conditioning of silicone rubber was evaluated. The authors observed over 90 % reduction in the initial deposition rates for most of the bacterial strains tested. The biosurfactants synthetized by *S. thermophilus* A proved to be the most effective against *Rothia dentocariosa* GBJ 52/2B, which is one of the microorganisms responsible for valve prosthesis failure. Other studies also reported the pre-treatment of silicone rubber with biosurfactants produced by *S. thermophilus*, showing that they were capable of inhibiting 85 % of *C. albicans* adhesion [97], whereas biosurfactants produced by probiotic bacteria, *L. fermentum* and *Lactobacillus acidophilus* adsorbed on glass could reduce in 77 % the adhering cells of *Enterococcus faecalis* [2]. Gan et al. [92] reported that *L. fermentum* biosurfactants inhibited *S. aureus* infection and adherence to surgical implants.

1.4.2. Microbial enhanced oil recovery (MEOR)

Crude oil recovery occurs in three main phases. Primary recovery, in which oil production occurs under natural pressure, leads to the recovery of 15 % of the oil in place. Secondary recovery, in which the oil well is flooded with water or other substances including CO_2 injection, alkaline surfactant polymers or solvents, leads to an additional 15–20 % oil recovery. The tertiary recovery or enhanced oil recovery is used to recover the remaining oil after primary and secondary recovery methods are exhausted or no longer economic. MEOR has been gaining significance as a tertiary process leading to an additional oil recovery up to 10 % [34]. This methodology used microorganisms and/or their metabolic end products for the recovery of residual oil that is hindered, due to low permeability of some reservoirs or high viscosity resulting in poor mobility. Specifically, biosurfactants have been proposed for MEOR in the recent years. The ability of biosurfactants to reduce oil/water interfacial tension and to form stable emulsions can help the oil move more freely away from rocks and crevices, so that it may travel more easily out of the well [1, 7, 34].

Rhamnolipids have been most frequently used in MEOR, although lipopeptides, such as surfactin and emulsan, have also been demonstrated to be very effective in enhancing oil recovery [1]. Biosurfactants produced by *Rhodococcus ruber* and *R. erythropolis* were tested to extract hydrocarbons from oil shale in flask experiments. The maximum recovery observed was 26 % and 25 % for the two strains, respectively [98]. Pornsunthorntawee et al. [99] observed that both biosurfactants produced by *P. aeruginosa* SP4 and *B. subtilis* PT2 were more effective than the chemical surfactants tested in the recovery of oil entrapped in a sand-packed column.

In conclusion, the high diversity and properties of biosurfactants have been the focus of interest of many researchers. Moreover, their versatility towards a wide range of applications has also aroused the industrial attention. For some biosurfactants and their specific producing-microorganisms, several advances have been achieved in order to reduce production costs, and therefore to make them industrially profitable products. Likewise, these studies should also be conducted for other producing-microorganisms that have not been deeply investigated, e.g. lactic acid bacteria. The applicability of new biosurfactants in different areas should be further explored, as well as their interaction with each other, in order to develop enhanced properties and activities. In other words, the investigation of biosurfactant mixtures aiming at the enhancement of their activity is pointed as a strategy to ease their applicability in different areas, thus competing with standard methodologies/compounds applied.

2. MATERIALS AND METHODS

2.1. Biosurfactants production

2.1.1. Strains and standard culture conditions

The following bacterial strains were screened for biosurfactant production: *Lactobacillus animalis* ATCC35046 and *Lactobacillus hamsteri* ATCC43851, obtained from the American Type Culture Collection (USA); *Lactobacillus agilis* CCUG31450, obtained from the Culture Collection of University of Göteborg (Sweden); *Lactobacillus paracasei* A20, isolated from a Portuguese dairy industry [100]; *S. thermophilus* A isolated from a Dutch dairy industry [101]; *P. aeruginosa* PX112 and *B. subtilis* PX573, isolated from crude oil samples obtained from a Brazilian oil field [102, 103]. The culture media used were: MRS medium (medium introduced by De Man et al. [104]) for cultivation of *Lactobacillus* species (OXOID, Basingstoke, England); M17 medium [105] (OXOID, Basingstoke, England) for cultivation of *S. thermophilus* A; Luria-Bertani (LB) medium for cultivation of *P. aeruginosa* PX112 and *B. subtilis* PX573. The composition of LB medium was (g/l): NaCl 10.0; tryptone 10.0; yeast extract 5.0; with pH adjusted to 7.0. All the media were sterilized at 121°C for 15 min.

For antimicrobial assays, the following strains kindly provided by the Faculty of Pharmacy, University of Porto (Portugal) were used: *E. coli*, *P. aeruginosa*, *S. aureus*, *Streptococcus agalactiae* and *C. albicans*. For anti-adhesive assays were only used the strains of *S. aureus* and *P. aeruginosa*, previously mentioned. These strains were cultured in LB medium at 37°C.

All the strains were stored at -80°C in the appropriate medium supplemented with 20 % (v/v) glycerol solution until use. Whenever required, frozen stocks were streaked on agar plates and incubated overnight at 37°C for further culturing. The agar plates were stored at 4°C no longer than 2 weeks.

2.1.2. Screening of biosurfactant-producing strains

Biosurfactant production by the different bacterial strains was studied in 500 ml flasks containing 200 ml of the culture medium established for each strain (Table 6). Each flask was inoculated with 2 ml of a pre-culture. Pre-cultures were prepared as follows: for *P. aeruginosa* PX112 and *B. subtilis* PX573 a single colony was taken from an agar plate and transferred to a

flask containing 25 ml of LB liquid medium; in the case of *Lactobacillus* strains and *S. thermophilus* A, 100 µl of a frozen stock were transferred directly into 25 ml of MRS or M17 medium, respectively. Pre-cultures were incubated overnight at 37°C and 100 rpm, except for *B. subtilis* PX573, which was incubated without shaking. The cultures were incubated in the same conditions than the corresponding pre-cultures.

Table 6. Media and culture conditions used for the different strains studied.

Strains	Medium	Temperature	Shaking
L. agilis CCUG31450	MRS	37°C	100 rpm
L. animalis ATCC35046	MRS	37°C	100 rpm
L. hamsteri ATCC43851	MRS	37°C	100 rpm
S. thermophilus A	M17	37°C	100 rpm
L. paracasei A20	MRS	37°C	100 rpm
B. subtilis PX573	LB	37°C	0 rpm
P. aeruginosa PX112	LB	37°C	100 rpm

In order to evaluate growth and biosurfactant production, samples (4 ml) were taken at different time points during the fermentation. Biomass concentration was determined by measuring the optical density at 600 nm using a multi-detection microplate reader Synergy™ HT (BioTek, USA). Whenever its value was higher than 0.7, the samples were diluted and the optical density was measured again. In the case of *B. subtilis* PX573, due to the formation of a pellicle in the surface of the medium, it was not possible to evaluate the growth by measuring the optical density during the fermentation. In this case, at the end of the fermentation, the cells were harvested by centrifugation (9000 rpm, 20 min) and cell dry weight (g/l) was determined (24h at 105°C).

After measuring the cell growth, the samples were centrifuged (9000 rpm, 20 min) and the cell-free supernatants were used to measure the surface tension and the emulsifying activity, as described below.

Biosurfactants can be excreted to the culture medium (extracellular biosurfactants) or remain attached to the cell wall (intracellular biosurfactants). Lactic acid bacteria usually produce mainly intracellular biosurfactants. In this case, the surface tension measured in the cell-free supernatants gives an indication of the extracellular biosurfactants produced; at the end of the fermentation the intracellular biosurfactants were extracted and the surface tension of the extract was measured to evaluate the intracellular biosurfactants production.

All the cultures were maintained until the maximum biosurfactant production was achieved (until the surface tension values remained constant). At the end of the fermentation, the biosurfactants were recovered as described below.

2.1.3. Alternative culture media

In order to reduce the production costs, biosurfactant production was investigated using industrial wastes as cheaper substrates, to replace synthetic media. Alternative low-cost media were prepared using molasses; cheese whey and corn steep liquor. All these substrates were supplied by local industries.

i. Corn steep liquor (CSL) medium

The CSL medium was prepared by dissolving CSL (10 % (v/v)) in demineralized water. The medium was adjusted to pH 7.0 and autoclaved (15 min at 121°C).

ii. CSL and molasses (CSLM) medium

A medium containing CSL and molasses (CSLM) was prepared by dissolving molasses (10 % (w/v)) and CSL (10 % (v/v)) in demineralized water. The medium was adjusted to pH 7.0 and autoclaved (15 min at 121°C).

iii. Cheese whey medium (CWM)

The CWM was prepared by dissolving cheese whey in demineralized water at a concentration of 100 g/l. The solution was sterilized (121°C for 15 min) and the precipitates formed were removed by centrifugation (9000 rpm, 40 min). The supernatants obtained were used as culture medium. The initial pH of this medium was 6.2.

2.1.4. Optimization of culture conditions

i. Effect of aeration on biosurfactant production

Environmental factors, namely aeration, can influence growth and biosurfactant production. In order to study the effect of aeration and improve biosurfactant production, different conditions were tested. *B. subtilis* PX573 was cultured without shaking (microaerophilic conditions) and at

200 rpm, whereas *P. aeruginosa* PX112 was grown at 100, 180 and 200 rpm. All the experiments were performed in 500 ml flasks containing 200 ml of culture medium. Regarding the lactic acid bacteria (*L. agilis* CCUG31450, *L. animalis* ATCC35046 and *L. hamsteri* ATCC43851), growth and biosurfactant production were studied under aerobic, anaerobic and microaerophilic conditions. Aerobic and microaerophilic cultures were performed in 500 ml flasks containing 200 ml of MRS medium at 100 rpm and without shaking, respectively. Anaerobic cultures were performed in 50 ml flasks containing 25 ml of MRS medium. After inoculation, the flasks were sealed with rubber stoppers, and oxygen was removed by aseptically bubbling oxygen-free nitrogen into the flasks for 10 min.

In all the cases, samples were taken at different time points during the fermentation to determine growth and biosurfactant production, except in the case of anaerobic cultures, where they were evaluated at the end of the fermentation.

2.1.5. Effect of trace elements on biosurfactant production

The effect of different trace elements (iron, manganese and magnesium) on biosurfactant production by *P. aeruginosa* PX112 and *B. subtilis* PX573 was evaluated. The culture media used for these assays were the ones previously optimized for each strain (CSLM for *P. aeruginosa* PX112 and CSL for *B. subtilis* PX573), which were supplemented with different trace elements at different concentrations as shown in Table 7 and Table 8. Control assays were performed using the media without trace elements.

Table 7. Concentrations of trace elements tested for biosurfactant production by *B. subtilis* PX573.

Condition		Concentration (mg/l)	References
B.A	$FeSO_4.7H_2O$	7.3	[80; 82]
B.B		73.1	[106]
B.C		548.7	[107]
B.D	$MnSO_4.H_2O$	0.0037	[81]
B.E		0.034	[106]
B.F		0.34	[80; 82]
B.G	$MgSO_4$	100	[80; 106; 82]
B.H		250	-
B.I		500	[108]

Table 8. Concentrations of trace elements tested for biosurfactant production by *P. aeruginosa* PX112.

Condition		Concentration (mg/l)	References
P.A	$FeSO_4.7H_2O$	0.3	[109]
P.B		0.6	[110]
P.C		7.4	[83]
P.D	$MnSO_4.H_2O$	150	[109]
P.E		300	[83]
P.F		500	-
P.G	$MgSO_4$	100	[110]
P.H		200	[83; 55; 109]
P.I		500	-

The cultures were performed in 250 ml flasks containing 100 ml of medium and incubated at the optimal conditions established previously for each strain. Growth and biosurfactant production were determined as previously described.

After establishing the optimal concentration of the different trace elements, in the case of *B. subtilis* PX573, the effect of different trace elements combinations on biosurfactant production was evaluated. The different combinations tested are shown in Table 9.

Table 9. Trace elements mixtures tested for biosurfactant production by *B. subtilis* PX573.

Condition		Concentration (mg/l)
B.J	$FeSO_4.7H_2O$	548.7
	$MnSO_4.H_2O$	0.034
B.K	$FeSO_4.7H_2O$	548.7
	$MgSO_4$	250
B.L	$MnSO_4.H_2O$	0.034
	$MgSO_4$	250
B.M	$FeSO_4.7H_2O$	548.7
	$MnSO_4.H_2O$	0.034
	$MgSO_4$	250

2.1.6. Biosurfactants recovery

Biosurfactants can be excreted to the culture medium (extracellular biosurfactants) or remain attached to the cell wall (intracellular biosurfactants). Therefore, depending on the biosurfactant, the processes used for biosurfactant recovery are different. For the extracellular biosurfactants, three different techniques were evaluated: acid precipitation (for biosurfactants which become insoluble at low pH values); solvent extraction (where organic solvents are used to extract the biosurfactants from the culture medium); and ammonium sulfate precipitation (the biosurfactants are precipitated through a salting-out process) [2]. Regarding the intracellular biosurfactants, they were extracted from the cell-wall as described below.

i. Extracellular biosurfactants

At the end of fermentation, the culture broth was centrifuged (9000 rpm, 20 min) to remove the cells and the cell-free supernatants were subjected to the different recovery techniques.

- *Acid precipitation*

The cell-free supernatants were adjusted to pH 2.0 with HCl 18 % (v/v) and incubated overnight at 4°C to promote the precipitation of the biosurfactants. Afterwards, the precipitates (crude biosurfactants) were collected by centrifugation (9000 rpm, 20 min, 4°C). The crude biosurfactants were dissolved in a minimal amount of demineralized water and the pH was adjusted to 7.0 using NaOH 1 M. Finally, the biosurfactant solutions were freeze-dried and the products obtained weighed and stored at -20°C for further studies.

- *Solvent extraction*

The cell-free supernatants were extracted with 3 volumes of chloroform or a mixture of chloroform:methanol:butanol (1:1:1 v/v/v). The mixtures were continuously shaken at room temperature for 5h. Subsequently, the mixtures was transferred to a separating funnel and allowed to separate overnight. The organic (lower) phase was recovered and the solvent was removed using a rotary evaporator at room temperature. The viscous product obtained was dissolved in a minimal amount of demineralized water, the surface tension was measured, and after that the solution was freeze-dried. The upper phase was extracted again in the same conditions to recover the maximum amount of biosurfactant.

- *Ammonium sulfate precipitation*

Ammonium sulfate was added to the biosurfactant-containing cell-free supernatants to a final concentration of 40 % (w/v) with stirring. This process was performed at 4°C and the mixture was incubated at 4°C overnight. The precipitate was collected by centrifugation (9000 rpm, 20 min, 4°C) and resuspended in demineralized water [111]. After measuring the surface tension, the solution was freeze-dried.

ii. Intracellular biosurfactants

Biosurfactants attached to the cell wall must be extracted from the cells. At the end of the fermentation, cells were harvested by centrifugation (9000 rpm, 20 min), washed once in the same volume of demineralized water, and resuspended in phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). 15 ml of PBS buffer were used per 100 ml of culture broth [101]. The suspension was left at room temperature for 2h with gentle stirring to promote biosurfactant release. Subsequently, the cells were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.2 µm pore-size filter (Whatman, GE Healthcare, UK). The supernatant was dialyzed against demineralized water at 4°C in a Cellu-Sep© membrane (molecular weight cut-off (MWCO) 6000–8000 Dalton; Membrane Filtration Products, Inc., USA) for 48h and freeze-dried. After being lyophilized the biosurfactants were stored at -20°C for further studies. In order to confirm biosurfactant production, during the extraction process the surface tension was routinely measured as described below.

2.2. Biosurfactants characterization

2.2.1. Surface-activity determination

Surface tension measurements of culture broth supernatants and biosurfactant solutions were performed according to the Ring method as described by Gudiña et al. [112]. A KRÜSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm De Noüy platinum ring

was used. Whenever required, the culture broth supernatants were diluted 10 times (ST^{-1}) or 100 times (ST^{-2}) with demineralized water and the surface tension was measured as described above. In order to increase the accuracy of the surface tension measurements, an average of duplicates was determined. All the measurements were performed at room temperature (20°C).

2.2.2. Emulsifying activity determination

Emulsifying activity was determined by the addition of 2 ml of *n*-hexadecane to the same volume of cell-free culture broth supernatants or biosurfactant solutions in glass test tubes. The tubes were mixed with vortex at high speed for 2 min and subsequently incubated at 25°C for 24h. The stability of the emulsion was determined after 24h, and the emulsification index (E_{24}, %) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) [103]. Whenever required, the culture broth supernatants were diluted 10 times (E_{24}^{-1}) or 100 times (E_{24}^{-2}) with demineralized water and the emulsifying activity was measured as described above.

2.2.3. Critical micelle concentration (CMC)

Critical micelle concentration is defined as the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. Therefore, it is important for several biosurfactant applications to establish the CMC, as above this concentration no further effect is expected in the surface activity. Crude biosurfactant solutions at different concentrations (depending on the microorganism and the culture medium used) were prepared in PBS buffer, and the surface tension of each sample was determined by the Ring method at room temperature (20°C) as described above. The CMC was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration and it was found at the point of intersection between the two lines that best fit through the pre- and post-CMC data [35]. All the measurements were done in duplicate.

2.2.4. Biosurfactant stability

The applicability of biosurfactants can be conditioned by their stability to different environmental parameters. Therefore, the effect of pH, temperature and salinity on the activity of biosurfactants produced by *B. subtilis* PX573 and *P. aeruginosa* PX112 was determined. Stability studies were performed using the cell-free broths obtained by centrifuging the cultures (at the end of the fermentation) at 9000 rpm for 20 min. In order to assess the effect of salinity on biosurfactant activity, the culture broth supernatants were supplemented with different NaCl concentrations (from 10 to 200 g/l). The surface tension and the emulsification indexes were measured as described above and compared with the corresponding values without addition of NaCl. To evaluate the stability of the biosurfactants to high temperatures, the broth samples were incubated at 121°C for 20 min and allowed to cool to room temperature. Surface tension and emulsification indexes were measured and compared to the corresponding values before heat treatment. The pH stability was studied by adjusting the cell-free broths to different pH values (2.0-13.0) using HCl or NaOH solutions. Then surface tension values and emulsification indexes were measured as described above. In the case of *L. agilis* CCUG31450 (that produces mainly intracellular biosurfactants) the stability assays were performed in the same way but using the freeze-dried biosurfactants dissolved in PBS buffer at a concentration of 7.5 mg/ml.

2.2.5. Thin layer chromatography (TLC)

A preliminary characterization of the different crude biosurfactants obtained was obtained by TLC. Lyophilized biosurfactant samples were dissolved in different solvents (methanol or acetonitrile) or PBS buffer. Between 4 and 8 µl of each sample were applied to a 10 x 20 cm silica gel TLC plate (Fluka® Analytical, Germany). The plates were developed using two different mobile phases: (a) chloroform-methanol-water (65:25:4 v/v/v) [113,114]; (b) acetonitrile-water (6:3 v/v) [115]. Subsequently the plates were air dried and visualized under ultra-violet (UV) light (254 and 366 nm) and the different spots detected were recorded. The retention factor (Rf) value for each spot was calculated as:

$$\mathbf{Rf} = \frac{\text{migration distance of substance spot}}{\text{migration distance of solvent front}}$$

2.3. Analytical techniques

The alternative culture media used and some of the biosurfactants produced were analyzed for total of carbohydrates and protein contents using the following methods:

2.3.1. Carbohydrate concentration

Total carbohydrates were determined using the phenol–sulfuric acid method as described by DuBois et al. [38]. Briefly, each sample (1 ml) was mixed with 20 µl of phenol solution (80 % (w/v)) in glass test tubes. Then 2.5 ml of concentrated sulfuric acid (95-97 %) were added to each tube with slight shaking. The samples were left at room temperature for 10 min, shaken again and allowed to stand at room temperature for another 20 min. Afterwards, the optical density of each sample was measured at 490 nm using a multi-detection microplate reader Synergy™ HT (BioTek, USA). The carbohydrates concentration was determined using a calibration curve prepared using glucose at concentrations ranging from 1 to 100 mg/l. All the assays were performed in triplicate.

2.3.2. Protein concentration

The protein concentration was determined according to the Bradford assay using a protein assay kit (Thermo Scientific, USA). Briefly, 30 µl of sample were mixed with 1.5 ml of Bradford reagent and incubated at room temperature for 5 min. Subsequently, the optical density at 595 nm was measured using a multi-detection microplate reader Synergy™ HT (BioTek, USA). The total amount of protein was determined using a calibration curve prepared using bovine serum albumin (BSA) at concentrations ranging from 0.1 to 1 mg/ml. All the assays were performed in triplicate.

2.4. Biosurfactants applications

2.4.1. Antimicrobial assays

The antimicrobial activity of the different biosurfactants against several microbial strains (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. agalactiae* and *C. albicans*) was determined using the micro-dilution method in 96-well plastic tissue culture plates (Orange Scientific, Belgium). The freeze-dried biosurfactants were dissolved in LB medium at different concentrations, and biosurfactant solutions were sterilized by filtration through a 0.2 µm pore-size filter (Whatman, GE Healthcare, UK). Subsequently, 250 µl of sterile biosurfactant solution were placed into the first column of the 96-well microplate, and 125 µl of sterile LB medium in the remaining wells. After that, 125 µl of biosurfactant solution from the first column were transferred to the second column and mixed with the medium. Serially, 125 µl were transferred to the subsequent wells, discarding 125 µl of the mixture in the tenth column, so that the final volume for each well was 125 µl. This process results in two-fold serial dilutions of the biosurfactant in the first 10 columns. Columns 11 and 12 do not contain biosurfactant and serve as growth and negative controls, respectively. All the wells (except for the 12th column) were inoculated with 5 µl of a pre-culture growth overnight in LB medium at 37°C diluted to an optical density (600 nm) of 0.3. Microplates were covered and incubated for 24h at 37°C. After 24h of incubation, 125 µl of demineralized water were added to each well and the optical density at 600 nm was determined using a multi-detection microplate reader Synergy™ HT (BioTek, USA). The growth inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\%\ Growth\ inhibition_c = \left[1 - \left(\frac{ODc}{ODo}\right)\right] \times 100$$

where OD_c represents the optical density of the well with a biosurfactant concentration c and OD_0 is the optical density of the control well (without biosurfactant) [112]. Triplicate assays were performed for the different microorganisms and biosurfactant concentrations.

2.4.2. Anti-adhesion assays

The anti-adhesive activity of the different biosurfactants against *P. aeruginosa* and *S. aureus* was determined according to the procedure described by Gudiña et al. [112]. Briefly, the wells of a sterile 96-well flat-bottomed plastic tissue culture plate (Orange Scientific, Belgium) were filled with 200 µl of biosurfactant solutions prepared in demineralized water at different concentrations. The plate was incubated for at least 18h at 4°C and subsequently washed twice with PBS buffer. Control wells contained distilled water. An aliquot of 200 µl of a washed bacterial suspension in PBS adjusted to an optical density (600 nm) of 0.6 was added to each well and incubated for 24h at 4°C. Unattached microorganisms were removed by washing the wells twice with PBS. The adherent microorganisms were fixed with 200 µl of 99 % methanol per well, and after 15 min the plates were emptied and left to dry. After that, the plates were stained for 5 min with 200 µl of 33 % crystal violet (used for Gram staining) per well. Excess stain was rinsed out by washing the wells three times with PBS. Subsequently the plates were air dried; the dye bound to the adherent microorganisms was re-solubilized with 200 µl of 33 % (v/v) glacial acetic acid per well and the optical density of each well was measured at 595 nm. The microbial inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\%\ Microbial\ inhibition_c = \left[1 - \left(\frac{ODc}{ODo}\right)\right] \times 100$$

where OD_c represents the optical density of the well with a biosurfactant concentration c and OD_0 is the optical density of the control well (without biosurfactant) [112]. Triplicate assays were performed at all the biosurfactant concentrations for the two strains. This procedure allows the estimation of the biosurfactant concentrations that are more effective in inhibiting the adhesion of the studied microorganisms.

2.4.3. Application of biosurfactants in the removal of crude oil from sand

The applicability of biosurfactants produced by *B. subtilis* PX573, *P. aeruginosa* PX112 and *L. agilis* CCUG31450, as well as the chemical surfactants Enordet and Petrostep (supplied by SNF FLOERGER (France)) in oil recovery was evaluated, using artificially contaminated sand containing 12.5 % (w/w) of Arabian Light oil (provided by GALP (Portugal)). Samples of 40 g of sand were

mixed with 5 g of crude oil in 100 ml Erlenmeyer flasks by shaking and allowed to age at room temperature for 24h. Afterwards, 40 ml of biosurfactants and chemical surfactants solutions (at concentrations ranging from 0.5 to 5 g/l) were added to each flask. The flasks were incubated at 90 rpm and 40°C for 24h and the oil removed was recovered from the surface and its volume was measured. The amount of oil recovered (grams) was calculated according to its density (0.837 g/ml). Control assays were performed using demineralized water at the same conditions. All the experiments were carried out in duplicate.

2.5. Biosurfactant mixtures

To evaluate the interaction between biosurfactants produced by the different microorganisms studied several mixtures with different composition and proportions were prepared. Those mixtures were evaluated in different applications, namely in antimicrobial and anti-adhesion assays as well as in removal of crude oil from sand. In this last case, two chemical surfactants were also tested, alone and in combination with different biosurfactants. In all the cases, the freeze-dried biosurfactants were dissolved in demineralized water (or in LB medium for the antimicrobial assays) at different concentrations, and then the different mixtures were prepared.

2.6. Statistical analysis

Results are presented as the mean ± standard deviation of at least two replicates. The analyses were carried out using Microsoft Office Excel software.

Statistically significant differences of the conditions tested in the different assays were evaluated by a one-way ANOVA ($P<0.05$) applying the Tukey multiple-comparisons. A significant difference was considered if $P<0.05$. Statistical analyses were performed using GraphPad (San Diego, USA) software.

3. RESULTS AND DISCUSSION

3.1. Screening of biosurfactant-producing strains

A total of seven isolates were screened for biosurfactants production under standard conditions. As described in the Materials and Methods section, both extracellular and intracellular biosurfactants production was evaluated through the measurement of surface tension and determination of emulsifying activity.

3.1.1. Lactic acid bacteria

Five out of the seven above mentioned isolates were lactic acid bacteria. As can be seen in Table 10, none of the microorganism evaluated was found to decrease the surface tension of the culture medium, thus no extracellular biosurfactants were produced. Nevertheless, a decrease in the surface tension of the PBS could be observed for the PBS extracts revealing the production of intracellular biosurfactants. The isolate *L. agilis* CCUG31450 showed the most interesting results either in relation to surface activity or emulsifying activity.

Table 10. Screening of bacterial isolates for biosurfactant production. Reduction of culture medium surface tension (–ΔST) (mN/m), corresponding to excreted biosurfactants; reduction of PBS surface tension (–ΔST of PBS) (mN/m), corresponding to cell-bound biosurfactants; emulsification index (E_{24}, %) of biosurfactants in PBS; biomass (OD 600 nm) and crude biosurfactants concentration (mg/l) obtained for different lactic acid bacteria strains, grown at 37°C in MRS medium (except *S. thermophilus* A (M17)). The control value of surface tension for the biosurfactants extracted with PBS was 71.9 mN/m. The results represent means of two independent experiments ± standard deviation (SD).

Microorganism	Time (h)	-ΔST of culture medium (mN/m)	- ΔST of PBS (mN/m)	Biomass (OD 600 nm)	E_{24} (%)	[Recovered Biosurfactants] (mg/l)
***L. paracasei* A20**	96	-1.0 ± 2.6	24.0 ± 3.1	2.752 ± 0.013	4	51.5 ± 12.2
***L. agilis* CCUG31450**	96	-1.8 ± 3.1	26.1 ± 3.2	1.152 ± 0.001	20	84.4 ± 1.6
***L. animalis* ATCC35046**	120	-0.5 ± 0.8	25.2 ± 1.7	-	-	27.6 ± 5.6
***L. hamsteri* ATCC43851**	96	-1.5 ± 2.0	18.3	4.065 ± 0.013	0	24.2 ± 1.6
***S. thermophilus* A**	96	0.9 ± 6.0	22.0	3.975 ± 0.015	12	29.4

Several researchers have reported the production of biosurfactants by lactic acid bacteria. Examples include the work by Saravanakumari and Mani [116] that reported an extracellular biosurfactant from *L. lactis* able to reduce the surface tension to 40.5 mN/m; and the work by Rodrigues et al. [77] that evaluated the ability of *L. pentosus* CECT-4023 and *L. casei* to produce biosurfactants and observed that both strains were biosurfactant producers. The surface tension values of biosurfactant extracted with PBS was 50.5, 53.0 mN/m respectively. They also characterized the biosurfactants produced by *L. lactis* 53 and reported a minimum surface tension value of 41.0 mN/m [117].

The lactic acid bacteria under study in the current work, namely *L. agilis* CCUG31450, *L. animalis* ATCC35046 and *L. hamsiteri* ATCC43851 showed surface tension values of 44.0, 47.7 and 53.7 mN/m, respectively. These values are quite similar to those previously described in the literature for other lactic acid bacteria cultured under the same conditions. Furthermore, the strain *L. paracasei* A20, previously studied by Gudiña et al. [118] was reported to show a surface tension value of 50.0 mN/m. The surface tension value obtained is this work (47.9 mN/m) for the same strain, is in good agreement with one reported before. Previously studies about biosurfactants production by *S. thermophilus* A, indicated that the crude biosurfactants showed three distinct fractions (A, B and C) with surface activity, surface tension values 50, 49 and 36 mN/m, respectively [101]. The surface tension value herein obtained for *S. thermophilus* A, 50.0 mN/m, are very close to the values previously reported.

The biosurfactants production by different lactic acid bacteria in MRS medium was also observed by Velraeds et al. [119]. From all strains studied, the most interesting results for the reduction of surface tension, determined relative to the surface tension of PBS (68 mN/m), were obtained for *L. acidophilus* ATCC4356 (27.0 mN/m); *L. casei* subsp. *rhamnosus* ATCC7469 and *L. casei* subsp. *rhamnosus* 81 (27.0 mN/m); *L. fermentum* B54 (29.0 mN/m); and *Lactobacillus plantarum* RC20 (26.0 mN/m). In general, the decrease in surface tension values obtained in this work (Table 10) are similar with the ones previously reported for lactic acid bacteria biosurfactants.

3.1.2. *Bacillus subtilis* PX573

B. subtilis PX573 was another isolate tested for biosurfactant production. This strain reduced the culture medium surface tension to 31.0 mN/m, thus it was considered a good extracellular biosurfactant producer (Figure 4).

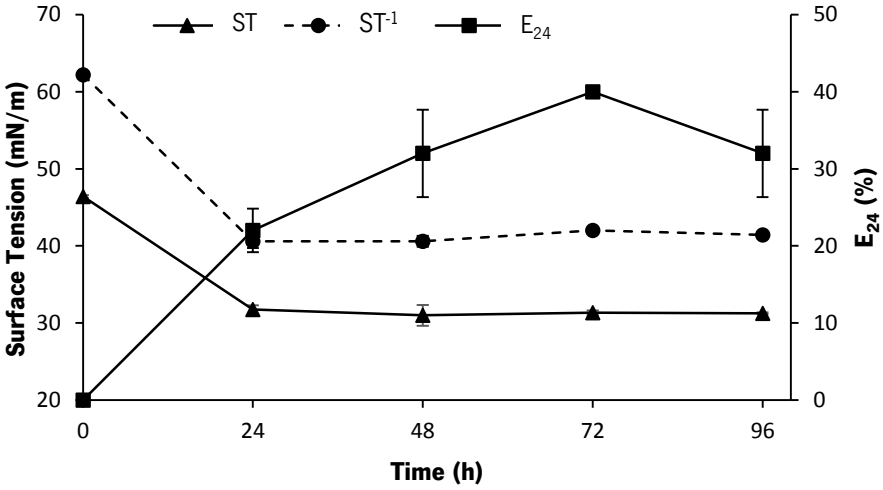

Figure 4. Time course of biosurfactant production by *B. subtilis* PX573 showing the profiles of surface tension (mN/m) and emulsification index (E_{24}, %). The strain was grown on LB medium at 37°C without shaking. Results represent the average of two independent experiments ± SD.

Analyzing the fermentative process it can be seen that in the first 24h it occurs a great reduction of the surface tension on the culture medium, from 46.9 to 31.6 mN/m. From this time point until the end of the fermentation the values of ST and ST^{-1} remained almost constant. In the case of the emulsifying activity, the maximum value, 40.0 %, was observed at 72h. Similar results were reported by Vaz et al.[35] using also a *B. subtilis* strain; in that case, the surface tension of the culture medium was reduced to 30.1 mN/m in the first 24h, and then remained nearly constant until the end of the fermentation (144h). These researchers also noticed that the E_{24} values contrary to the surface tension continued increasing after the first 24h, similar results was observed in the current work with *B. subtilis* PX573.

In order to assess the most favorable fermentation interval for the production of biosurfactant, its concentration in culture medium was measured over time. The results presented

in Table 11 show that the highest biosurfactant concentration is obtained at 48h, although the biomass concentration reached its maximum at 72h. These results suggest that the production of biosurfactants is probably not associated with cellular growth.

Table 11. Biomass concentration (g dry weight/l) and crude biosurfactant concentration (mg/l) obtained for *B. subtilis* PX573 grown in LB medium at 37°C without shaking. Results represent the average of two independent experiments ± SD.

Time (h)	[Biomass] (mg/l)	[Crude biosurfactant] (mg/l)
48	0.914 ± 0.03	726.7
72	1.159 ± 0.08	533.3

In these experiments, in which the *B. subtilis* PX573 strain was cultured without agitation, we noticed that after 48h of fermentation the biomass was very difficult to separate from the culture broth by centrifugation since the cells do not sediment easily, thus compromising the total amount of biosurfactant being recovered (most probably underestimated). In summary, although the surface tension remained constant until the end of fermentation, the amount of biosurfactant recovered was lower at 72h than at 48h of fermentation, not due biosurfactant degradation but due to the recovery limitations.

Ghribi et al. [120] studied the production of biosurfactants by *B. subtilis* SPB1 in a mineral medium with glucose as the carbon source. The bacterium produced 720 mg of biosurfactant/l at the end of the fermentation. The amounts of biosurfactant obtained in our study for 48h of fermentation are in good agreement with that study. Makkar and Cameotra [121] studied the production of biosurfactants by *B. subtilis* MTCC1427 in a minimum medium supplemented with 2 % sucrose, at 30°C and pH 9. The biosurfactant concentration obtained in those conditions was 808.0 mg/l at 48h of fermentation. The same authors also studied the production of biosurfactants by a different *B. subtilis* strain in a mineral salt medium. After 72h of fermentation, the biosurfactants production was 342 mg/l, which is quite low as compared with the concentrations obtained in the current study (Table 11). Similar results were reported for the strain *B. subtilis* 28 studied by Toledo and co-workers [122], that provided 430 mg/l of biosurfactant.

3.1.3. *Pseudomonas aeruginosa* PX112

P. aeruginosa PX112 was the last isolate being evaluated. Figure 5 illustrates the fermentation profiles of growth and biosurfactant production.

In the case of this strain, the biosurfactant production was found to be associated with cellular growth, as an increase in the biomass concentration led to a decrease in the surface tension, especially in the first 48h. The surface tension continued dropping until 72h reaching its lowest value (33.0 N/m), and then remained nearly constant until de end of fermentation (96h). The E_{24} values also reached the highest value at 72h, 60.0 %. After this time point the E_{24} values remained constant until the end of fermentation. At the end of fermentation the biosurfactants present in the culture medium were recovered by acid precipitation. The amount of biosurfactant obtained was 136.1 ± 41.6 mg/l.

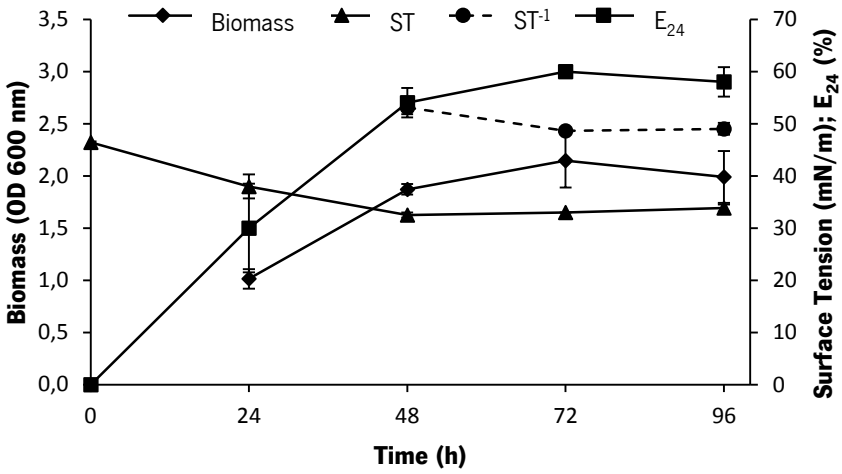

Figure 5. Time course of growth and biosurfactant production by *P. aeruginosa* PX112 showing the profiles of biomass (OD 600 nm), surface tension (mN/m) and emulsification index (E_{24}, %). The strain was grown in LB medium at 37ºC and 100 rpm. Results represent the average of two independent experiments ± SD.

The biosurfactant production by *P. aeruginosa* BS-161R in Bushnell-Haas medium was evaluated by Kumar et al. [55]. In this case the yield of rhamnolipid produced was 369 mg/l, slightly higher than the one that we obtained using the *P. aeruginosa* PX112 strain (136 mg/l). Wei et al. [123] studied the rhamnolipid production by *P. aeruginosa* J4 grown in LB at 30ºC, 200

rpm. The yield obtained, 773 mg/l, was much higher than the one obtained for *P. aeruginosa* PX112 in LB medium, at 100 rpm, thus suggesting that an increase in agitation could lead to an improvement on the biosurfactant production. The production of biosurfactants by *P. aeruginosa* MR01 in a minimum salt medium was also described in literature [124]. The results showed that the biosurfactant production was affect by the NaCl concentration, and reported biosurfactants yields between 300 and 500 mg/l. These values are higher than ours probably as a result of the higher agitation used or due to the NaCl concentration present in LB medium.

3.2. Optimization of biosurfactants production

3.2.1. Alternative culture media

In order to reduce the production costs, biosurfactant production was investigated using industrial wastes as cheaper substrates, to replace synthetic media. Alternative low-cost media were prepared using molasses; cheese whey and corn steep liquor. All these substrates were supplied by local industries. Before analyzing the biosurfactants production using the alternative media, their characterization regarding protein and carbohydrates concentrations was conducted (Table 12).

Table 12. Concentration of carbohydrates and protein (mg/ml) present in the industrial wastes used to prepare the different alternative media. Results represent the average of three measurements ± SD.

	[Carbohydrates] (mg/ml)	**[Protein] (mg/ml)**
CSL (10% (v/v))	7.5 ± 0.01	0.5 ± 0.02
Molasses (10% (w/v))	49.0 ± 0.92	0.06 ± 0.04
Cheese whey (100 g/l)	34.9 ± 0.79	8.0 ± 0.9

3.2.2. Lactic acid bacteria

An optimization strategy was adopted for the biosurfactant production by *L. agilis* CCUG31450, using alternative media based on low-cost substrates suggested by the literature [1, 68]. Previously was optimized the culture conditions for the acid lactic bacteria studied.

i. Optimization of culture conditions

In order to evaluate the most favorable conditions of oxygenation towards an increased biosurfactant production, the acid lactic bacteria *L. agilis* CCUG31450, *L. animalis* ATCC35046 and *L. hamsteri* ATCC43851 were cultured in anaerobic and microaerophilic conditions. The fermentations were ended at the time point in which the cells stopped growing. These results were compared with the ones obtained for aerobic conditions during the screening process (Section 3.1). The results suggest that aerobic conditions (Table 10) are more favorable for biosurfactant production by these lactic acid bacteria comparing to anaerobic and microaerophilic conditions (Table 13), except for the case of *L. animalis* ATCC35046, for which similar results were obtained under aerobic and microaerophilic conditions. For *L. agilis* CCUG31450, the surface tension decrease in aerobic conditions (26.1 mN/m) was higher than in anaerobic and microaerophilic conditions (15.0 and 17.4 mN/m, respectively).

Table 13. Effect of aeration on the biosurfactants production. Reduction of PBS surface tension (–ΔST) (mN/m) obtained for the crude biosurfactants produced by different isolates grown under anaerobic and microaerophilic conditions in MRS medium at 37°C. Biomass results represent the average of two measurements ± SD.

Microorganisms	Anaerobic			Microaerophilic		
	- ΔST of PBS	Biomass (OD 600 nm)	Time (h)	- ΔST of PBS	Biomass (OD 600 nm)	Time (h)
***L. agilis* CCUG31450**	15.0	2.305 ± 0.01	48	17.4	2.590 ± 0.01	48
***L. animalis* ATCC35046**	21.6	-	120	23.8	2.315 ± 0.00	120
***L. hamsteri* ATCC43851**	11.5	3.400 ± 0.01	48	16.0	3.605 ± 0.01	48

ii. Alternative culture media

Three different alternative media, CWM, CWM+CSL and CSLM, were used to evaluate the biosurfactant production by *L. agilis* CCUG31450. Similarly to the results presented in Table 10, also in this case no reduction of the supernatants surface tension values (Table 14) was observed, thus demonstrating that *L. agilis* CCUG31450 does not excrete biosurfactants into the culture medium. On the other hand, the analysis of the surface tension reduction of the PBS showed a good production of cell-bound biosurfactants for all the alternative media evaluated. Comparing the –ΔST values of PBS, obtained for the different alternative media and for the synthetic MRS medium, it is possible to observe a higher reduction of the PBS surface tension in the case of biosurfactants produced in MRS medium. Statistical analysis demonstrated that these differences were significant (*p*-value = 0.0114). On the other hand, regarding the amount of biosurfactants recovered in the different media, a clearly higher amount was observed for the CWM (959 mg/l), contrarily to the amount obtained for the MRS medium (87.0 mg/l), after 120h of fermentation. In general, a higher concentration of biosurfactants was obtained for all the alternative media comparing with the MRS medium. However, these results must be carefully considered, since the recovered compounds can eventually include other components from the culture medium that are not surface active molecules.

Table 14. Biosurfactant production by *L. agilis* CCUG31450 in different alternative media. Reduction of culture medium surface tension (–ΔST) (mN/m) reduction of PBS surface tension (–ΔST of PBS) (mN/m)) and crude biosurfactants concentration (mg/l) obtained in the different media. The results represent the average of two measurements ± SD, except the results of MRS medium that represent the average of two independent experiments ± SD.

Culture Media	Time (h)	-ΔST of culture medium (mN/m)	- ΔST of PBS (mN/m)	[Recovered biosurfactant] (mg/l)
CWM	96	-	21.2 ± 0.3	623.5 ± 43.1
	120	-1.3 ± 0.4	22.7 ± 0.1	959.0 ± 9.90
CWM+CSL	96	-1.5 ± 2.0	21.0 ± 0.1	294
	120	-2.1 ±0.4	21.7 ± 0.1	492.5 ± 29.0
CSLM	120	0.3 ± 0.0	21.0 ± 0.9	356.3
MRS	96	-1.8 ± 3.1	26.1 ± 3.2	84.4 ± 1.6
	120	-3.6	24.0	87.0 ± 4.0

In the literature, cheese whey is described as a good substrate for biosurfactant production, once it contains high levels of lactose, protein, organic acids and vitamins [69, 70]. Cheese whey can be an interesting substrate for biosurfactant production by lactic acid bacteria, which have the ability of using lactose as a carbon source. As can be seen the bacterium *L. agilis* CCUG31450 was able to produce biosurfactants in CWM, as confirmed by the decrease of PBS surface tension (Table 14), thus demonstrating that CWM could be used alternatively to MRS medium. Biosurfactant production by *L. pentosus* CECT-4023, a strong biosurfactant producer strain, using cheese whey as substrate was studied by Rodrigues et al. [77]. Optimization of the biosurfactant production led to a maximum biosurfactant concentration of 1.4 g /l. This value is higher than the one obtained in the current study (959.0 mg/l).

Rodrigues et al. [78] also studied the potential of other alternative media for biosurfactants production. Cheese whey and/or molasses combined with peptone and yeast extract have been used. The results obtained showed that both cheese whey and molasses media can be used as a relatively inexpensive and economical alternative to the synthetic media commonly used for biosurfactant production by probiotic bacteria. Specifically, an increase about 2 times in the mass of produced cell-bound biosurfactant per gram cell dry weight could be obtained when compared to the synthetic medium. In the current study, a higher amount of biosurfactants was obtained using CWM when compared with the medium composed by molasses, contrary to the findings of Rodrigues et al [78].

Furthermore, in the current study we attempted to replace the yeast extract by cheaper nitrogen sources (CSL) also mentioned in other reports. Lee and co-workers [125] reported the use of CSL (5 % (v/v)) as a suitable option to replace yeast extract to produce lactic acid using Lactobacilli strains. Although in our work, the -ΔST of PBS was quite similar for the biosurfactants produced in CWM and CWM+CSL, the supplementation with CSL did not improve the amount of biosurfactants produced, once the results obtained for CWM (959.0 mg/l) are higher than with CWM+CSL (492.5 mg/l) after 120h of fermentation.

It was interesting to notice that a change in the carbon source (from glucose to lactose) induced the cells to produce more biosurfactant. However, a higher amount of biosurfactants did not imply a higher or equal surface activity. The different carbon sources yielded varying amounts of by-products [126]. Therefore, it can be speculated that the use of lactose as carbon source instead of glucose induced the cells to use another metabolic pathway, and therefore the amount

of cell-bound biosurfactant produced per gram of cell varied. Moreover, it should be noticed that biosurfactants produced in different media can have different properties and structures. Therefore, further studies were conducted to analyze the activity of the biosurfactants produced by *L. agilis* CCUG31450 in CWM and MRS media.

3.2.3. *Bacillus subtilis* PX573

The high biosurfactant production that was found for *B. subtilis* PX573 in LB medium makes this microorganism an interesting choice to further optimize the biosurfactant production while reducing the production costs. Therefore, an alternative medium based on industrial wastes, such as CSL, as a low-cost substrate was studied. This alternative medium was supplemented with different trace elements and the agitation conditions were also optimized.

i. Alternative culture media

Afterwards the biosurfactant production was evaluated as previously mentioned through the measurement of surface tension and emulsifying activity along the time. Whenever required the cell-free supernatants were diluted 10 or 100 times, and the corresponding surface tension values and emulsification indexes (ST^{-1}/E_{24}^{-1} and ST^{-2}/E_{24}^{-2}) were measured. The lowest surface tension value (30.6 mN/m) was observed at 24h, showing a high decrease in the surface tension when compared with the CSL medium itself (52.9 mN/m) (Figure 6A). After the first 24h, the surface tension values started increasing; this effect is particularly observed for the ST^{-2} values. In the case of the emulsifying activity (Figure 6B), the E_{24} values remained constant throughout the fermentation and no significant changes could be observed comparing with the emulsifying activity of the culture medium. However, analyzing the results obtained for the E_{24}^{-2}, an evident increase of the emulsifying activity at 48h (20 %) was found.

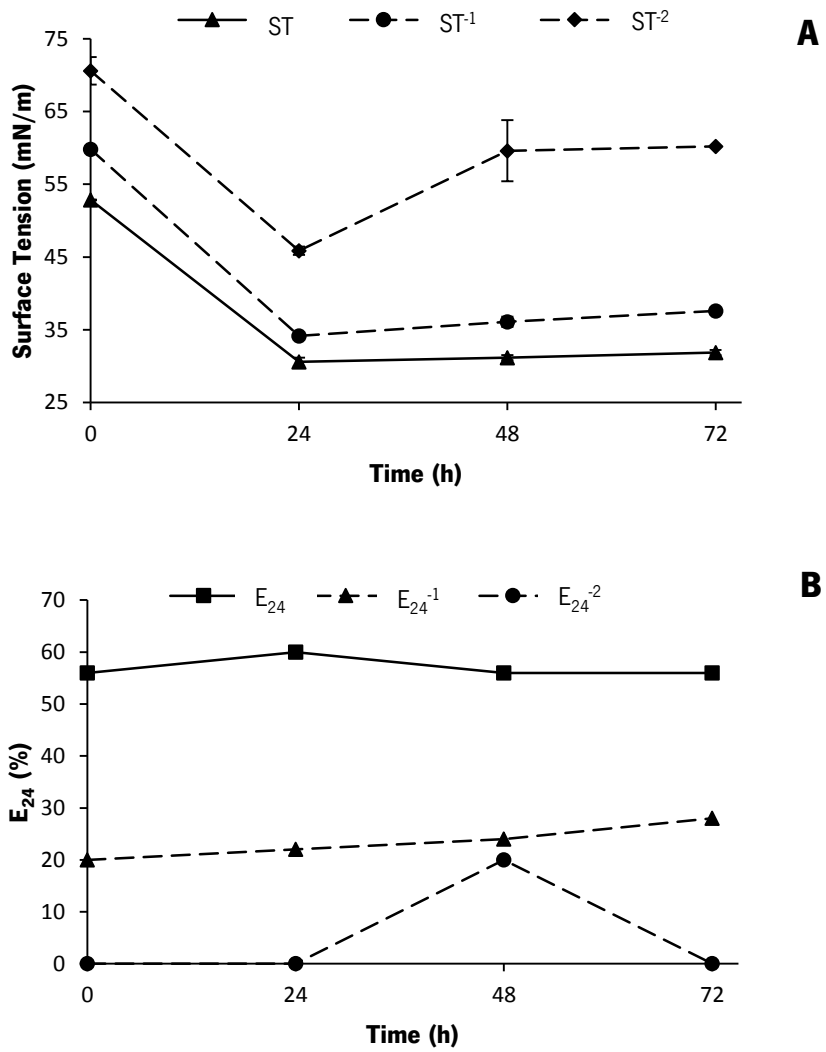

Figure 6. Profiles of the culture broth supernatant surface tension (mN/m) **(A)** and emulsification index (E_{24}, %) **(B)** during the time course of the fermentation by *B. subtilis* PX573. The strain was cultured in CSL medium at 37°C without shaking. Results represent the average of two measurements ± SD.

Furthermore, the biosurfactants concentration was also determined at two different fermentation time points. As can be seen in Table 15, a higher production of biosurfactants in CSL medium was reached at 48h.

Table 15. Biosurfactants concentration (mg/l) obtained using *B. subtilis* PX573 grown in CSL medium at 37°C and without shaking.

	Time (h)	
	24	**48**
[Recovered biosurfactants] (mg/l)	1236.0	2060.0

Comparing the biosurfactant concentrations obtained for the different media studied, it was found that the CSL medium is the most favorable for biosurfactant production, when compared with LB medium, which is in agreement with the ST^{-1} values previously obtained. Specifically, the growth of *B. subtilis* PX573 in CSL medium showed a biosurfactants production (2060.0 mg/l) 2.8 times higher comparing with the values obtained using LB medium (726.7 mg/l). Although a higher amount of biosurfactants is produced in CSL medium, the efficiency of these molecules being produced using different media should also be considered, as described later.

Several researchers described the production of biosurfactants by *B. subtilis* strains using low-cost substrates. Al-Bahry et al. [127] studied the biosurfactant production by *B. subtilis* B20 grown in a low-cost molasses-based mineral media. Acidic precipitation allowed recovering 2.29 g/l crude biosurfactant using a medium containing 80 g/l molasses. These biosurfactants reduced the surface tension from 58 to 27 mN/m. Nitschke et al. [15] obtained a crude biosurfactant concentration of 3.0 g/l after 48h of fermentation growing *B. subtilis* LB5a in cassava waste. These biosurfactants reduced the surface tension of the medium to 26.6 mN/m. The biosurfactant yields obtained in the current work (2.06 g/l, 48h) are in agreement with the values reported in the literature. In this way, CSL can be considered an interesting alternative low-cost substrate for the production of biosurfactants.

ii. Optimization of culture conditions

After studying the biosurfactants production using the alternative media CSL, a further optimization step was conducted regarding the agitation rates (from 0 to 200 rpm). Similarly to the experiments without agitation, the most interesting results regarding the emulsifying activity were achieved at 48h (Figure 7B), principally for the E_{24}^{-1} (36 %) and E_{24}^{-2} (16 %) values comparing with the culture medium (E_{24}^{-1}, 20 %, and E_{24}^{-2}, 0 %). A pronounced decrease of the surface tension, from 52.9 to 29.7 mN/m, was observed in the first 24h (Figure 7A). Nevertheless, the ST^{-1} and ST^{-2} values were found to continuously decrease until 48h of fermentation. After 48h of fermentation, the concentration of biosurfactants was 2171.8 ± 18.0 mg/l.

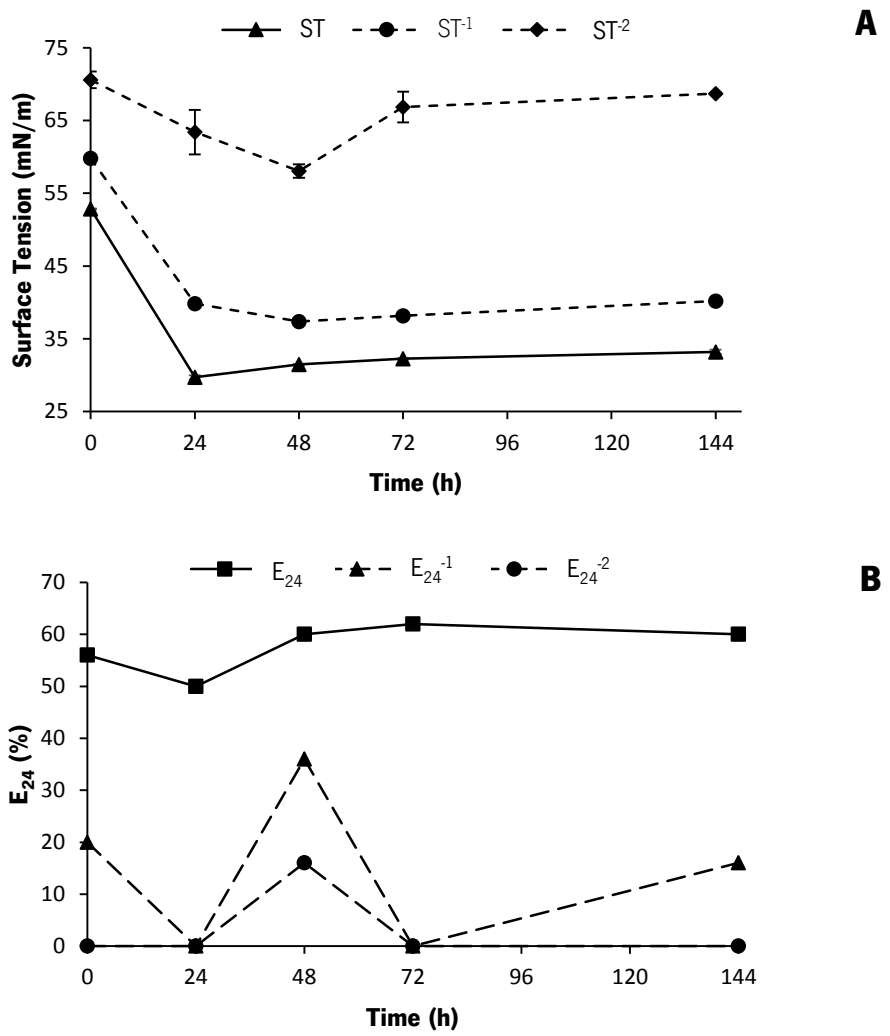

Figure 7. Profiles of the culture both supernatant surface tension (mN/m) **(A)** and emulsification index (E_{24}, %) **(B)** during the time course of the fermentation by *B. subtilis* PX573. The strain was grown on CSL medium at 37°C and 200 rpm. Results represent the average of two measurements ± SD.

iii. Effect of trace elements

The alternative CSL medium was supplemented with three metal ions, Fe^{2+} (conditions B.A, B.B and B.C), Mn^{2+} (conditions B.D, B.E and B.F) and Mg^{2+} (conditions B.G, B.H and B.I) at different concentrations (Table 7). From Figure 8 it can be seen that almost all the conditions studied led to a decrease of surface tension as compared with the control (CSL without supplementation). The conditions B.C, B.E and B.H stand out as the best ones, presenting the most notable effect in the decrease of surface tension, mainly showed by the ST^{-2} values at 48h (Figure 8C), 48.5 mN/m, 48.7 mN/m and 49.0 mN/m, respectively. These results were analyzed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test. The

differences observed between the surface tension values of the control and B.C, B.F and B.H conditions were found to be statistically significant (*p*-value = 0.0108).

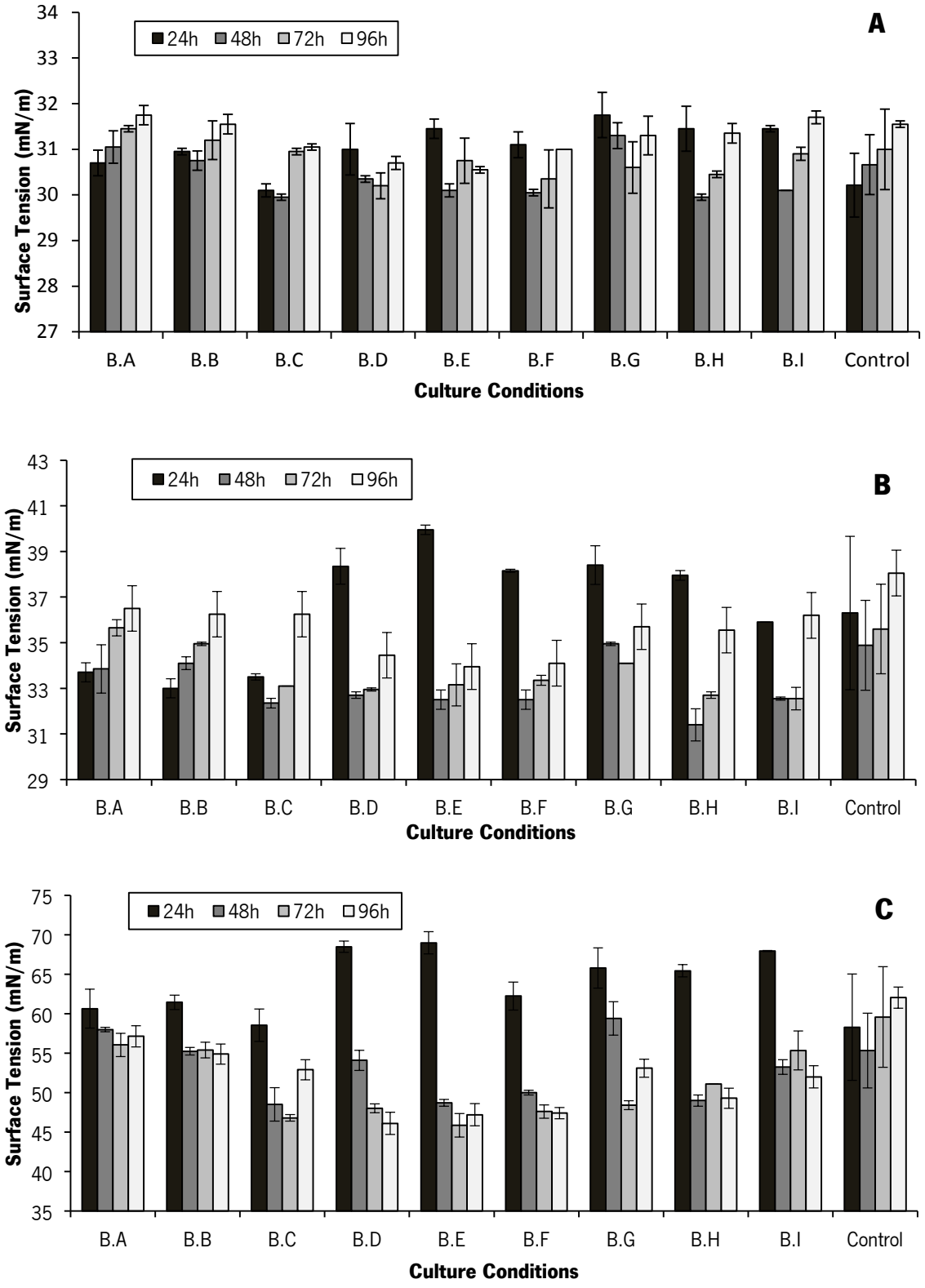

Figure 8. Effect of trace elements (Fe^{2+}, Mn^{2+} and Mg^{2+}) at different concentrations on the biosurfactant production by *B. subtilis* PX573. Profile of surface tension (ST) **(A)** ST^{-1} **(B)** and ST^{-2} **(C)** along the time. The strain was cultured in CSL medium at 37°C and 200 rpm. Results represent the average of two measurements ± SD.

Although the differences in the surface tension values were obvious, this was not reflected in the amount of biosurfactants being recovered (Table 16). Indeed, a slightly higher biosurfactant production was found in the control comparing with the experiments in which trace elements have been supplemented. Thus, a higher amount of biosurfactants recovery did not correspond to a higher surface activity. It is well-known that biosurfactants produced in different media can present different properties, which might have been the case. On the other hand, the presence of impurities in the media may also differ, thus leading to different biosurfactant efficiencies.

Table 16. Biosurfactant production (mg/l) by *B. subtilis* PX573 grown in CSL medium supplemented with different trace elements at 37°C and 200 rpm after 72h of fermentation.

Culture conditions	[Recovered biosurfactants] (mg/l)
Control	2361.6
B.C	2280.5
B.E	2085.4
B.H	2062.7

Several researchers showed that in some cases the optimal concentration determined individually for each trace element could not be the optimal for the biosurfactants production when all the trace elements are present in the culture medium [106]. Specifically, the results reported by Wei et al. [106] suggest an interaction between the metal ions in terms of their effects on biosurfactants production. Therefore, in the current work, after determining the best concentration of each element, the interaction between them and their effect on biosurfactants production was further explored. Four combinations of the three metal ions at their optimal individual concentration were evaluated: conditions B.J (Fe^{2+} and Mg^{2+}), B.K (Fe^{2+} and Mn^{2+}), B.L (Mg^{2+} and Mn^{2+}), and B.M (Fe^{2+}, Mg^{2+} and Mg^{2+}) (Table 9, Materials and Methods section). From Figure 9 it can be seen that the best results regarding surface tension decrease were obtained in the first 24h. The combination of trace elements was found to be more effective in the surface tension reduction then the elements themselves. The four conditions tested showed quite similar behaviors, as demonstrated by the ST^{-1} values in the first 24h, B.J: 31.6 mN/m, B.K: 31.5 mN/m, B.L: 31.3 mN/m and B.M: 31.3 mN/m. However, only slight decreases of the surface tension could be observed comparing with the control (36.3 mN/m). The statistical analysis showed that the differences between the control and the different combinations of trace elements were not significant (p-value >0.05).

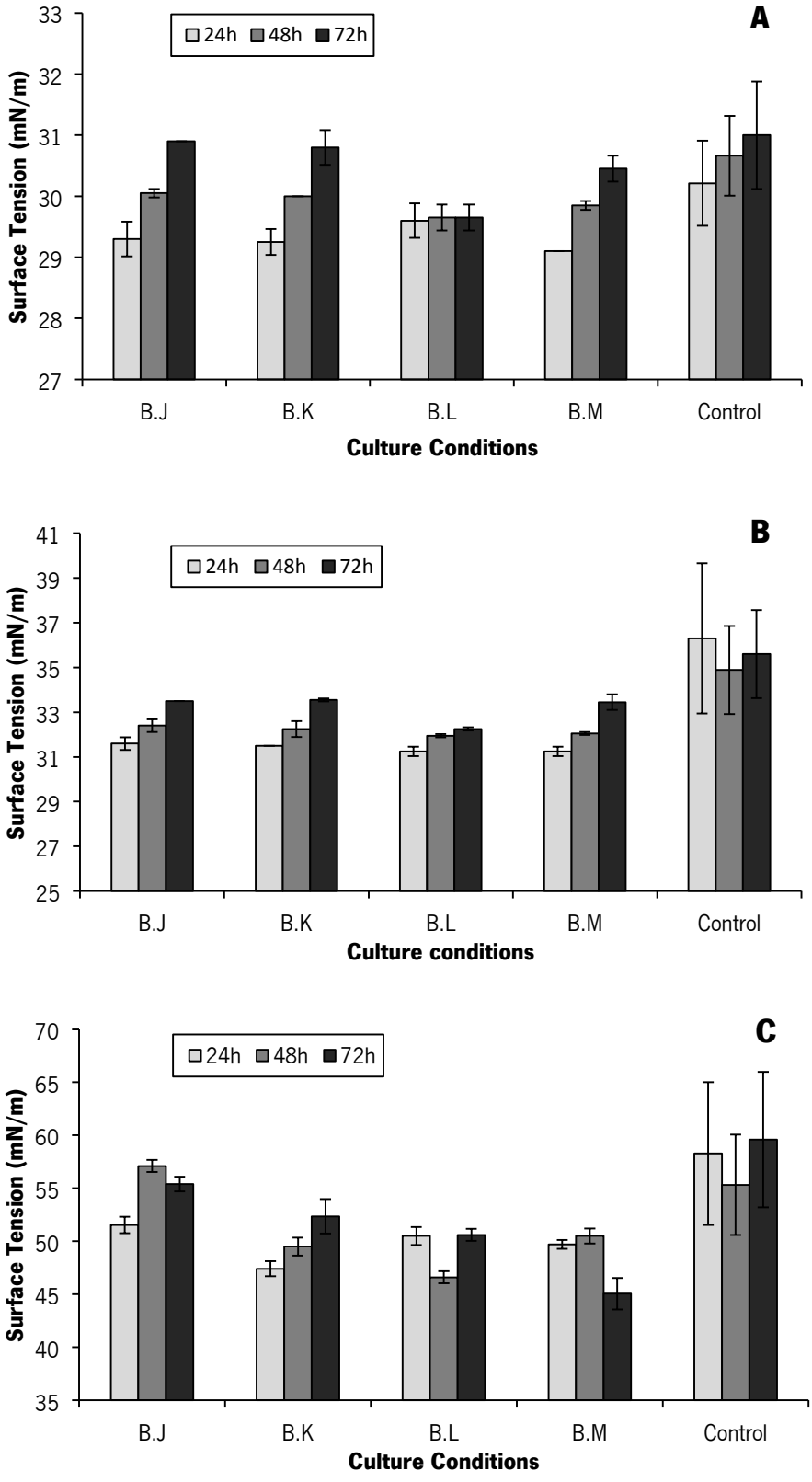

Figure 9. Effect of different metal ions combinations (Fe^{2+}, Mn^{2+} and Mg^{2+}) on the biosurfactant production by *B. subtilis* PX573. Profile of surface tension (ST) **(A)** ST^{-1} **(B)** and ST^{-2} **(C)** along the time. The strain was cultured in CSL medium at 37°C and 200 rpm. Results represent the average of two measurements ± SD.

3.2.4 *Pseudomonas aeruginosa* PX112

- Optimization of culture conditions

As previously mentioned, several environmental factors, such as aeration, can influence the bacterial cell growth and/or the biosurfactant production [3, 55, 128]. Therefore, in the current work the aeration conditions that maximize the biosurfactants production by *P. aeruginosa* PX112 in LB medium were studied (from 100 to 180 rpm). Figure 10 shows that a maximum biomass concentration is reached after 48h using 180 rpm, in contrast with the previous results using 100 rpm (Figure 5), in which the maximum biomass concentration was reached later. Furthermore, it was found that this biomass concentration corresponds to the lowest surface tension (34.1 mN/m) and highest E_{24} (60 %) values obtained. After 48h, a strong decrease in the biomass concentration was observed, which corresponds to cellular death. Nevertheless, the surface tension and emulsification index values remained constant until the end of the fermentation (120h), i.e. these results suggest that no biosurfactant degradation occurred until the end of the fermentation. At the end of the fermentation, the biosurfactants present in the culture medium were recovered by acid precipitation and were further freeze-dried. The biosurfactant concentration at this time point was 1596.6 mg/l, which represents a great increase of biosurfactant production, i.e. 12 times higher than the concentration obtained in the fermentation conducted at 100 rpm (136.1 mg/l).

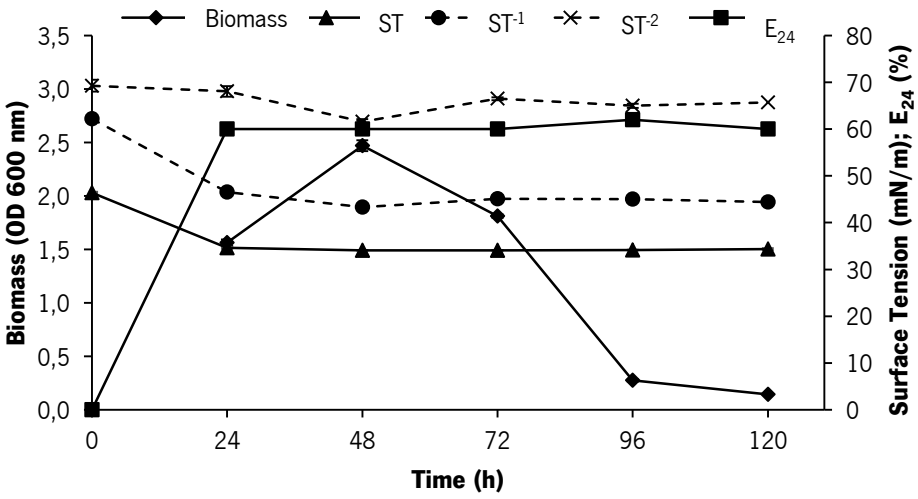

Figure 10. Time course of growth and biosurfactant production by *P. aeruginosa* PX112 showing the profiles of biomass (OD 600 nm), surface tension (mN/m) and emulsification index (E_{24}, %). The strain was grown on LB medium at 37°C and 180 rpm. Results represent the average of two measurements ± SD.

i. Alternative culture media

The initial screening tests showed that *P. aeruginosa* PX112 produced high amounts of biosurfactants in LB medium. Therefore, aiming at reducing the production costs, an alternative media based in industrial wastes, specifically molasses and CSL, was studied.

The production of biosurfactants was performed in CSLM medium at 37ºC and 180 rpm and evaluated through surface tension and emulsification indexes measurements (Figure 11). The lowest surface tension value, 30.3 mN/m, was observed at 72h (Figure 11A). In the case of the emulsifying activity, the E_{24}^{-1} and E_{24}^{-2} values increased until the end of the fermentation (144h), meaning that the biosurfactant is being continuously produced. The medium CSLM itself (control) showed a very high emulsifying activity (E_{24}=60 %), therefore we cannot conclude that the E_{24} values determined in the supernatant along the fermentation are due to the production of biosurfactants, but most probably correspond to other medium components that also exhibit emulsifying activity. For instance, in the first 24h it was observed a decrease in the E_{24} values probably due to the consumption of such medium components. However, regarding the E_{24}^{-1} values, a decrease was observed in the first 24h (to 0 %), after which an increase was observed and this increase could be due to biosurfactant production since those medium components must have been already exhausted. At 120h, the E_{24}^{-1} values reached their maximum (equal to the E_{24} value). At the end of fermentation, the biosurfactants present in the culture medium supernatant were recovered by acid precipitation and were further freeze-dried. The concentration of biosurfactants was 4076.6 mg/l, corresponding to a concentration 2.5 times higher than the one obtained using the LB medium.

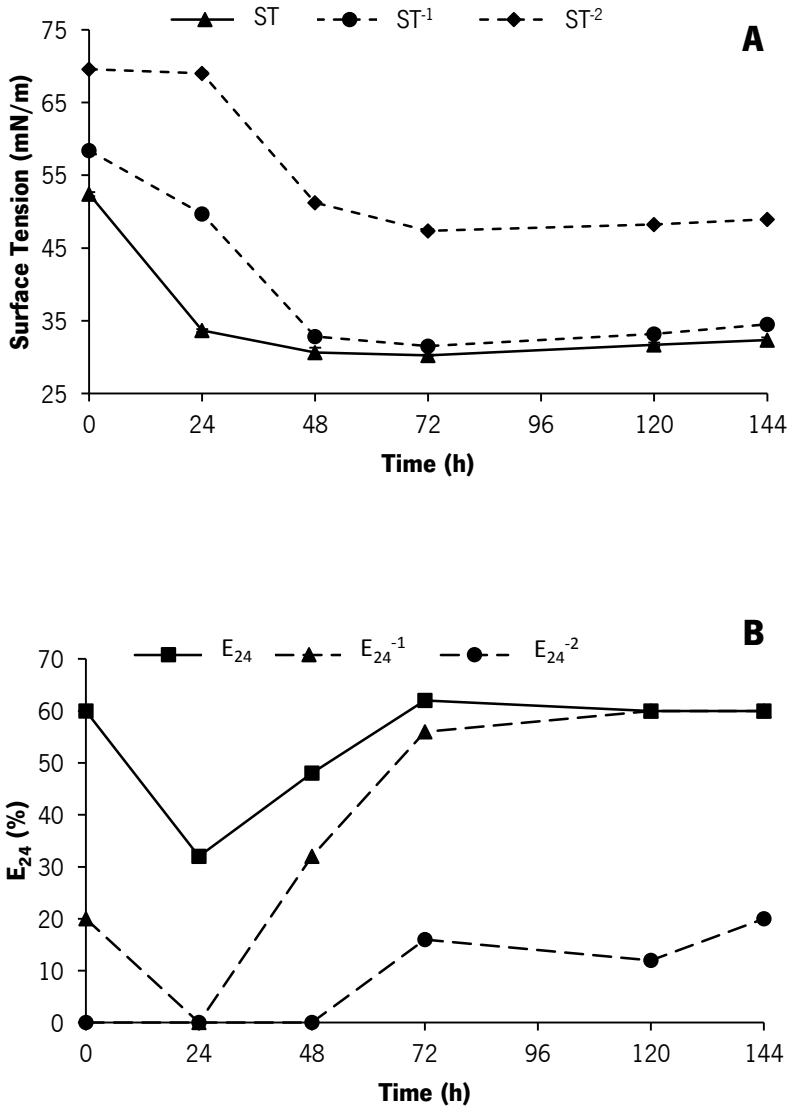

Figure 11. Profiles of the culture both supernatant surface tension (mN/m) **(A)** and emulsification index (E_{24}, %) **(B)** during the time course of the fermentation by *P. aeruginosa* PX112. The strain was grown on CSLM medium at 37°C and 180 rpm. Results represent the average of two measurements ± SD.

Based on the positive results obtained for biosurfactants production using the alternative media CSLM, a second optimization of the process was conducted, namely regarding the agitation rate that was increased from 180 to 200 rpm (Figure 12). Similarly to the results obtained at 180 rpm, the most interesting results obtained for 200 rpm were a surface tension value of 31.5 mN/m and an E_{24} of 60 % at 72h of fermentation. These values remained approximately constant until de end of fermentation (144h).

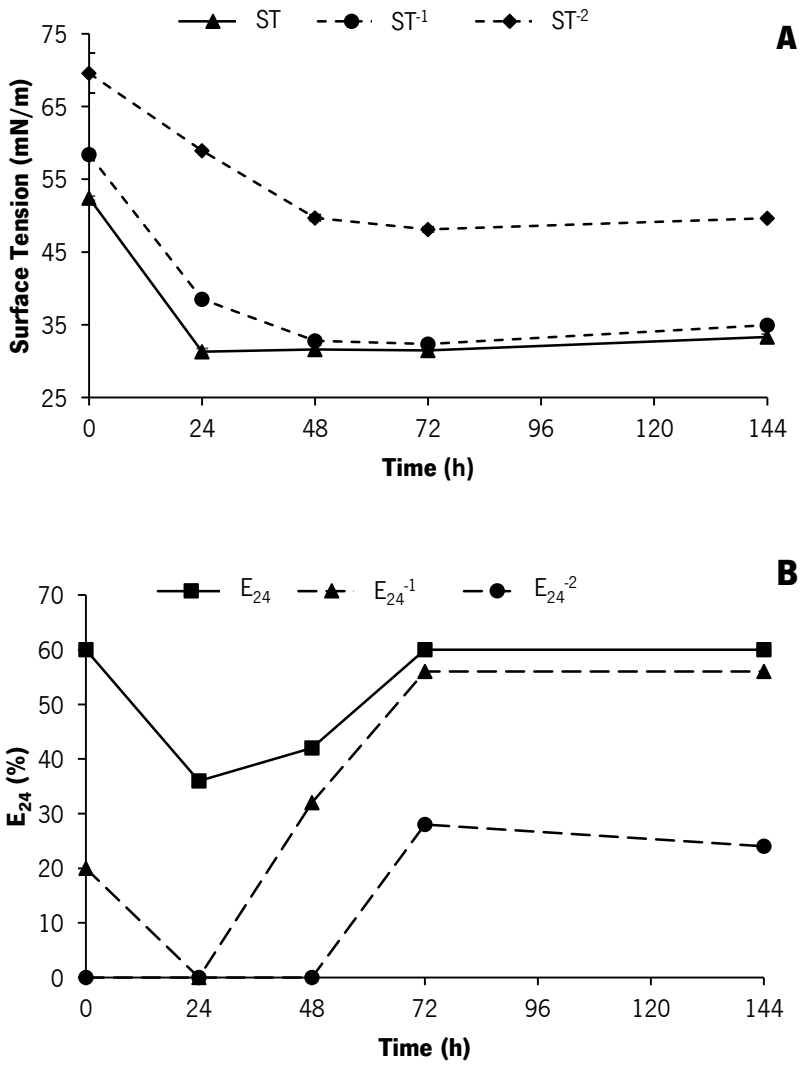

Figure 12. Profiles of the culture both supernatant surface tension (mN/m) **(A)** and emulsification index (E_{24}, %) **(B)** during the time course of the fermentation by *P. aeruginosa* PX112. The strain was grown on CSLM medium at 37°C and 200 rpm. Results represent the average of two measurements ± SD.

The biosurfactants produced under a higher agitation rate were recovered at 96 and 120h (Table 17). A higher biosurfactant production was recovered after 120h of fermentation. The amount of biosurfactant produced at 200 rpm was also found to be higher than the amount previously obtained for 180 rpm, although the surface tension and emulsification indexes obtained were similar in both cases.

Table 17. Biosurfactants concentration (mg/l) along the time. Biosurfactants were produced by *P. aeruginosa* PX122 in CSLM medium at 37°C and 200 rpm.

	Time (h)	
	96	**120**
[Recovered biosurfactants] (mg/l)	4270.3	5185.4

There is a great variety of low cost substrates that have been described in literature and could be used for the production of rhamnolipids by *P. aeruginosa* strains. Mercade et al. [129] evaluated the potential use of olive oil mill effluent (OOME) for the production of rhamnolipids. In Mediterranean countries, such as Portugal, the OOME is a major pollutant of the agricultural industry being produced in large quantities [130], therefore this substrate could also be a suitable choice for our study. The biosurfactants produced with this substrate (6.4 g/l) reduced the surface tension from 42 to 30 mN/m [130]. Furthermore, Patel and Desai [131] described the use of renewable water-soluble by-products (molasses, corn steep liquor) to produce rhamnolipids from *P. aeruginosa* GS3. Benincasa et al. [132] evaluated a number of water-immiscible substrates in mineral medium, namely sunflower (4.9 g/l biosurfactant), soybean (4.8 g/l biosurfactant), olive oil (5.3 g/l biosurfactant). The results obtained in the current study (Table 17) are in good agreement with previous reports from the literature.

ii. Effect of trace elements

As previously mentioned, in this work, the composition of trace elements in the medium was optimized. In order to define the optimal concentration of metal ions, Fe^{2+} (conditions P.A, P.B and P.C), Mn^{2+} (conditions P.D, P.E and P.F) and Mg^{2+} (conditions P.G, P.H and P.I), several shake flasks with CSLM medium were inoculated with *P. aeruginosa* PX112 and supplemented with different concentration of these elements (Table 8, Materials and Methods section). As can be seen in Figure 13, the conditions P.C, P.D and P.F showed a slightly higher reduction of surface tension, mainly in the first 72h, when compared to the other conditions tested. However, analyzing the ST^{-2} values at 72h of fermentation (Figure 13C), for the conditions P.C (46.8 mN/m), P.D (46.3 mN/m) and P.F (46.0 mN/m) it was possible to see that they were quite similar to the control (46.6 mN/m), thus no further improvement of the biosurfactant could be observed under those conditions.

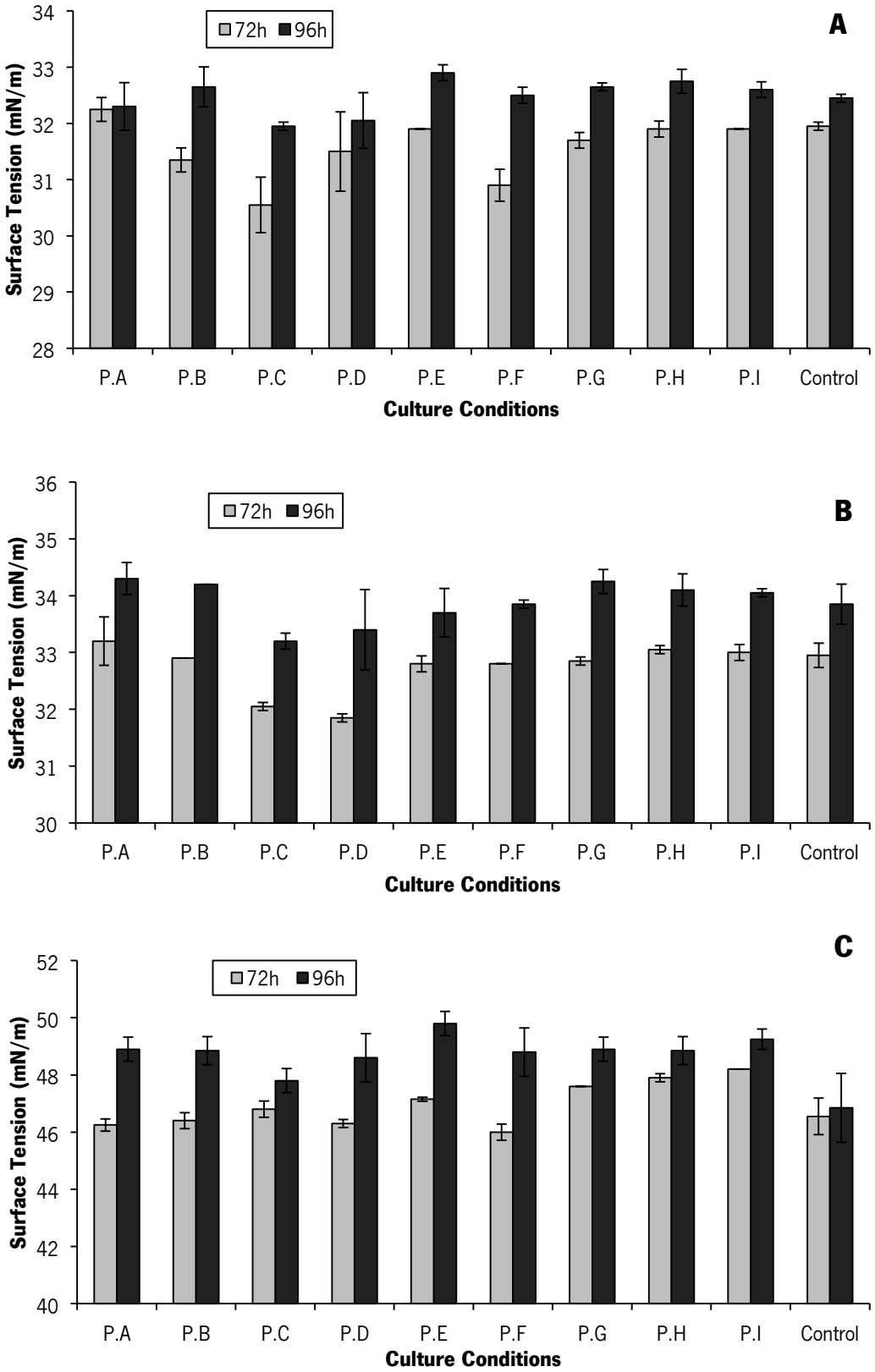

Figure 13. Effect of trace elements (Fe^{2+}, Mn^{2+} and Mg^{2+}) at different concentrations on the biosurfactant production by *P. aeruginosa* PX112. Profile of surface tension (ST) **(A)** ST^{-1} **(B)** and ST^{-2} **(C)** along the time. The strain was cultured in CSLM medium at 37°C and 200 rpm. Results represent the average of two measurements ± SD.

iii. Improvement of biosurfactants recovery

The total amount of biosurfactants being recovered at the end of fermentation is highly influenced by the recovery technique used. In this work, we also aimed at exploring other techniques that could lead to a maximum biosurfactant recovery and at the same time that are simple to perform and inexpensive.

Acid precipitation is one of the methods commonly used to recover biosurfactants. However, in the case of the biosurfactants from *P. aeruginosa* PX112, the surface tension values of the supernatants after biosurfactants recovery indicated that only a small fraction was being recovered. Therefore, as a first approach, different times of acid precipitation were studied. From Table 18 it can be seen that the higher concentration of biosurfactants recovered (710 mg/l) was obtained after 1 day of acid precipitation. The extension of the exposure time to acid conditions did not show a positive effect in the total amount of biosurfactant being recovered.

The surface tension values of the supernatant (Table 18), before and after acid precipitation, were found to be similar, thus meaning that the biosurfactants recovered represent just a small fraction of the existing surface active compounds in the medium supernatant.

Table 18. Effect of different acid precipitation times on the amount of biosurfactant recovered. Surface tension values (mN/m) of supernatants before and after acid precipitation, and the concentration of recovered biosurfactants (mg/l). The supernatants were obtained by growing *P. aeruginosa* PX112 at 37°C and 180 rpm for 120h. The control surface tension (LB medium) was ST=49.4 mN/m, ST^{-1}=62.2 mN/m and ST^{-2} =69.3 mN/m. The supernatant after acid precipitation corresponds to the liquid obtained after centrifugation and removal of the precipitated biosurfactants. Results represent the average of two measurements ± SD.

Supernatant	Surface Tension (mN/m)			Biosurfactant recovered (mg/l)
	ST	ST^{-1}	ST^{-2}	
Before acid precipitation	32.5 ± 0.4	41.2 ± 0.1	66.1 ± 0.6	-
After acid precipitation				
1 day	33.0 ± 0.0	43.9 ± 0.1	66.1 ± 1.2	710
4 days	32.0 ± 0.0	41.2 ± 0.4	64.0 ± 1.3	450
11days	-	-	-	400 ± 20

Since the surface tension value obtained for the supernatant remaining after biosurfactants removal is still quite low we can conclude that most of the biosurfactants being produced stay in the supernatant and that acid precipitation was ineffective. Therefore, the evaluation of possible

secondary recovery methods to be applied after the acid precipitation is required to assure a maximum recovery of biosurfactants. In this work, the recovery of biosurfactants by extraction with solvents (chloroform and a mixture of chloroform:methanol:butanol (1:1:1 v/v/v)) and by precipitation with ammonium sulfate (40 % (w/v)) were evaluated. The analysis of the surface tension values of the supernatant after biosurfactants recovery was generally found to be increased; especially after the precipitation with ammonium sulfate. Therefore, most of the methods studied were showed to be more efficient as compared to the sole use of acid precipitation. This conclusion is supported by the results on the concentration of recovered biosurfactants shown on Table 19. Although the surface tension values were lower for the freeze-dried biosurfactants recovered by chloroform extraction (29.4 mN/m) than the ones recovered by ammonium sulfate precipitation (32.5 mN/m), the concentration of these biosurfactants (4.45 mg/ml) was 2.3 times higher as compared to the chloroform extraction (1.93 mg/ml). Regarding the extraction with chloroform, a second subsequent extraction was performed, but in this case no further biosurfactants could be recovered. In the case of the mixture of chloroform:methanol:butanol was not possible recover the biosurfactants, i.e the mixture was not efficient. The optimization of the biosurfactants recovery procedure allowed an increase of 6.3 times of the amount of biosurfactants recovered by ammonium sulfate precipitation as compared with sole use of acid precipitation (standard methodology). Therefore, the results showed that the use of both recovery techniques together (acid precipitation flowed by ammonium sulfate precipitation) will improve the amount of biosurfactants being recovered, thus making the process more efficient.

Table 19. Different methods for the recovery of biosurfactants produced in LB medium by *P. aeruginosa* PX112. Concentration of the biosurfactant recovered (mg/l), surface tension values (mN/m) of supernatants after being subjected to different recovery methods (control (LB medium): ST=49.4 mN/m and ST^{-1}=62.2 mN/m)) and of freeze-dried biosurfactant (concentration 1.25 mg/ml) (control PBS: ST=71.9 mN/m). Results represent the average of two measurements ± SD.

	Surface Tension (mN/m)		**Recovered biosurfactants (mg/l)**
	ST	**ST^{-1}**	
After acid precipitation	33.0 ± 0.0	43.9 ± 0.1	-
Chloroform extraction			
Freeze-dried biosurfactant	29.4 ± 0.1	44.0 ± 0.0	1930
Supernatant after extraction	35.0 ± 0.0	40.3 ± 0.4	
Ammonium sulfate precipitation			
Freeze-dried biosurfactant	31.5 ± 0.6	57.4 ± 1.1	4450
Supernatant after extraction	45.3 ± 1.1		

Likewise, the secondary recovery methods were also evaluated for the biosurfactants produced by the same strain but using the CSLM alternative medium. The results gathered in Table 20 show that the biosurfactants recovery with ammonium sulfate is more efficient comparing to the chloroform extraction, similarly to the previous findings with LB medium. Specifically, although the surface tension values (28 mN/m) for the freeze-dried biosurfactants recovered by chloroform extraction were lower than the ones obtained by ammonium sulfate precipitation, the amount of biosurfactants recovered was higher when ammonium sulfate precipitation was used (10 times). In conclusion, together with the biosurfactants recovered by ammonium sulfate precipitation it may exist also a great amount of impurities, thus leading to higher surface tension values.

Table 20. Different methods for the recovery of biosurfactants produced in CSLM medium by *P. aeruginosa* PX112 at 200 rpm during 120h. Concentration of the biosurfactant recovered (mg/l), surface tension values (mN/m) of supernatants after being subjected to different recovery methods (control (CSLM medium): ST=52.4 mN/m and ST^{-1}=58.4 mN/m)) and of freeze-dried biosurfactant (concentration 1.25 mg/ml) (control PBS: ST=71.9 mN/m). Results represent the average of two measurements ± SD.

	Surface Tension (mN/m)	**Recovered biosurfactants (mg/l)**
After acid precipitation	33.0 ± 0.0	**-**
Chloroform extraction		
Supernatant after extraction	30.5 ± 0.4	690
Freeze-dried biosurfactant	28.0 ± 0.1	
Ammonium sulfate precipitation		
Supernatant after precipitation	43.0 ± 0.0	6830
Freeze-dried biosurfactant	30.0 ± 0.0	

As previously mentioned, the method most commonly used to recover rhamnolipids is the acid precipitation. However, when a complex carbon source is used, such as molasses or CSL, the downstream recovery of biosurfactants is a delicate step because of the similar polarity of the product and the substrate components [133]. Therefore, the combination of acid precipitation with another recovery technique as suggested in the current study could be helpful in reducing the presence of impurities in the crude biosurfactant and in improving the biosurfactant yields. Banat [134] also suggested the use of two combined methods to recover rhamnolipids from *P. aeruginosa*, namely acidification of culture media followed by extraction with chloroform/methanol.

3.3. Biosurfactants characterization

One of the issues in any new biosurfactant production process is the fact that in the end a crude biosurfactant mixture is obtained and the downstream processes usually retrieve very low yields and sometimes are inefficient. As a consequence, the characterization of the pure molecules is hard and sometimes not feasible. Nevertheless, it is important for several reasons, e.g. to improve the recovery but also the production, to know the structures or at least the functional groups that are involved in these biosurfactant mixtures. On the other hand, it is of utmost important to have a functional characterization of the biosurfactants since this will open application opportunities.

3.3.1. TLC characterization

The freeze-dried biosurfactants recovered from all isolates under study were characterized by TLC. Two different mobile phases were tested: (a) chloroform-methanol-water (65:25:4 v/v/v) and (b) acetonitrile-water (6:3 v/v). The mobile phase (b) was the most efficient, being able to separate more components from the biosurfactants mixture produced by lactic acid bacteria. Different solvents were used to dissolve the freeze-dried biosurfactants. Methanol and acetonitrile were not able to dissolve completely the biosurfactants; however, all of them were soluble in PBS buffer, thus demonstrating their dominant hydrophilic character. Table 21 summarizes the results obtained for the seven isolates studied. *L. paracasei* A20, *L. agilis* CCUG31450 and *L. animalis* ATCC35046 show a few Rf values that are quite similar (in bold), which suggests that these biosurfactants might have some structural similarities. However, despite some similar Rf values, in general, the different biosurfactants showed distinct Rf profiles, meaning that different structures are present in the biosurfactant mixtures (it is important to notice that these biosurfactants have not been purified).

Table 21. TLC analysis of the crude biosurfactants recovered from the seven isolates studied. Solvent system used was acetonitrile-water (6:3 v/v). The freeze-dried biosurfactants were dissolved in PBS. The spots were visualized under ultraviolet light at 254 and 366 nm.

Retention factor (Rf) (254 nm)						
L. paracasei A20	*L. agilis* CCUG31450	*L. animalis* ATCC35046	*L. hamsteri* ATCC43851	*S. thermophilus* A	*P. aeruginosa* PX112	*B. subtilis* PX573
0.23	**0.29**	**0.29**	0.58	0.25	0.42	0.37
0.50	0.32	**0.50**		0.35		
				0.44		
Retention factor (Rf) (366 nm)						
L. paracasei A20	*L. agilis* CCUG31450	*L. animalis* ATCC35046	*L. hamsteri* ATCC43851	*S. thermophilus* A	*P. aeruginosa* PX112	*B. subtilis* PX573
0.34	**0.39**	**0.38**	0.58	0.39	0.86	0.37
0.41	**0.45**	**0.46**		0.44		0.73
0.50	0.56					

3.3.2. Critical micelle concentration (CMC)

To determine the CMC of the freeze-dried biosurfactants, the relationship between their concentration and the corresponding surface tension was determined. As described in the literature, the most important interfacial properties (as detergency and solubilization) are affected by the existence of micelles in solution [35]. Therefore, the CMC is widely used as an index to evaluate the surface activity of a given surfactant. At biosurfactant concentrations higher than the CMC, the surface tension becomes stable, and there no further significant reduction of the surface tension will occur. The results showed, as expected, a progressive decrease in surface tension values with the increase of the biosurfactants concentration.

i. *Lactobacillus agilis* CCUG31450

The biosurfactants produced by *L. agilis* CCUG31450 in MRS and CWM medium were tested at different concentrations. The CMC was found to be 7500 mg/l for biosurfactants produced in MRS medium (Figure 14A) with a minimum surface tension value of 42.8 mN/m, and 13000 mg/l

for those produced in CWM (Figure 14B) with a minimum surface tension value of 47.5 mN/m. The lowest CMC value for *L. agilis* CCUG31450 biosurfactants was obtained with the standard medium and not with the alternative. Moreover, this biosurfactants produced in MRS medium were able to reduce the surface tension more effectively than the ones produced in CWM. The effectiveness is defined as the minimum value to which the surface tension value can be reduced, whereas efficiency is defined by the surfactant concentration required to produce a significant reduction in the surface tension of water, i.e. the CMC [35].

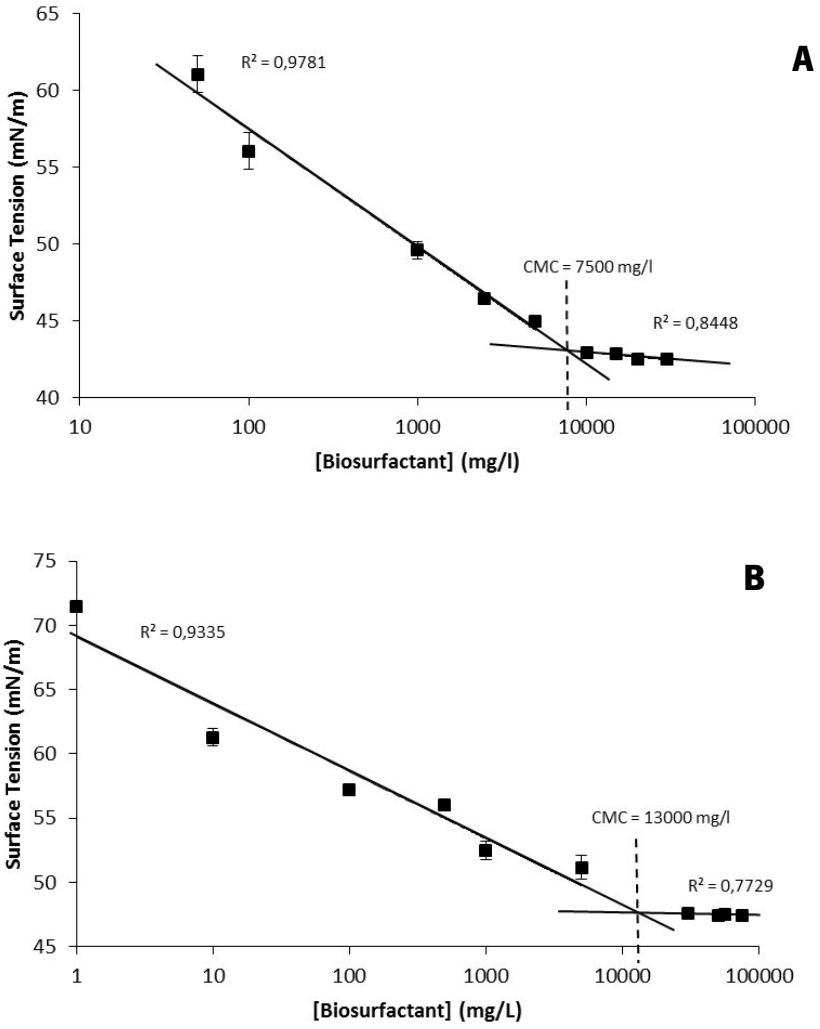

Figure 14. Critical micelle concentration of the biosurfactant produced by *L. agilis* CCUG31450 in MRS medium **(A)**, or in CWM **(B)** at 100 rmp. The plot represents surface tension (mN/m) of crude biosurfactants versus logarithm of biosurfactant concentration. The CMC was determined from the intersection of the regression lines better describe the two parts of the curve, below and above the CMC. The reference surface tension value was 71.9 mN/m. Results represent the average of two measurements and error bars represent SD of the mean values.

Regarding the biosurfactant from *L. rhamnosus* CCM1825, the highest decrease of surface tension was registered within the range from 6.0 to 4.5 g/l, namely 43.6 and 45.4 mN/m, respectively. For *L. fermenti* 126 the most pronounced decrease in surface tension ranges from 45.1 to 46.3 mN/m for a concentration of 9.0 and 6.0 g/l, respectively [135]. As can be seen, the CMC values for the biosurfactants produced by *L. agilis* CCUG31450 in MRS are quite similar to the ones obtained with the biosurfactants from *L. rhamnosus* CCM1825 and *L. fermenti* 126. Moreover, these values were found to be even lower than the ones reported for *S. thermophilus* A biosurfactant, 20 g/l [101]. Nevertheless, Gudiña et al. [112] reported for *L. paracasei* sbsp. *paracasei* A20 a quite lower value of CMC (2.5 mg/ml) corresponding to a surface tension value of 41.8 mN/m.

The CMC values herein found for *L. agilis* CCUG31450 biosurfactants are in good agreement with previous values reported in the literature. It is important to notice that the biosurfactants studied in the current study were not as pure as the ones described by other authors; nevertheless it seems that the dialysis process used after recovery is efficient in the removal of impurities. On the other hand, the biosurfactants produced in CWM showed a much lower efficiency, this could be justified by the presence of impurities.

ii. *Bacillus subtilis* PX573

Both biosurfactant mixtures produced by *B. subtilis* PX573 in LB and CSL medium were tested at different concentrations. The CMC was found to be 250 mg/l for the biosurfactant produced in LB medium (Figure 15A), with a minimum surface tension value of 32.6 mN/m; and 160 mg/l for the biosurfactant produced in CSL medium (Figure 15B), with a minimum surface tension value of 29.9 mN/m. Contrarily to was observed for *L. agilis* CCUG31450, for this strain the results suggest that the biosurfactant produced in the low cost medium CSL is able to reduce the surface tension more effectively than the one produced in LB medium.

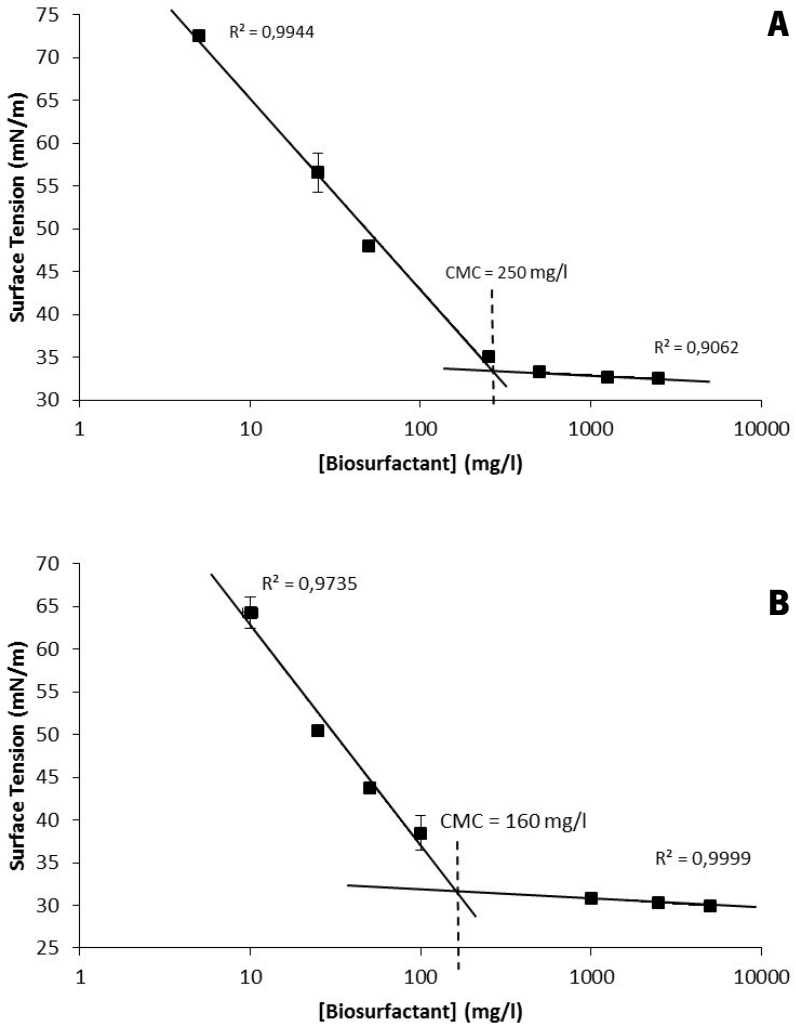

Figure 15. Critical micelle concentration of the biosurfactant produced by *B. subtilis* PX573 in LB medium without shaking **(A)**, or in CSL medium at 200 rpm **(B)**. The plot represents surface tension (mN/m) of crude biosurfactants versus logarithm of biosurfactant concentration. The CMC was determined from the intersection of the regression lines that better describe the two parts of the curve, below and above CMC. The reference surface tension value was 71.9 mN/m. Results represent the average of two measurements and error bars represent SD of the mean values.

Surfactin, the main biosurfactant produced by *B. subtilis,* is the most effective biosurfactant known so far, being able to decrease the surface tension of water from 72 to 26 - 30 mN/m [127, 136]. Different CMC values have been reported for surfactin by several authors. Vaz et al. [35] reported a CMC of 40 mg/l for biosurfactants produced by *B. subtilis* EG1 in LB medium, with a minimum surface tension value of 29.0 mN/m. Similarly, Arutchelvi et al. [137] showed that crude surfactin produced by *B. subtilis* YB7 reduced the surface tension of water from 72 to 30 mN/m with a CMC of 40 mg/l. Nitschke and Pastore [70] reported for the biosurfactant from *B. subtilis* LB5a a minimum surface tension of 26.6 mN/m and a CMC of 33 mg/l. Cooper et al. [10] reported

a CMC of 25 mg/l and a minimum surface tension of 27.0 mN/m for surfactin from *B. subtilis* strains. Pereira et al. [138] also described the CMC values obtained for different *B. subtilis* isolates: 20 mg/l for isolates #309 and #311; and 30 mg/l for isolate #573. These authors concluded that biosurfactants produced by isolates #309 and #311 were more efficient than the ones produced by isolate #573, which explained why the isolate #573, although producing higher amounts of biosurfactant, provided similar or higher (i.e. less active surfactant) surface tension values.

In our study, the characterization of the biosurfactants produced by *B. subtilis* PX573 in different media showed similar reductions of the PBS surface tension, from 71.9 to 32.9 mN/m (biosurfactants produced in LB medium), and from 71.9 to 29.9 mN/m (biosurfactants produced in CSL medium). These results were found to be in accordance with the above mentioned studies. In contrast, the CMC values obtained herein are substantially higher when compared with the ones referred in literature. These differences are largely due to the purity of biosurfactants since no purification process was conducted.

As previously mentioned, *B. subtilis* PX573 produced higher amounts of biosurfactants in CSL than in LB medium showing a slightly higher reduction of the PBS surface tension and a higher efficiency, as seen by the lower CMC value. Although the biosurfactants produced in CSL medium exhibited a higher efficiency it is necessary to confirm if the biosurfactants obtained from different media display the same activity, such as antimicrobial, anti-adhesive and capacity to recovery oil. The evaluation of such activities will be discussed further in the sub-section 3.4 (Biosurfactants applications).

iii. *Pseudomonas aeruginosa* PX112

The biosurfactants produced by *P. aeruginosa* PX112 in both LB and CSLM media were tested at different concentrations. The CMC was found to be 1250 mg/l for the biosurfactants produced in LB medium (Figure 16A) with a minimum surface tension value of 33.9 mN/m; and 650 mg/l for the biosurfactants produced in CSLM medium (Figure 16) with a minimum surface tension value of 30.2 mN/m. Similarly to the previous results for *B. subtilis* PX573, the biosurfactants from *P. aeruginosa* PX112 were also found to have higher CMC values in LB medium as compared to CSLM. Specifically, the CMC of the biosurfactants produced in CSLM medium was

found to be 2 times lower than the ones produced in LB. These results also indicate that the biosurfactants produced in CSLM medium are able to reduce the surface tension more effectively.

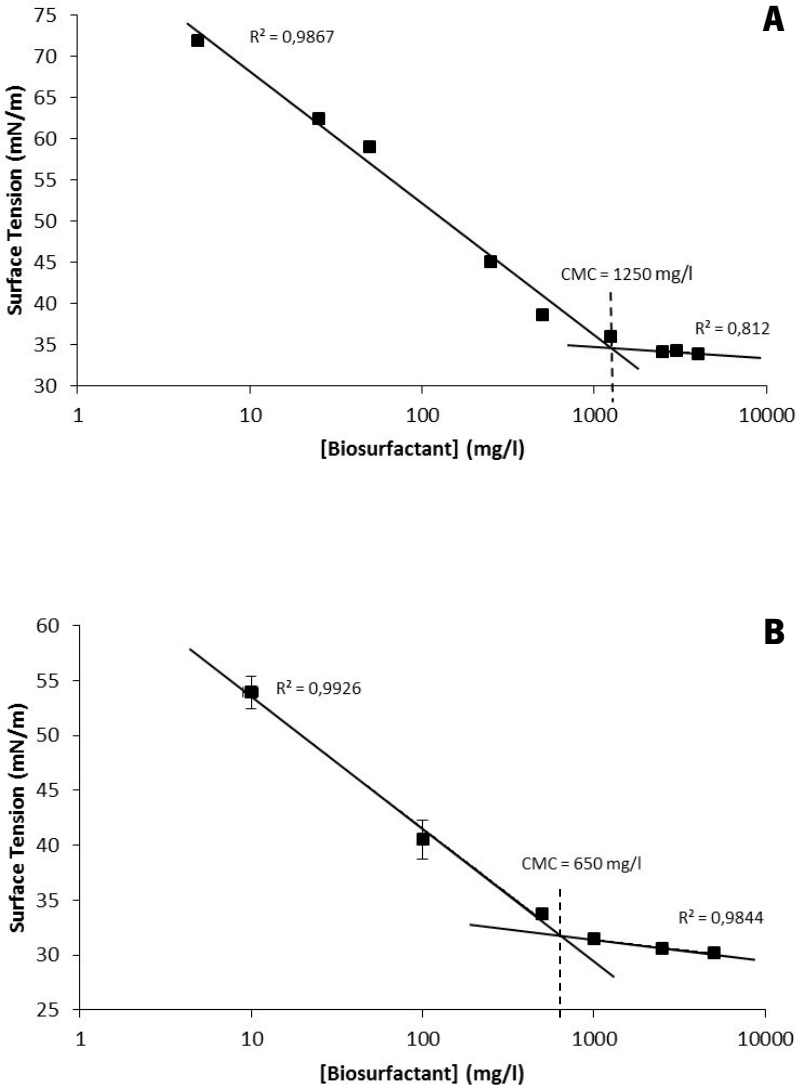

Figure 16. Critical micelle concentration of the biosurfactant produced by *P. aeruginosa* PX112 in LB medium at 100 rpm **(A)**, or in CSLM medium at 200 rpm **(B)**. In both cases, the biosurfactants were recovered by acid precipitation. The plot represents surface tension (mN/m) of crude biosurfactants versus logarithm of biosurfactant concentration. The CMC was determined from the intersection of the regression lines that better describe the two parts of the curve, below and above CMC. The reference surface tension value was 71.9 mN/m. Results represent the average of two measurements and error bars represent SD of the mean values.

The CMC values herein obtained for the *P. aeruginosa* PX112 biosurfactants were found to be superior to the ones reported previously in the literature. CMC values for rhamnolipids produced by *P. aeruginosa* have been reported in the range from 5 mg/l to 200 mg/l depending on the

nature of the rhamnolipid and its producer [133, 139]. In particular, the variability of the CMC values for pure rhamnolipids and its mixtures greatly depends on the chemical composition of the various types of rhamnolipids and also on the hydrophilic/hydrophobic ratio of the rhamnolipids constituting the crude biosurfactant [140, 141]. For instance, Nitschke et al. [130] described a CMC value of 5 mg/l for the di-rhamnolipid $Rha_2C_{10}C_{10}$ that was lower than the CMC value determined for $RhaC_{10}C_{10}$ (40 mg/l) and for the species more hydrophilic ($RhaC_{10}$ and Rha_2C_{10}) which showed CMC values of 200 mg/l [142, 143]. Besides, the differences in CMC values could also be related with the presence of impurities [144] or to different microbial sources.

Since the biosurfactants produced by *P. aeruginosa* PX112 did not undergo specific purification processes, the presence of impurities lead to increased CMC values as compared to the ones reported in the literature. Abdel-Mawgoud et al. [141] described the influence of impurities in the CMC value of rhamnolipids. In that study, the authors suggested that the low purity (25 %) of the tested rhamnolipid could be responsible for the difference observed in the CMC values comparing with standard rhamnolipids.

Moreover, the interference of medium components on the CMC values has also been widely reported in the literature. Depending if the biosurfactant is from the culture supernatant, crude or purified, the CMC values could be different, in part due to the medium components that could interfere with micellization of biosurfactants, influencing positively or negatively the CMC values. Pornsunthorntawee et al. [99] showed that the crude biosurfactant produced by a *P. aeruginosa* strain reduced the surface tension to 29.0 mN/m with a CMC of 200 mg/l. Furthermore, the authors showed that the CMC value for the biosurfactants present in the culture supernatant, free of cells (120 mg/l), was lower than that of the crude biosurfactant (200 mg/l), probably due to the impurities existent in the culture supernatant. In that study, the culture supernatant contained a small amount palmitic acid, which is able to interact with the biosurfactant molecules, thus affecting the micellization. Therefore, the micellization could occur more easily, leading to a CMC value of the culture supernatant lower than that of the extracted biosurfactant [99]. This study clearly showed that CMC values are strongly affected by the medium components. In our study, and as previously mentioned, the lower CMC values obtained for the biosurfactants produced in CSLM could be due to some medium components that might precipitate or sediment during the recovery process. Accordingly, these biosurfactants could have different properties comparing to the ones produced in LB.

Despite of the biosurfactants produced in CSLM medium exhibited a more pronounced efficiency it is required to confirm if the biosurfactants obtained from distinct media display the same activity, such as antimicrobial, anti-adhesive and capacity to recovery oil. The evaluation of such activities will be discussed further in the sub-section 3.4 (Biosurfactants applications).

3.3.3. Biosurfactants stability

The applicability of biosurfactants in several fields greatly depends on their stability at different temperatures, salinities and pH values.

i. *Lactobacillus agilis* CCUG31450

In this case, as the biosurfactants are cell-bound, a biosurfactant solution in distilled water was prepared at a concentration of 7.5 mg/ml (CMC value) using the biosurfactants produced in MRS medium. The pH of the biosurfactants solution was changed from 2.0 to 13.0 and the surface tension and emulsification indexes were measured. As illustrated in Figure 17A, both acid and alkaline conditions led to a slight decrease of the surface and emulsifying activity. The most favorable conditions regarding surface tension and emulsification index were observed at pH 7.0.

The effect of salts on the biosurfactant activity was evaluated. From Figure 17B it can be seen that these biosurfactants, mainly their emulsifying activity, were affected by NaCl concentrations higher than 50 g/l.

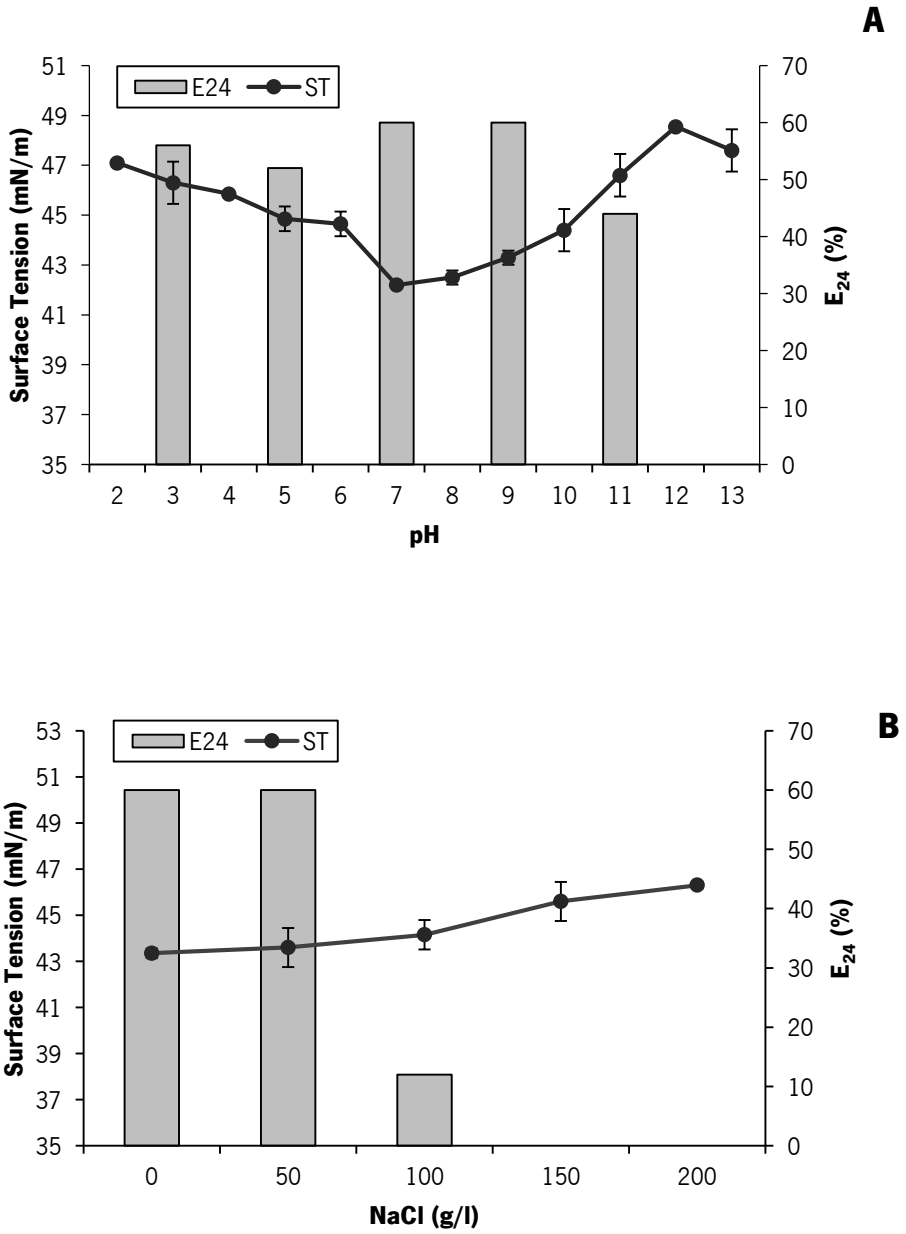

Figure 17. Effect of pH **(A)** and salinity **(B)** on the stability of the biosurfactants isolated from *L. agilis* CCUG31450 in MRS medium. Surface tension values (mN/m) and emulsification indexes (E_{24}, %) obtained with freeze-dried biosurfactants dissolved in PBS (pH 7.0) at 7.5 mg/ml under different pH values and NaCl concentrations. Measurements were conducted at room temperature. Results represent the average of two measurements and error bars represent the SD.

Regarding the biosurfactants stability to an extreme temperature, no negative effect was observed (Table 22), i.e. the biosurfactants produced by *L. agilis* CCUG31450 preserved their emulsifying and surface activities.

Table 22. Effect of high temperature on the stability of the biosurfactants isolated from *L. agilis* CCUG31450 in MRS medium. Surface tension values (mN/m) and emulsification indexes (E_{24}, %) were obtained with freeze-dried biosurfactants dissolved in PBS (pH 7.0) at a concentration of 7.5 mg/ml before and after exposure to 121ºC for 20 min. Results represent the average of two measurements ± SD.

	Before treatment	**After treatment**
Surface Tension (mN/m)	43.4 ± 0.2	43.4 ± 0.6
E_{24} (%)	60	60

Gudiña et al. [112] analyzed the sensitivity of the biosurfactants produced by *L. paracasei* sbsp. *paracasei* A20 to different values of pH. The surface activity of the crude biosurfactants remained relatively stable between pH 6.0 and 10.0, with a higher stability at alkaline conditions. Similarly, in the current work the *L. agilis* CCUG31450 biosurfactants were more stable in the same pH range, although some instability was found for pH 2.0 and 13.0 at which surface tension values was about five units higher than at pH 7.0.

The instability of the biosurfactants produced by some lactobacilli mainly in acidic conditions has been previously described by other researchers, and has been related to the presence of negative charged groups at the polar ends of the molecules which are protonated under those conditions [112]. The characterization of the intracellular biosurfactants produced by some lactobacilli has revealed the presence of proteic fractions probably associated to phosphate groups [112], which could also be negatively affected by the denaturalization at acidic pH. Accordingly, in our work, for pH values lower than 6.0, precipitation of some of the biosurfactant components was observed contributing to an increase of the surface tension.

Regarding the experiments conducted with different NaCl concentrations, it was found that surface tension did not suffer any pronounced change with increasing NaCl concentrations, the variations observed were very small, from 43.4 mN/m (control) to 46.3 mN/m (corresponding to the higher concentration of NaCl tested) (Figure 17A). On the other hand, the emulsifying activity is highly affected by salinity, as can be seen (Figure 17B) a NaCl concentration higher than 50 g/l lead to an abrupt loss of emulsifying activity.

ii. *Bacillus subtilis* PX573

The pH of the culture broth supernatants obtained at the end of the fermentation (LB medium, without shaking, 48h) was changed from 2.0 to 13.0 and the surface tension and emulsification indexes were measured. The results in Figure 18A show that the biosurfactant activity was retained in the pH range of 6.0-13.0 with a minimum deviation in surface tension and emulsification indexes. The minimum surface tension value was observed at pH 6.0 (30.4 mN/m) and the higher emulsification index at pH 13.0 (46 %). Below pH 6.0 the surface tension increased and no emulsification activity could be observed, probably due to the biosurfactant precipitation. Therefore, it could be established that biosurfactants produced by *B. subtilis* PX573 in LB medium exhibited a higher stability at alkaline than acidic conditions.

Regarding the effect of salinity on the biosurfactant activity, the culture broth supernatant obtained at the end of the fermentation was supplemented with different NaCl concentrations, ranging from 50 to 200 g/l. Both surface tension and emulsifying activity were measured (Figure 18B). The highest emulsification index (44 %) and surface tension reduction (31.8 mN/m) were reached at 50 g/l NaCl. The biosurfactant activity was not affected by NaCl concentrations up to 50 g/l. The behavior of biosurfactants at 50 g/l of NaCl is similar to the control (without NaCl), thus demonstrating that this biosurfactant is stable at increasing concentrations of salt.

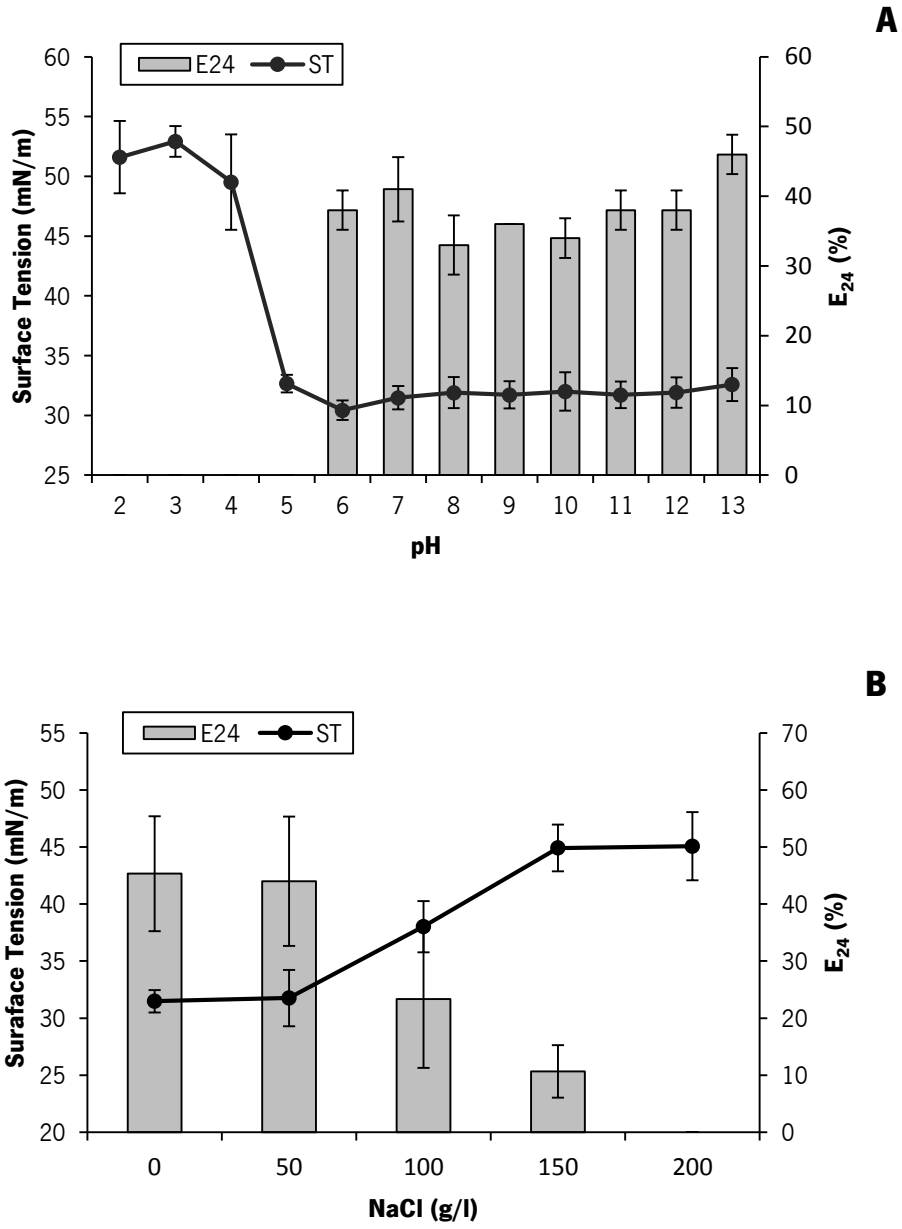

Figure 18. Effect of pH **(A)** and salinity **(B)** on the stability of biosurfactants produced by *B. subtilis* PX573 in LB medium. Surface tension values (mN/m) and emulsification indexes (E_{24}, %) were obtained with the culture broth supernatants at different pH values and NaCl concentrations. Results represent the average of three independent measurements and error bars represent the SD.

To study the stability of biosurfactants at high temperatures, the culture broth supernatants obtained at the end of fermentation were incubated at 121°C for 20 min. Surface tension and emulsification indexes were measured before and after heating. From Table 23, it can be seen the high temperatures do not negatively influence the biosurfactant properties.

Table 23. Effect of high temperature on the stability of biosurfactants produced by *B. subtilis* PX573 in LB medium. Surface tension values (mN/m) and emulsification indexes (E_{24}, %) were obtained with the culture broth supernatants before and after exposure to 121°C for 20 min. Values represent the average of three independent experiments ± SD.

	Before treatment	**After treatment**
Surface Tension (mN/m)	31.1 ± 0.9	30.9 ± 1.9
E_{24} (%)	50.0 ± 8.5	53.0 ±4.2

Al-Bahry et al. [127] reported a higher stability of biosurfactants produced by *B. subtilis* strains at alkaline conditions, specifically in pH values ranging from 6.0 to 12.0. The maximum activity was observed at pH of 7.0. At pH 2.0 and 4.0, they observed higher surface tension values, since these biosurfactants are not soluble at such acidic conditions and they tend to precipitate. They also described that acidic conditions promote structural distortions in the precipitated biosurfactants that leads to loss of their capability of reducing surface tension. This effect could likewise explain why the biosurfactants produced by *B. subtilis* PX573 precipitated at acidic conditions.

The biosurfactants produced by *B. subtilis* PX573 in the current work showed a good surface and emulsifying activity under extreme conditions of temperature, thus these results are in accordance with the ones described in the literature. Furthermore, Al-Bahry et al. [127] tested the *B. subtilis* B20 biosurfactants stability over a wide range of temperatures (40 - 100°C for 60 min; 121°C for 20 min) and could not found relevant changes in the surface tension, thus demonstrating the biosurfactants stability. Additionally, four different Bacilli isolates were reported to produce biosurfactants that were stable for nine days at 80°C [76, 145]. Desai and Banat [3] observed that heat treatment of some biosurfactants caused no considerable changes in their properties even after autoclaving them at 120°C for 15 min.

The analysis of the biosurfactants stability to different NaCl concentrations was also studied by Gudiña et al. [103]. Their results are similar to the ones obtained in this work, showing the minimum surface tension value at 50 g/l NaCl. Other authors, namely Yakimov et al. [146], Abdel-Mawgoud et al. [147] and Ghojavand et al. [148], also reported similar results.

The *B. subtilis* biosurfactants stability compared with several chemical surfactants, namely Glucopone®215, Glucopone®650, Findet®1214N/23 and linear alkylbenzene sulfonates was studied by Vat el al. [35]. Independently of the temperature tested, the surface tension value

obtained for the biosurfactant (at a concentration of 1000 mg/l) was found to be similar to those obtained for the commercial chemical surfactants, except for Findet®1214N/23 for which the surface tension values were slightly higher. Therefore, it can be assumed that the surface activity of that biosurfactant is comparable to the commercial products and it is stable at extreme temperature conditions. On the other hand, the commercial chemical surfactants were found to be more stable to pH changes as compared to the biosurfactant over the whole range of pH values studied. In this study, the authors suggested that the instability of the biosurfactant to acidic pH was probably due to the presence of proteinaceous contaminants. Nevertheless, it is important to notice that above pH 5.0 the biosurfactant showed a better surface activity than the chemical ones.

The biosurfactants produced by *B. subtilis* PX573 showed a similar stability to the biosurfactants described by Vaz et al. [35]. Therefore, these biosurfactants could also represent a viable alternative to the chemical surfactants used in a number of applications.

In summary, the *B. subtilis* PX573 biosurfactants were found to be stable to heat treatement and over a wide range of pH values, which together with their excellent surface and emulsifying activities, make them suitable candidates to be used in bioremediation of contaminated sites and in the petroleum industry (MEOR), in which extreme conditions commonly prevail [149].

iii. *Pseudomonas aeruginosa* PX112

The same experimental setup was used as described above. Figure 19A shows that the biosurfactant activity was retained at pH values between 2.0 and 5.0. Contrarily to the biosurfactants produced by *B. subtilis* PX573, the ones produced by *P. aeruginosa* PX112 exhibited a higher stability at acid than at alkaline conditions, showing a minimum surface tension value (30.8 mN/m) at pH 4.0 and 5.0. On the other hand, the emulsifying activity was found to be more stable at alkaline conditions, with a maximum emulsification index (61 %) being obtained for pH values of 7.0 and 8.0. The biosurfactants produced by *P. aeruginosa* PX112 were found to be highly stable to acid conditions, which explain why the acid precipitation is not efficient for the complete removal of the biosurfactant present in the culture supernatants.

Regarding the biosurfactant stability to different NaCl concentrations, the same procedure as for *B. subtilis* PX573 biosurfactant was used. From Figure 19B it can be seen that the increase in NaCl concentration has a minimum effect in the surface and emulsifying activity. The

biosurfactants produced by *P.* aeruginosa PX112 were found to be very stable in conditions with high salinity.

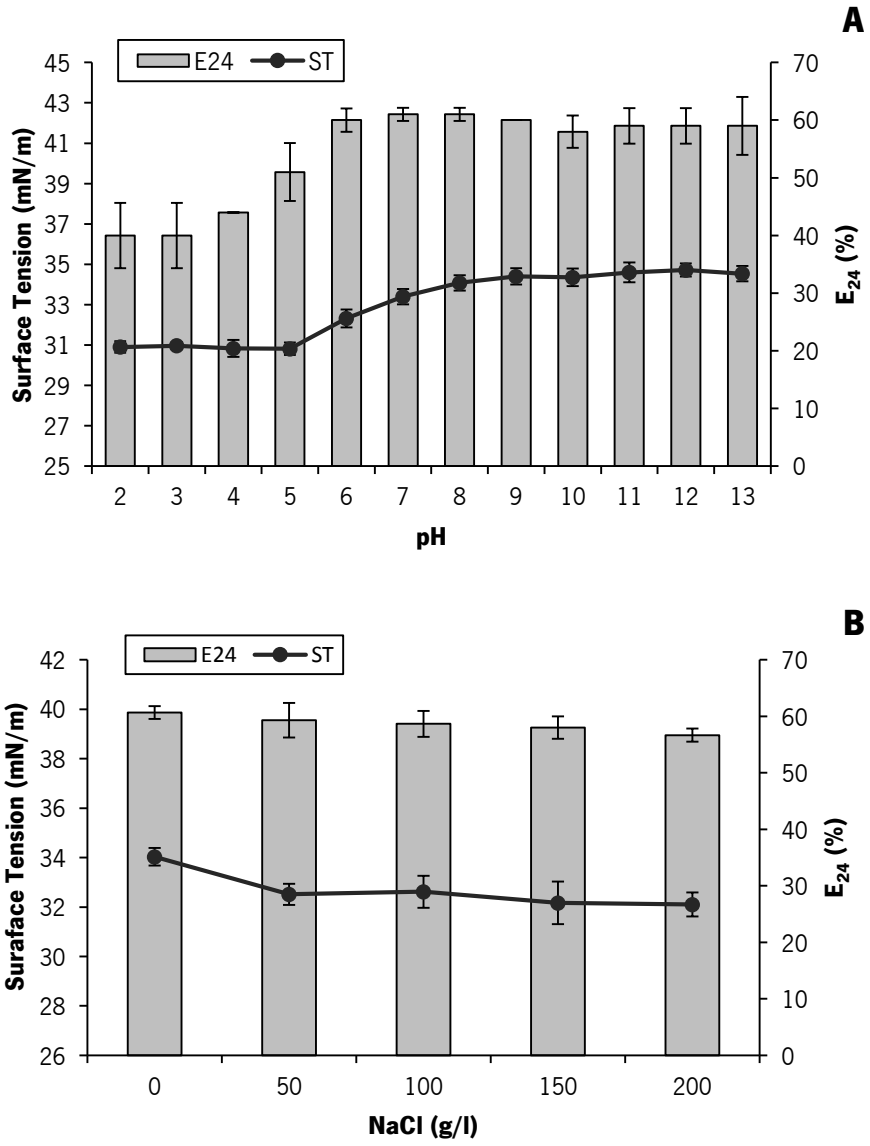

Figure 19. Effect of pH **(A)** and salinity **(B)** on the stability of biosurfactants produced by *P. aeruginosa* PX112 in LB medium. Surface tension values (mN/m) and emulsification indexes (E_{24}, %) were obtained with the culture broth supernatants at different pH values and NaCl concentrations. Results represent the average of three independent measurements and error bars represent the SD.

The stability of these biosurfactants at high temperatures was also investigated. Culture broth supernatants obtained at the end of fermentation were incubated at 121°C for 20 min. Results gathered in Table 24 showed no negative effect in the biosurfactant properties with the temperature increase.

Table 24. Effect of a high temperature on the stability of the biosurfactants produced by *P. aeruginosa* PX112 in LB medium. Surface tension values (mN/m) and emulsification indexes (E_{24}, %) were obtained with the culture broth supernatants before and after exposure to 121°C for 20 min. Values represent the average of three independent experiments ± SD.

	Room Temperature	**121°C**
Surface Tension (mN/m)	34.0 ± 0.35	33.5 ± 0.52
E_{24} (%)	60.7 ± 1.15	59.3 ± 3.06

Rhamnolipids, produced by *P. aeruginosa*, have been widely studied regarding their stability in order to find novel applications in which extreme conditions are commonly used. Abel-Mawgoud et al. [141] reported that the rhamnolipids surface activity was stable over a wide range of pH values (from 2 to 13) with a maximum surface activity reached at pH 7.0 to 8.0. Pirôllo et al. [150] also reported that the *P. aeruginosa* LBI rhamnolipids surface activity remained unaltered over a broad pH range (from 5 to 10). In the pH range tested, the biosurfactants herein produced by *P. aeruginosa* PX112 also showed a small variation in surface activity from 30.9 to 34.5 mN/m.

Given the stability to salts herein found for *P. aeruginosa* biosurfactants, these can be considered as suitable candidates to be applied in the bioremediation of contaminated marine environments where high salinities prevail.

Moreover, the exposure of these biosurfactants to a high temperature did not have a negative impact on the surface and emulsifying activities. These results showed to be in agreement with other studies reported about rhamnolipids stability. In particular Mohammad et al. [141] observed an exceptional stability of the rhamnolipids at high temperatures (heating at 100°C for 1h and autoclaving at 121°C for 10 min). Similarly, Borodoli and Konwar [151] exposed the *P. aeruginosa* biosurfactants to 100°C for different time periods, and found that their activity remained unaffected. In the same way, the rhamnolipids from *P. aeruginosa* LBI were reported to exhibit a good stability when heated [150]. Similarly to the biosurfactants produced by *L. agilis* CCUG31450 and *B. subtilis* PX573, the ones produced by *P. aeruginosa* PX112 also showed a good stability to high temperatures.

The stability of these biosurfactants at different pH values, salinities and high temperatures is an important issue that can affect its application spectrum. Our results show that *P. aeruginosa*

PX112 biosurfactants, similarly to the rhamnolipids tested by Lovaglio et al. [152], could replace chemical surfactants in different industrial fields, in which extreme conditions are used.

3.3.4. Chemical characterization

In order to have a preliminary chemical characterization of the components present in the biosurfactant mixture produced in MRS medium by *L. agilis* CCUG31450, the protein and carbohydrates concentration was determined. The results gathered in Table 25 show that this biosurfactant has 10 times more carbohydrates than proteins.

Table 25. Chemical characterization of the biosurfactants produced by *L. agilis* CCUG31450 in MRS medium. Concentration of carbohydrates and protein were obtained with freeze-dried biosurfactants dissolved in demineralized water at a concentration of 1 mg/ml and expressed as %. Results represent the average of three measurements ± SD.

Biosurfactants	**% Carbohydrates**	**% Protein**
L. agilis CCUG31450	33.0 ± 3.0	3.5 ± 0.1

The biosurfactants produced by lactic acid bacteria have not been so widely studied compared to surfactin or rhamnolipids. The most well-known biosurfactants from lactic acid bacteria have been described as mixtures containing proteins and polysaccharides [101, 119, 153].

In this work, the content in carbohydrates and protein of the biosurfactants produced by *L. agilis* CCUG31450 was also determined and it was found that they were mainly composed of carbohydrates. These results are similar to the ones reported for the biosurfactants produced by *L. casei* subsp. *rhamnosus* 36 and *L. casei* subsp. *rhamnosus* ATCC7469T that possess a higher content in polysaccharides [119]. On the other hand, the biosurfactants from *L. acidophilus* RC14 and *L. fermentum* B54 appear to contain more protein than the ones from *L. casei* subsp. *rhamnosus* 36 and *L. casei* subsp. *rhamnosus* ATCC7469T and a lower content in polysaccharides [119].

In summary, the biosurfactants produced by lactic acid bacteria may have the same components but their proportions seem to be very different depending on the producing strain. Therefore, the study of this biosurfactants by FTIR will clearly and elucidate the composition and structure of the biosurfactants produced by *L. agilis* CCUG31450. For instance, a FTIR analysis of the biosurfactants from *L. pentosus* clearly indicated the presence of -OH and -NH groups, that are commonly present in glycoproteins. This type of biosurfactant structures was also proposed for the biosurfactants produced by *L. lactis* and *L. paracasei* [135]. Additionally, a glycolipid-like structure has previously been proposed for the biosurfactants produced by *S. thermophilus* [135].

It is important to notice that in this work *L. agilis* CCUG31450 was also able to produce biosurfactants in CWM, and that differences in the CMC values comparing to the standard medium have been observed, which suggested differences in the biosurfactants composition. Golek et al. [153] showed interesting results regarding the influence of medium composition on the growth phase of *L. casei* 8/4 and consequently on the biosurfactants composition, namely the ratios of protein and polysaccharides. The highest proportion of protein was noted for the case of surface-active compounds obtained using the MRS medium. In contrast, the highest proportion of polysaccharides was reported for biosurfactants synthesized by *L. casei* 8/4 on a medium prepared from whey permeate enriched with mineral salts. The results obtained suggested that the chemical structures of the biosurfactants were not homogeneous and greatly depend on the culture medium used. Furthermore, it should be noted that some biosurfactants produced by lactic acid bacteria showed high contents of lipids in their composition. For instance, the biosurfactant produced by *Lactobacillus delbrueckii* was classified as a glycolipid showing a higher lipid content as compared to its composition in carbohydrates (30 %:70 % (w/w)) [154]. Therefore, the biosurfactants produced from CWM should also be characterized. Moreover, besides this preliminary characterization, further studies should be conducted to infer the lipid contents of the biosurfactants produced by *L. agilis* CCUG31450.

3.4. Biosurfactants applications

3.4.1. Antimicrobial activity

i. Individual biosurfactants

The antimicrobial activity of the crude biosurfactants isolated from several lactic acid bacteria was determined by measuring the growth inhibition percentages obtained for several microorganisms. Table 26 shows that only the biosurfactants produced in MRS medium by *L. animalis* ATCC35046, *L. paracasei* A20 and *L. agilis* CCUG31450 have antimicrobial activity, specifically against *S. aureus*. The biosurfactants recovered from *L. agilis* CCUG31450 were efficient against other two microorganisms, *S. agalactiae* and *P. aeruginosa*. In this last case, the effect in the microbial growth inhibition was smaller when compared with the one observed for *S. aureus.*

As mentioned before, the properties of the biosurfactants produced by the same microorganism can differ depending on the media in which they are produced. Therefore, the antimicrobial activity of *L. agilis* CCUG31450 biosurfactants produced in MRS and CWM was evaluated. From Table 26, it is possible to observe a clear difference in the antimicrobial activity of the biosurfactants produced in different media. Indeed, the biosurfactants produced in CWM did not present any activity. Therefore, the results once more suggest that biosurfactants being produced in different media may have distinct activities and probably different structures. The biosurfactants produced in CWM presented a higher CMC value comparing to the standard medium, which could also explain why no antimicrobial activity could be observed

The biosurfactants produced by *L. agilis* CCUG31450 in MRS medium were also tested at a lower concentration (2.5 mg/ml); however, no antimicrobial activity was observed.

Table 26. Antimicrobial activity of the biosurfactants isolated from different lactic acid bacteria in MRS medium against several pathogenic bacterial and yeast strains. Percentages of growth inhibition were obtained with freeze-dried biosurfactants dissolved in LB medium at a concentration of 5mg/ml. Results represent the average of three experiments ± SD. Biosurfactants produced in a) MRS medium; b) CWM.

Microorganisms	**Microbial growth inhibition (%)**				
	L. animalis ATCC35046	*L. hamsteri* ATCC43851	*L. paracasei* A20	*L. agilis* CCUG31450 **a)**	*L. agilis* CCUG31450 **b)**
S. aureus	16.9 ± 4.9	0.0	5.2 ± 1.0	20.0 ± 8.1	0.0
S. agalactiae	0.0	0.0	0.0	10.7 ± 2.7	0.0
P. aeruginosa	0.0	0.0	0.0	13.5 ± 0.7	0.0
E. coli	0.0	0.0	0.0	0.0	0.0
C. albicans	0.0	0.0	0.0	0.0	0.0

The biosurfactants produced by *B. subtilis* PX573 in LB medium were tested against the same microorganisms. These biosurfactants showed a good antimicrobial activity against *S. agalactiae* (54.5 %) (Figure 20A), for the highest biosurfactant concentration tested (5 mg/ml). The antimicrobial activity against other microorganisms was lower, namely *E. coli:* 16.4 %, *P. aeruginosa:* 14.5 % and *C. albicans:* 10.4 %.

The biosurfactants produced by the same strain in CSL medium were also tested. A much stronger antimicrobial activity against all the microorganisms tested was found (Figure 20B). These biosurfactants showed antimicrobial activity against *S. aureus* (48.0 %), and it is important to notice that such activity was not observed for the biosurfactants obtained from LB medium. From the results obtained, it can be concluded that the biosurfactants produced by *B. subtilis* PX573 in CSL medium exhibited a higher antimicrobial activity than the ones produced in LB medium. As mentioned before, this can be due to the different compositions of the biosurfactants, but also to the fact that the biosurfactants produced in CSL medium are more efficient (as shown by the CMC value).

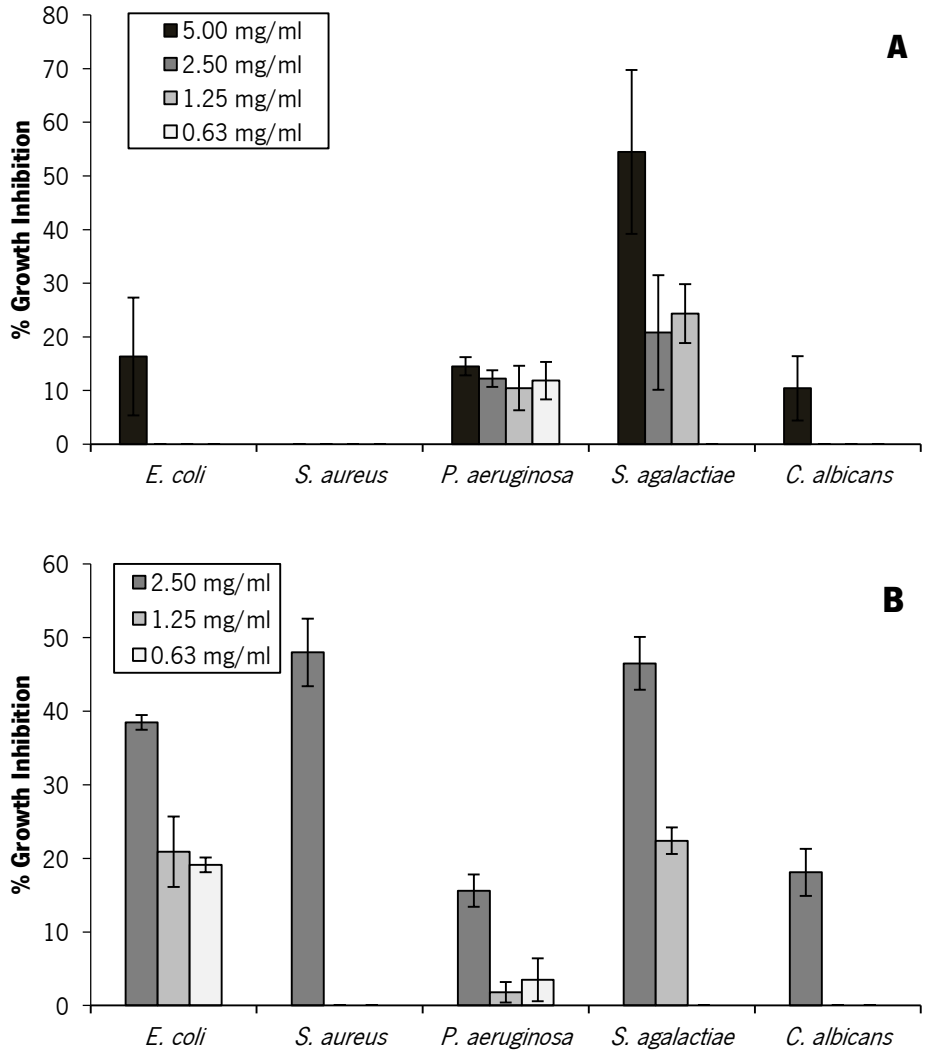

Figure 20. Antimicrobial activity of biosurfactants produced by *B. subtilis* PX573 in LB medium **(A)** and in CSL medium **(B)** against several pathogenic bacterial and yeast strains. Percentages of growth inhibition were obtained with freeze-dried biosurfactants dissolved in LB medium at different concentrations. Results represent the average of three experiments and error bars represent the SD.

The biosurfactants from *P. aeruginosa* PX112 in LB medium were found to inhibit the growth of all microorganisms tested. Antimicrobial activity against both Gram-positive and Gram-negative bacteria and the yeast *C. albicans* was demonstrated (Figure 21A). A slightly higher antimicrobial activity against Gram-positive than Gram-negative bacteria was observed. Specifically, for *S. agalactiae* a complete growth inhibition was achieved even at the lowest biosurfactant concentration used.

The biosurfactants produced by the same strain in CSLM medium were shown to possess a higher antimicrobial activity comparing to the biosurfactants from LB medium, except against *C. albicans*, i.e. only the biosurfactants produced in LB medium showed antimicrobial activity against this yeast.

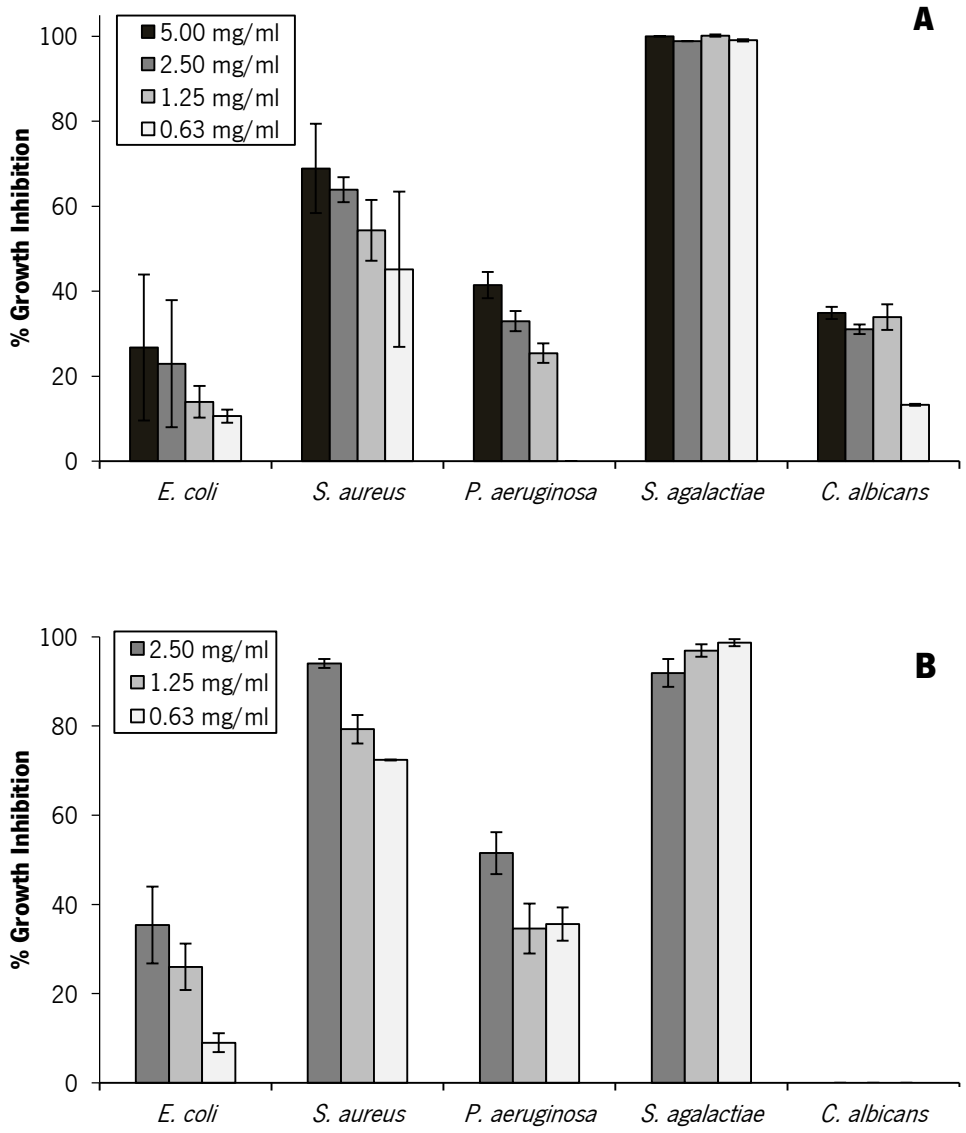

Figure 21. Antimicrobial activity of biosurfactants produced by *P. aeruginosa* PX112 in LB medium **(A)** or in CSLM medium **(B)** against several pathogenic bacterial and yeast strains. Percentages of growth inhibition were obtained with freeze-dried biosurfactants dissolved in LB medium at different concentrations. Results represent the average of three experiments and error bars represent the SD.

These antimicrobial assays were also conducted with the biosurfactants produced by *P. aeruginosa* PX112 in LB medium that were recovered by different strategies, in order to verify if the recovered biosurfactants showed the same activity independently of the recovery process. Comparing the results obtained for the biosurfactants recovered by acid precipitation (Figure 21A), chloroform extraction (Figure 22A), and ammonium sulfate precipitation (Figure 22B), clear differences could be observed in the antimicrobial activity, especially against *E. coli*, *S. aureus* and *C. albicans*. These results revealed that the biosurfactants recovered by ammonium sulfate

precipitation did not have any antimicrobial activity against *E. coli* and *C. albicans,* showing the smaller antimicrobial activity spectrum as compared to the biosurfactants recovered using other strategies.

The biosurfactants recovered by chloroform extraction at a concentration of 5.0 mg/ml showed the highest antimicrobial activity (56.1 %) against *E. coli,* when compared with the ones recovered by acid precipitation (26.8 %), or by ammonium sulfate precipitation (0 %). All biosurfactants were effective against *S. aureus*, although the ones recovered by acid precipitation revealed an antimicrobial activity significantly lower (*p*-value = 0.003) than the others. Again, the different activities observed for the biosurfactants recovered by different techniques support the hypothesis that these could have different structures and properties.

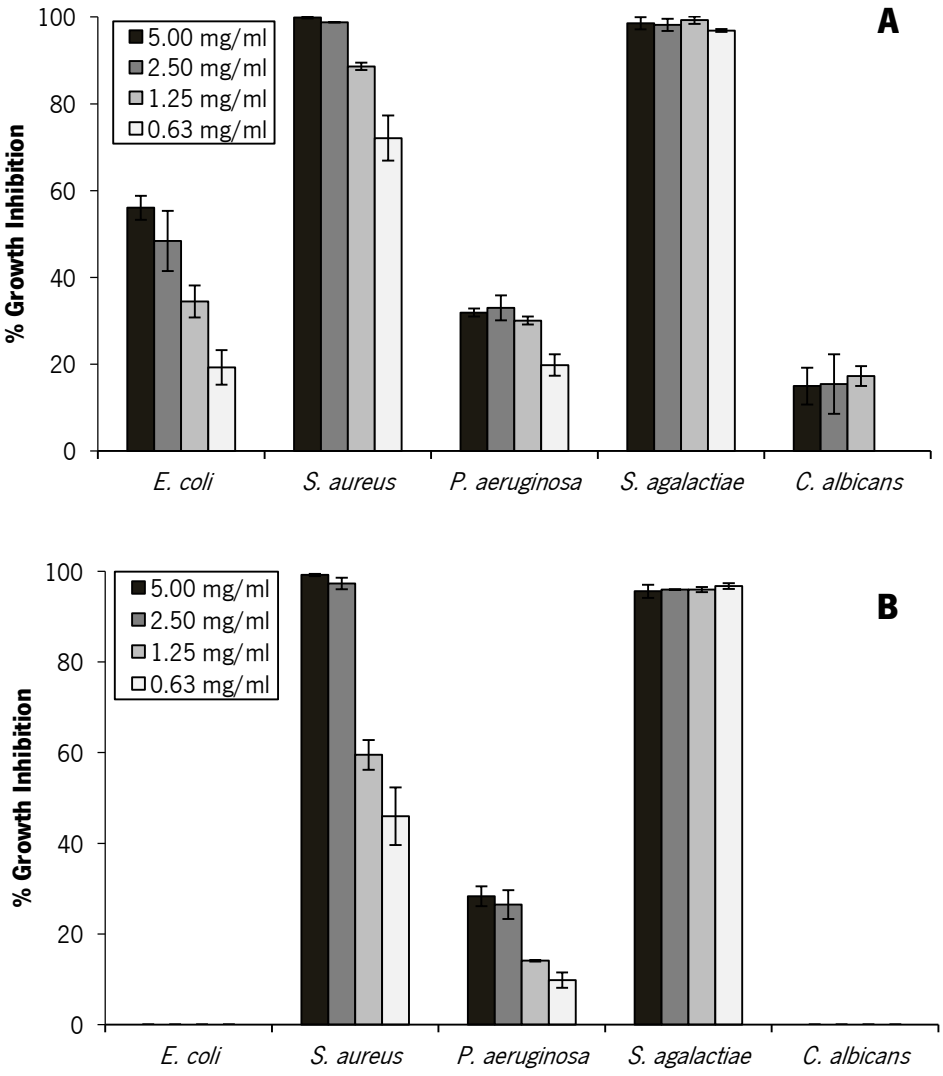

Figure 22. Antimicrobial activity of biosurfactants recovered from *P. aeruginosa* PX112 cultures by chloroform extraction **(A)** or by ammonium sulfate precipitation **(B)** against several pathogenic bacterial and yeast strains. Percentages of growth inhibition were obtained with freeze-dried biosurfactants dissolved in LB medium at different concentrations. Results represent the average of three experiments and error bars represent the SD.

Ghribi et al. [155] demonstrated the antimicrobial activity of biosurfactants produced by *B. subtilis* SPB1 against several bacteria and fungi strains, some of which with multidrug-resistant profiles. Specifically, their biosurfactants were effective against *S. aureus*, a strain that is known to be resistant to at least two β-lactams. Moreover, the authors demonstrated that these biosurfactants were more active against Gram-positive than against Gram-negative bacteria, and also showed an important antifungal activity, especially against *C. albicans.* Fernandes et al. [156] also studied the activity of *B. subtilis* R14 biosurfactants against multidrug-resistant bacteria. All strains were sensitive to the surfactants, in particular *E. faecalis* (Gram-positive bacteria). The activity against Gram-negative bacteria was lower comparing to Gram-positive ones. The *B. subtilis* C1 strain was described to produce a lipopeptide N1 which revealed antimicrobial activity against several Gram-positive bacteria, including *S. aureus* and *Mycobacterium smegmatis* [157]. Similarly to these reports, the biosurfactants produced by *B. subtilis* PX573 in the current work showed a similar antimicrobial behavior, being more aggressive against Gram–positive (*S. aureus* and *S. agalactiae*) than Gram–negative bacteria (*E. coli*). Furthermore, the biosurfactants produced in CSL medium by this strain showed antifungal activity against *C. albicans*, which is in agreement with previous reports [155].

Regarding the biosurfactants produced by *P. aeruginosa* strains also several studies have been conducted to infer their potential antimicrobial activity. Abalos et al. [88] showed that the rhamnolipids from *P. aeruginosa* AT10 were effective against Gram-negative (*E. coli* and *A. faecalis*) and Gram-positive bacteria (*S. aureus* and *Clostridium perfringes*). In general, the MIC (the minimum concentration of product to inhibit the growth of a microorganism) for Gram-positive bacteria was lower than for Gram-negative ones, demonstrating their high sensibility to rhamnolipids. The authors showed that these rhamnolipids were excellent antifungal agents against *A. niger* and *Gliocadium virens*. Haba et al. [133] evaluated the antimicrobial activity of *P. aeruginosa* 47T2 NCBIM40044 rhamnolipids. These rhamnolipids inhibited the growth of both Gram-positive (*S. epidermidis* ATCC11228 and *S. aureus* ATCC6538) and Gram-negative (*E. coli* ATCC8739 and *P. aeruginosa* ATCC9027) bacteria. Moreover, these rhamnolipids were effective against *C. albicans* ATCC10231 although the MIC value was much higher compared with the ones calculated for bacteria. Accordingly, in this work, the biosurfactants produced by *P. aeruginosa* PX112 exhibited potent antimicrobial and antifungal activities. These biosurfactants were more effective against Gram-positive bacteria (*S. agalactiae* and *S. aureus*) than against Gram-negative

ones (*E. coli* and *P. aeruginosa*). Besides, although these biosurfactants showed antifungal activity against *C. albicans* this activity was less pronounced as compared to the activity against bacteria.

Generally speaking, cationic surfactants are the most toxic and have historically been used as antimicrobials, while anionic surfactants are less toxic but more active against Gram-positive than Gram-negative bacteria, and non-ionic surfactants are often considered non-toxic [158]. Surfactin and rhamnolipids produced by *B. subtilis* and *P. aeruginosa* strains, respectively, have been described as anionic surfactants [159, 160]. Therefore, the biosurfactants produced by *B. subtilis* PX573 (Figure 20) and *P. aeruginosa* PX112 (Figure 21), which most probably are anionic biosurfactants, showed a higher antimicrobial activity again the Gram-positive strains, which is in agreement with was has been described before.

Depending on the biosurfactants nature, the toxic effect on the microorganisms could be distinct. For instance, biosurfactants may exert toxic effects by causing membrane disruption leading to cellular lysis, by increasing the membrane permeability causing metabolite leakage, by altering the physical membrane structure or by disrupting protein conformations thus interfering with important membrane functions such as energy generation and transport. The response of a given microorganism to a biosurfactant will depend on a variety of factors such as cellular ultrastructure, capacity for biodegradation or efflux, biosurfactant concentration and bioavailability, and other environmental and culture conditions [158].

The biosurfactants produced by *B. subtilis* strains was found to incorporate into membranes at low concentrations and induce slow leakage due to changes in membrane ultrastructure [66]. Specifically, the incorporation of surfactin in the cell membrane induced pores promoting membrane permeabilization. This effect occurs because the biosurfactant interacts with the phospholipid acyl chains, resulting in considerable membrane fluidization. Similarly to what is observed for surfactin, rhamnolipids have also been reported to interact with the cell membrane [140]. Ortiz et al. [161] showed that di-rhamnolipids intercalate into the phosphatidylcholine bilayers and produce structural perturbations in the membrane which might affect its function. The biosurfactants disrupt cell membranes through the formation of a transient pore. The permeabilizing effects of rhamnolipids on Gram-positive and Gram-negative bacteria was also studied by Sotirova et al. [162]. These researchers hypothesized that rhamnolipids can act against bacteria by two different mechanisms. The biosurfactant forms molecular aggregates in the surface of the Gram-positive bacteria or could release lipopolysaccharides from the outer membrane of Gram-negative bacteria, thus leading to the formation of transmembrane pores as channels to the

periplasm. In both cases it results in a modification of the cell permeability. In this study it was found that the biosurfactant PS produced by *Pseudomonas* sp. PS-17 promoted a higher release of proteins in *B. subtilis* 168 cells compared with those of *P. aeruginosa*, which confirmed the higher susceptibility of Gram-positive cells to the effect of the biosurfactant [162].

The mechanism of action of surfactin and rhamnolipids described above can also explain the antimicrobial effects observed in this work for the biosurfactants produced by *B. subtilis* PX573 and *P. aeruginosa* PX112.

If in one hand these biosurfactants produced by *B. subtilis* and *P. aeruginosa* strains have the capacity to interfere with the integrity of the membrane of Gram-positive bacteria, the same effect normally is not observed for Gram-negative bacteria. Hamounda et al. [163] postulated that the resistance of the Gram-negative bacteria may be attributable to its cell wall LPS [164] and their negative surface charge. Assuming that the biosurfactants produced by *B. subtilis* PX573 and *P. aeruginosa* PX112 have an anionic nature, they could also exert a repulsive effect against the Gram-negative bacteria. However, it is likely that more than a single mechanism is involved in the resistance observed.

In summary, the results showed that, in general, the individual biosurfactants exert a small or any antimicrobial activity against *C. albicans*. The resistance of yeast cells to biosurfactants is most likely due to its rigid cell wall structure [163], which prevents the biosurfactants penetration. In this work the biosurfactants with the most promising action against the yeast *C. albicans* were the ones produced by *P. aeruginosa* PX112 in LB medium and by *B. subtilis* PX573 in CSL, showing an antimicrobial activity of 34.9 and 18.1 %, respectively.

ii. Biosurfactants mixtures

In this task, different biosurfactants were mixed at different concentrations in a 1:1 proportion in order to evaluate their interaction and synergistic or antagonistic effect on the microorganisms tested. The interaction between biosurfactants is synergistic when their combined activity is greater than the additive effect of the single biosurfactants; the synergistic interaction allows the use of lower dosages and in some cases can extend the range of actuation [159].

The first mixture tested was prepared with the biosurfactants from *P. aeruginosa* PX112 in CSLM medium and biosurfactants from *L. agilis* CCUG31450 in MRS medium. As can been seen in Figure 23, this mixture was able to completely inhibit the growth of *S. agalactiae*, thus corresponding to a slight increase in the overall antimicrobial activity as compared to the individual biosurfactants performance. Statistical analysis demonstrated that the differences observed were significant (*p*-value <0.0001). In contrast, the mixture showed a strong negative interaction between the biosurfactants against *S. aureus*, demonstrated by the abrupt reduction of the overall antimicrobial activity. The mixture showed only 21.3 % of antimicrobial activity against *S. aureus* when compared with the biosurfactant produced by *P. aeruginosa* PX112 (94 %) at the same concentration.

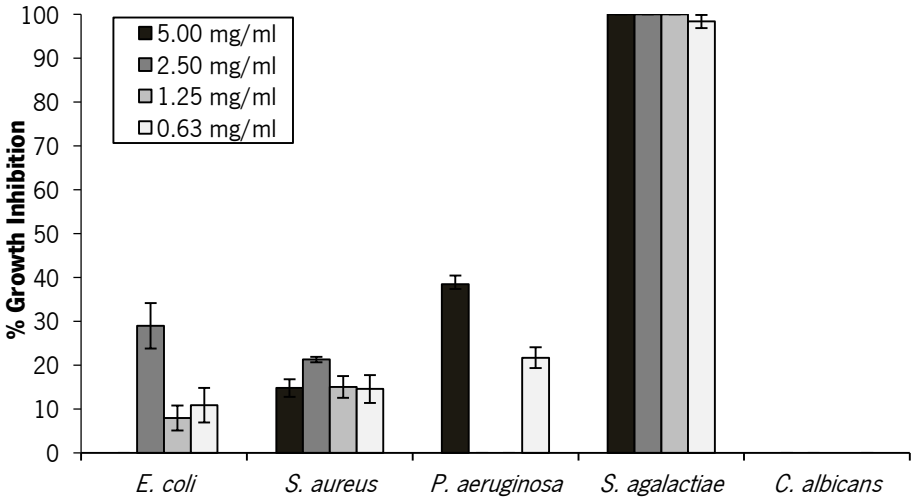

Figure 23. Antimicrobial activity of biosurfactant mixtures against several pathogenic bacterial and yeast strains. The mixtures were prepared at 1:1 proportion with freeze-dried biosurfactants recovered from *P. aeruginosa* PX112 (produced in CSLM medium) and *L. agilis* CCUG31450 (produced in MRS medium). Percentages of growth inhibition were obtained with biosurfactant mixtures at different concentrations. The legend shows the individual concentration of each biosurfactant present in the mixture. Results represent the average of three experiments and error bars represent the SD.

The biosurfactants obtained from alternative media were also mixed and tested for their antimicrobial activity. The mixture was effective against *S. agalactiae* (Figure 24) completely inhibiting its growth (at 2.5 mg/ml biosurfactant) corresponding to a positive interaction between the individual biosurfactants. Statistical analysis again showed that the differences observed between mixture and individual performances were significant (*p*-value <0.0001). Contrarily, the mixture was not effective against the other microorganisms studied, suggesting an antagonist effect

between the biosurfactants present in the mixture. In some cases this antagonist effect was found to be very strong. For instance, the individual biosurfactants from *B. subtilis* PX573 (produced in CSL medium) and *P. aeruginosa* PX112 (produced in CSLM medium) showed antimicrobial activity against *E. coli* of 38.5 % (Figure 20B) and 35.4 % (Figure 21B), respectively. However, the mixture of these two biosurfactants showed a very low percentage of growth inhibition (16.3 %) (Figure 24). The same was observed for *S. aureus* and *P. aeruginosa.*

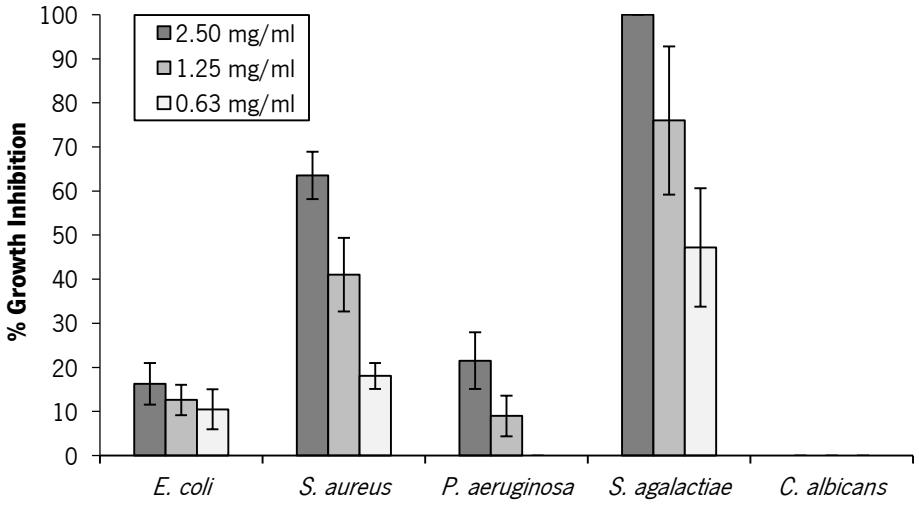

Figure 24. Antimicrobial activity of biosurfactant mixtures against bacterial and yeast strains. The mixtures were prepared at 1:1 proportion with freeze-dried biosurfactants recovered from *P. aeruginosa* PX112 (produced in CSLM medium) and *B. subtilis* PX573 (produced in CSL medium). Percentages of growth inhibition were obtained with biosurfactant mixtures at different concentrations. The legend shows the individual concentration of each biosurfactant present in the mixture. Results represent the average of three experiments and error bars represent SD.

The antimicrobial assays performed with the individual biosurfactants produced by lactic acid bacteria showed a common activity against *S. aureus*. Therefore, mixtures combining biosurfactants from *L. paracasei* A20, *L. animalis* ATCC35046 and *L. agilis* CCUG31450 were prepared, and their activity against *S. aureus* was evaluated. From Figure 25, the antimicrobial activity of the mixture (55.3 %) showed a great increase as compared to the individual biosurfactants from *L. paracasei* A20 (5.2 %) and *L. animalis* ATCC35046 (16.9 %) (Table 26). The mixture of these biosurfactants produced a remarkable synergistic effect since the individual biosurfactants activity was greatly enhanced. The same synergistic effect was observed for the mixture of biosurfactants from *L. paracasei* A20 and *L. agilis* CCUG31450 in MRS medium. The antimicrobial activity of the mixture is significantly higher (28.9 %) comparing to the individual

biosurfactants. Statistical analysis confirm the significance of the differences observed (*p*-value = 0.04). However, in the case of the mixture of *L. animalis* ATCC35046 and *L. agilis* CCUG31450, the antimicrobial activity of the mixture was similar to the one obtained for each biosurfactant at a concentration of 5 mg/ml.

The individual biosurfactants tested in the mixtures that are presented in Figure 25 did not show any antimicrobial activity against *S. aureus* at a concentration of 2.5 mg/ml. However, mixing the individual biosurfactants at 2.5 mg/ml led to 17.4 – 26.0 % growth inhibition of *S. aureus*. In summary, a clear synergistic effect could be observed for the mixtures of lactic acid bacteria biosurfactants. Actually, these mixtures potentiated the individual performance even at low concentrations. The interaction between the different biosurfactants might have led to complex structures with more affinity to the microbe's cell surface where the biosurfactants could exert a more potent antimicrobial activity. Although the percentages of growth inhibition were generally low, the potential of these biosurfactants as antimicrobial agents should be further explored.

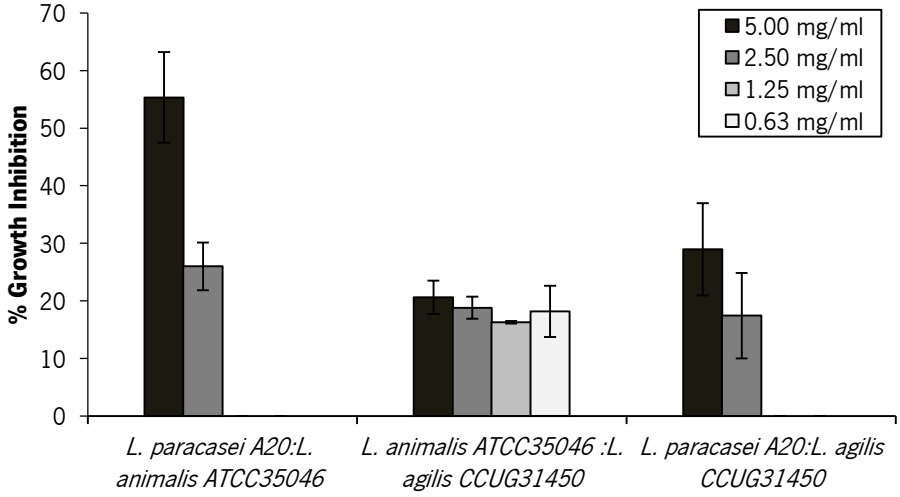

Figure 25. Antimicrobial activity of biosurfactant mixtures against *S. aureus*. The mixtures were prepared at 1:1 proportion with freeze-dried biosurfactants recovered from different lactic acid bacteria cultivated in MRS medium. Percentages of growth inhibition were obtained with biosurfactant mixtures at different concentrations. The legend shows the individual concentration of each biosurfactant present in the mixture. Results represent the average of three experiments and error bars represent the SD.

Although the biosurfactants of *B. subtilis* PX573 and *P. aeruginosa* PX112 as individuals demonstrated positive results, their combination with other biosurfactant did not always showed a

positive or even a synergistic effect as could be expected, but contrarily in some cases an antagonist effect was observed.

As mentioned before, the biosurfactants produced either by *B. subtilis* PX573 or by *P. aeruginosa* PX112 are most likely anionic [159, 160]. Therefore, as both are negatively charged, a repulsive effect could prevent the interaction between them, which could explain the antagonist effect observed. On the other hand, since the bacterial surface has an anionic charge, [165] the negative species on the cell surface could induce an electrostatic repulsion between the anionic biosurfactants and the cell surface. Since the mixture of both biosurfactants should have an extremely anionic nature, the electrostatic repulsion between the cell surface of microorganisms and the mixture is amplified comparing to the individual biosurfactants. Kihara et al. [165] studied the interaction of anionic surfactants with polyvalent metal cations. In this study it was observed that the presence of polyvalent metal cations increases the activity of the anionic surfactants. The authors found that the anionic surfactant was remarkably effective against *E. coli* in the presence of only a few polyvalent cations. This effect is observed because polyvalent cations neutralize the negative charge of the cell surface in the presence of the surfactant, indicating that the interaction between the hydrophobic NaOS-polycation complex and the cell surface is promoted.

In order to develop more efficient mixtures, an mixture between anionic and cationic biosurfactants could promote a better interaction between the mixture and the cell surface of microorganisms similarly to the report by Kihara et al. [165].

On the other hand, the mixture of biosurfactants produced by *L. paracasei* A20 and *L. animalis* ATCC35046 showed a high synergistic effect. Since the yield of biosurfactants produced by lactic acid bacteria is very low, the mixture of these biosurfactants would reduce the amount of biosurfactants required to promote a better effect comparing to the amounts required for the individual biosurfactants to produce the same effect. The results suggest that these biosurfactants in the mixture might have a better affinity to the microbe's cell surface, thus enhancing their antimicrobial activity. However, in order to understand more clearly the interaction between these biosurfactants, and consequently their effect on a given microorganism, a more profound study on the structure of these biosurfactants should be conducted.

The individual biosurfactants and the mixtures that presented a good antimicrobial activity could be further used as possible substitutes of antibiotics, in particular against microorganisms

that are resistant to conventional treatment agents. *S. aureus* is a common cause of community and hospital-acquired infections. Moreover, the clinical impact of *S. aureus* is rising due to the global increase in the incidence of multidrug-resistant strains and its growing prevalence as a major cause of surgical infections [92]. As a result, there is an urgent need to identify new antimicrobial agents and develop preventive strategies to help in the management of these types of infections. Therefore, the biosurfactants and their mixtures herein studied could be an answer to the necessity of efficient antimicrobial agents against antibiotic resistant microorganisms. The biosurfactants that cannot act by themselves and that did not show a positive interaction with other biosurfactants could be further evaluated in combination with antibiotics towards an enhanced activity, reduction of antibiotics concentrations, and reduction of pathogenic microorganism's resistance to those pharmaceutical agents. For instance, Ortiz et al. [161] suggested that bacteria with more hydrophobic surface will be more susceptible to the action of hydrophobic antibiotics, thus future research should focus on the combined use of rhamnolipids with antibiotics to potentiate the antibiotics effects. Rivardo et al. [166] showed that V9T14 biosurfactant produced by *B. licheniformis* V9T14 in association with antibiotics (ampicillin) led to a synergistic increase of the antibiotic efficacy in biofilm killing, and in some combinations led to the total eradication of *E. coli* CFT073 biofilm. Moreover, it was showed that the concentration of antibiotic required to obtain the same effect was decreased by the presence of the biosurfactant. The authors hypothesize that the action of V9T14 biosurfactant lies in its interaction with the bacterial membrane, increasing the activity of antimicrobial agents by forming pores in the outer membrane and thus facilitating the entrance of the antibiotics. The use of surfactin to increase the effect of enrofloxacin against planktonic *Mycoplasma pulmonis* was also reported in literature [167].

3.4.2. Anti-adhesion activity

i. *Staphylococcus aureus*

The anti-adhesive activity of a variety of individual biosurfactants produced by several bacteria in different media were evaluated against *S. aureus* (Figure 26). The biosurfactants produced by *L. agilis* CCUG31450 in MRS medium showed the greatest anti-adhesive activity, 64.5 % at 10 mg/ml, in contrast with the ones produced in CWM, in which no anti-adhesive activity was detected. Regarding the biosurfactants produced by *P. aeruginosa* PX112, either in LB or in CSLM medium, the anti-adhesive activity against *S. aureus* was similar and low, 10.6 % and 11.2 %, respectively, for the highest concentration tested. The biosurfactants from *L. paracasei* A20 in MRS medium and *B. subtilis* PX573 in CSL medium revealed also an interesting anti-adhesive activity, 29.6 % and 46.8 %, respectively.

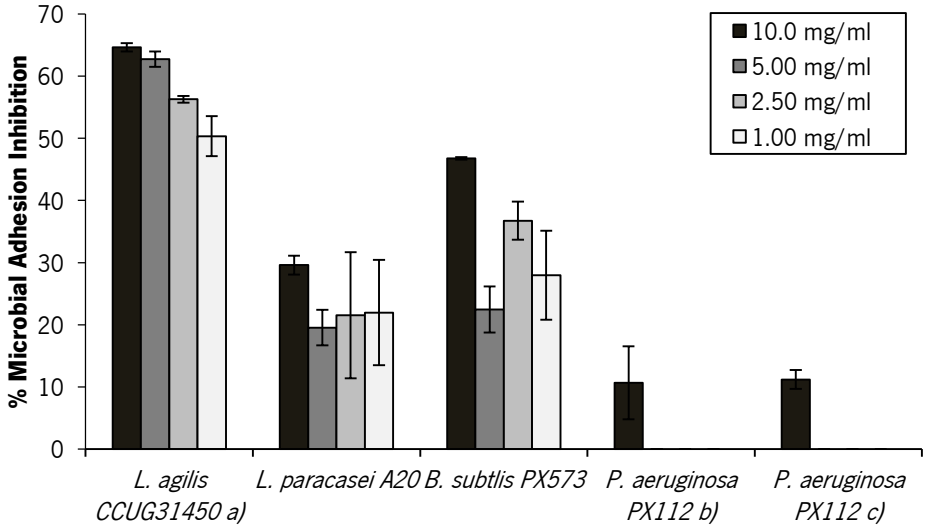

Figure 26. Anti-adhesive activity of individual biosurfactants isolated from different bacterial strains against *S. aureus*. Percentages of microbial adhesion inhibition were obtained with freeze-dried biosurfactants dissolved in demineralized water at different concentrations. Results represent the average of three experiments and error bars represent SD. Biosurfactants produced in a) MRS medium, b) LB medium and c) CSLM medium.

Also in this case different biosurfactants combinations were explored towards an enhanced activity. Mixtures were performed using freeze-dried biosurfactants from *B. subtilis* PX573, *P. aeruginosa* PX112, *L. paracasei* A20 and *L. agilis* CCUG31450 at different proportions. The mixture of biosurfactants produced by *B. subtilis* PX573 in CSL medium and biosurfactants produced by *L. aiglis* CCUG31450 in MRS medium at 1:1 proportion (Figure 27), showed the most favorable results, 68.4 % of microbial adhesion inhibition, marginally higher than the best result obtained using individual biosurfactants (64.6 % for the *L. agilis* CCUG31450 biosurfactant obtained in MRS). Likewise the mixture containing the biosurfactants from *P. aeruginosa* PX112 in CSLM medium and *L. aglis* CCUG31450 in MRS medium showed a good anti-adhesive activity (60.7 %). For both mixtures above mentioned a positive interaction could be found between the biosurfactants used. On the other hand, the other mixtures tested showed antagonist effects, i.e. the anti-adhesive activity of the mixtures were lower than the activity of the individual biosurfactants, at the same concentration.

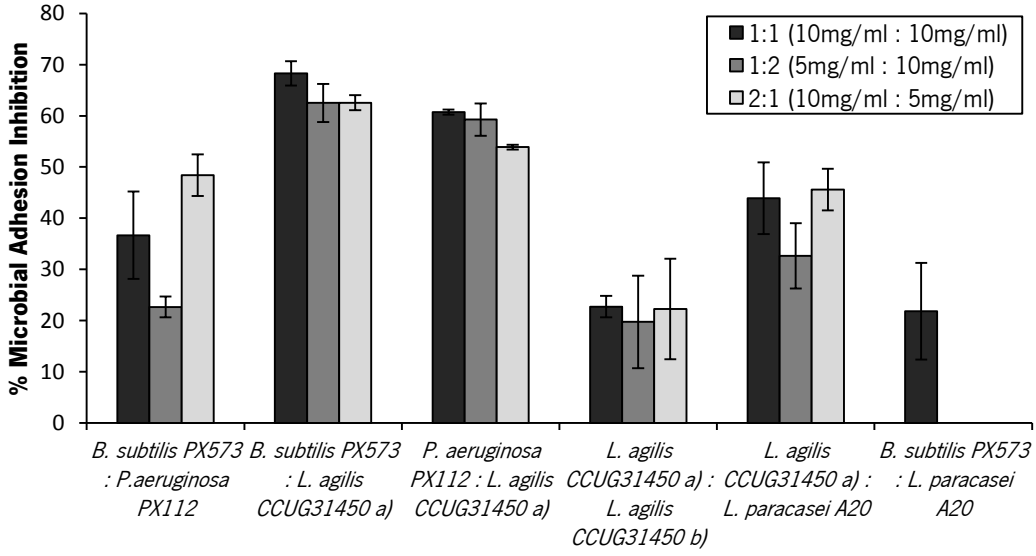

Figure 27. Anti-adhesive properties of several biosurfactant mixtures against *S. aureus*. Percentages of microbial adhesion inhibition were obtained with freeze-dried biosurfactants recovered from different bacterial strains and dissolved in demineralized water at different proportions. The mixture *B. subtilis* PX573:*L. paracasei* A20 was only tested at 1:1 proportion. Results represent the average of three experiments and error bars represent SD. Biosurfactants produced in a) MRS medium and b) CWM.

ii. *Pseudomonas aeruginosa*

In the same way, several biosurfactants produced in this work were test against *P. aeruginosa* in order to evaluate their anti-adhesive activity (Figure 28). The biosurfactants from *L. agilis* CCUG31450 in CWM showed the greatest anti-adhesive activity, 46.2 % at 1 mg/ml, although no anti-adhesive effect was previously found against *S. aureus*. Regarding the biosurfactants from *P. aeruginosa* PX112 in LB or in CSLM medium, it was observed a small difference in their anti-adhesive activity against *P. aeruginosa,* 17.3 % and 27.4 %, respectively, for the highest concentration tested. No anti-adhesive activity could be found for the biosurfactants from *L. paracasei* A20, contrarily to what was previously seen for *S. aureus*. These distinct results suggest that each biosurfactant have a unique effect that could be different from microorganism to microorganism.

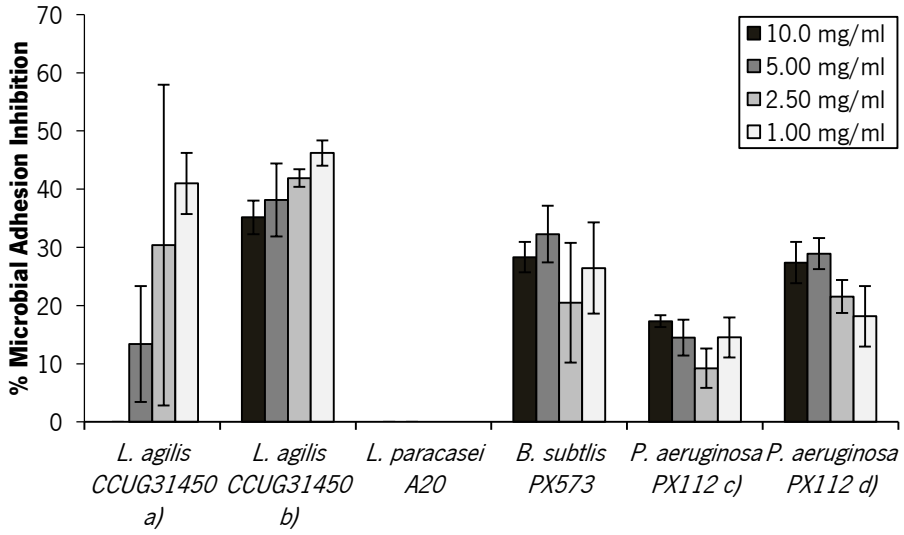

Figure 28. Anti-adhesive properties of biosurfactant isolated from different bacterial strains against *P. aeruginosa*. Percentages of microbial adhesion inhibition were obtained with freeze-dried biosurfactants dissolved in demineralized water at different concentrations. Results represent the average of three experiments and error bars represent SD. Biosurfactants produced in a) MRS medium, b) CWM, c) LB medium and d) CSLM medium.

The mixtures tested against *P. aeruginosa* were prepared in the same way as described before. In Figure 29, the mixture of biosurfactants from *P. aeruginosa* PX112 produced in CSLM and *L. agilis* CCUG31450 produced in MRS medium, in a 1:2 proportion, was found to be the best

for inhibiting the adhesion of *P. aeruginosa*, 62.5 %. The mixture of biosurfactants from *B. subtilis* PX573 produced in CSL medium and *P. aeruginosa* PX112 was found to be very interesting, since a positive interaction between both biosurfactants was observed. The anti-adhesive activity of the mixture, 40.7 % (1:1 proportion), is superior to the activity of each biosurfactant, 28.3 % for biosurfactants from *B. subtilis* PX573 and 27.3 % for biosurfactants from *P. aeruginosa* PX112, at 10 mg/ml.

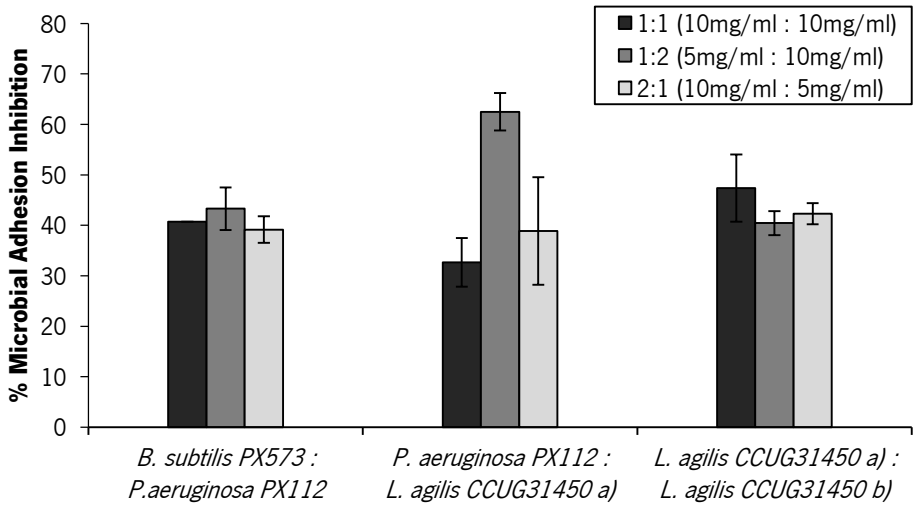

Figure 29. Anti-adhesive properties of several biosurfactant mixtures against *P. aeruginosa*. Percentages of microbial adhesion inhibition were obtained with freeze-dried biosurfactants recovered from different bacterial strains and dissolved in demineralized water at different proportions. Results represent average three experiments and error bars represent SD. Biosurfactants produced in a) MRS medium and b) CWM.

To test the properties of the *P. aeruginosa* PX112 biosurfactants produced in LB medium and recovered by different strategies, anti-adhesive assays were performed against *S. aureus* and *P. aeruginosa*. The results are gathered in Table 27 and no anti-adhesive activity against *P. aeruginosa* could be observed either for the biosurfactants recovered by chloroform extraction or by ammonium sulfate precipitation. In contrast, the biosurfactants recovered by acid precipitation inhibited the adhesion of *P. aeruginosa*, 17.3 % (Figure 28). On the other hand, for *P. aeruginosa* PX112 biosurfactants produced in LB medium tested against *S. aureus*, the best results were obtained for the ones recovered by chloroform extraction and by ammonium sulfate precipitation showing similar anti-adhesive activities, 21.9 % and 28.4 %, respectively, for the higher concentration tested. Again, these results suggest that biosurfactants recovered by different techniques could have distinct natures. Moreover, the reduced activity of the biosurfactants

recovered by acid precipitation could be due to the greater amount of impurities present that will naturally interfere with biosurfactants activity.

Table 27. Anti-adhesive properties of biosurfactants isolated from *P. aeruginosa* PX112 (produced in LB medium) with different recovery methods. Percentages of microbial adhesion inhibition against *S. aureus* and *P. aeruginosa* were obtained with freeze-dried biosurfactants dissolved in demineralized water at different concentrations. Results represent the average of three experiments ± SD.

	% Microbial Adhesion Inhibition	
	S. aureus	***P. aeruginosa***
Biosurfactants recovered by chloroform extraction (mg/ml)		
10	21.9 ± 5.4	0.0 ± 0.0
5	20.0 ± 5.6	0.0 ± 0.0
2.5	29.4 ± 6.6	0.0 ± 0.0
1.25	19.1 ± 7.7	0.0 ± 0.0
Biosurfactants recovered by Ammonium sulfate precipitation (mg/ml)		
10	28.4 ± 4.7	0.0 ± 0.0
5	22.4 ± 7.5	0.0 ± 0.0
2.5	23.9 ± 2.5	0.0 ± 0.0
1.25	19.7 ± 5.6	0.0 ± 0.0

The anti-adhesive activity of the biosurfactants has been widely described in the literature. Microorganisms produce biosurfactants, which prevent the adhesion of other microorganisms, as a mechanism of defense from environmental assaults. In other words, the biosurfactants are responsible for conditioning the surface causing physical and chemical alterations that can prevent bacteria from adhering [168]. This ability to decrease the bacterial attachment to a given surface has been reported for biosurfactants produced by a large range of microorganisms such as *Lactobacillus* spp., *Bacillus* spp., *P. aeruginosa*, *Streptococcus* spp., *Candida* spp. [94–96,119,169–171]. For instance, the biosurfactant released by an oral *Streptococcus mitis* strain reduced the adhesion of *Streptococcus mutans* [168]. Similarly, *L. fermentum* RC-14 releases components with surface activity that can inhibit the adhesion of uropathogenic bacteria, including *E. faecalis* [172].

In this work, the most interesting anti-adhesive activity, either against *S. aureus* or *P. aeruginosa*, was observed for the biosurfactants from lactic acid bacteria, mostly *L. agilis* CCUG31450. Several studies already reported the efficiency of other lactic acid bacteria in decreasing the bacteria and yeast adhesion, namely *L. lactis*, *L. acidophilus*, *L. fermentum*, *L. casei* and *L. rhamnosus* strains [173]. Walenka et al [173] described the inhibition of *S. aureus* adhesion due to biosurfactants obtained from three strains of *L. acidophilus*. The authors suggested that the anti-adhesive effect of the biosurfactant mixture could be due to the changes in the cell-to-surface interactions causing alterations in the hydrophobicity of the surface. The same changes in the properties of the surface could also explain the anti-adhesive effect that was observed for the biosurfactants from *L. agilis* CCUG31450.

However, the exact mechanisms of anti-adhesive activity has not yet been explained, it seems to be highly dependent on the properties of the target bacteria, surface properties and biosurfactant type [174].

Regarding biosurfactants produced by *L. paracasei* A20, clear differences in the anti-adhesive activity were observed. A 29.6 % inhibition was observed against *S. aureus,* while no activity was observed against *P. aeruginosa*. The biosurfactants from *L. agilis* CCUG31450 in CWM showed an opposite effect, being more effective against *P. aeruginosa* (35.2 %) than against *S. aureus* (0 %). This distinct effect of the biosurfactants could be due to the differences in the bacteria, since *S. aureus* is a Gram-positive and *P. aeruginosa* is a Gram-negative. In order to better understand the influence of cell surface properties on the anti-adhesive activity of biosurfactants, Zeraik and Nitschke [174] performed a physicochemical characterization of cell surfaces of some microorganisms. All bacteria studied, *S. aureus*, *Listeria monocytogenes*, and *M. luteus*, were classified as strongly hydrophobic. *S. aureus* showed the highest hydrophobic characteristic, thus clarifying its great affinity to the hydrophobic polystyrene surface. Similarly, Makin et al. [175] studied the characteristics of the surface of *P. aeruginosa,* in order to understand its influence in the action of the biosurfactants. The authors showed that *P. aeruginosa* has two chemically and immunologically distinct LPS O-polysaccharide species, termed the A and B. The presence or absence of species A and B and their proportion influence the surface characteristics of the strains, thus affecting their ability to adhere to hydrophilic (glass) and hydrophobic (polystyrene) surfaces. Therefore, the differences in the activity of the same biosurfactants against distinct bacteria could also be explained due to the hydrophobic/hydrophilic characteristics of the cells surface.

In the case of surface properties, is known that biosurfactants could adsorb to a substratum surface and alter the hydrophobicity of the surface interfering with the microbial adhesion processes [168]. Rodrigues et al. [117] postulated that biosurfactants reduce the hydrophobic interactions, and consequently the microbial adhesion. Hydrophobic surfaces have shown to be particularly colonized by microorganisms, probably because these surfaces facilitate the close approach between microorganism and solid substratum. Consequently, when a surface is conditioned with biosurfactant, it becomes more hydrophilic, with an expected decrease of microbial attachment. Zeraik and Nitschke [174] observed a decrease in the hydrophobicity of surfaces treated with surfactin and also a substantial decrease of bacterial attachment, which is in agreement with the aforementioned explanation. Correspondingly, the results obtained in this work showed that biosurfactants from *B. subtilis* PX573 in CSL medium, probably surfactin, were responsible for a microbial adhesion decrease of 46.8 % for the Gram-positive *S. aureus* and of 28.6 % for the Gram-negative *P. aeruginosa*. According to the previous explanation, these biosurfactants probably altered the hydrophobicity of the surface causing a decrease in the microbial adhesion. Furthermore, Zeraik and Nitschke [174] showed that polystyrene becomes more hydrophilic after being conditioned with surfactin, while conditioning with rhamnolipids increased the surface hydrophobicity. Being the microorganism *S. aureus* extremely hydrophobic that could explain why the biosurfactants from *B. subtilis* PX573 showed a stronger anti-adhesive activity (46.8 %) than the biosurfactants from *P. aeruginosa* PX112 in LB medium (10.6 %), and in CSLM medium (11.2 %) at 10 mg/ml. The differences observed could also be attributed to the chemical composition of the biosurfactants (one is a lipopeptide, while the other is a glycolipid), their molecular orientation on the surfaces, as well as their surface properties.

As previously mentioned, the chemical structure of biosurfactants, mainly the proportions of their components, seems to significantly affect their anti-adhesive properties [135]. Velraeds et al. [119] revealed that biosurfactants containing a higher fraction of proteins, synthesized by *L. acidophilus* RC14 and *L. fermentum* B54, are more effective in impeding the adhesion of *E. faecalis* 1131 to glass than those containing high polysaccharide and phosphate contents. These effects can explain the differences observed for biosurfactants produced by different lactic acid bacteria, or by the same microorganisms but in different media. Also, it should be noted that the more hydrophilic or hydrophobic character of the biosurfactants will influence its activity. Rodrigues et al. [101] showed that the most active fraction isolated from a biosurfactant mixture produced by

S. thermophilus A exhibited a high hydrophilic character. Furthermore, the authors showed that adsorbing the biosurfactant from *L. lactis* 53 onto the surface of silicone rubber turned the surface more hydrophilic. Likewise the biosurfactants from *L. agilis* CCUG31450 produced in MRS may have a good hydrophilic character and consequently lead to a more hydrophilic surface that is traduced in a higher anti-adhesive activity. This hydrophilic character of the biosurfactants showed a more pronounced effect against *S. aureus* (64.6 %) (Figure 26) (most probably the strain with the stronger hydrophobic character), comparing to *P. aeruginosa*, 0 % (Figure 28) (most probably this strain possesses a more hydrophilic cell surface) at the highest concentration tested.

An interesting result obtained in this work was observed for the mixture of biosurfactants produced by *L. agilis* CCUG31450 in different media. As can been seen the biosurfactants produced in MRS medium showed a stronger anti-adhesive activity against *S. aureus*, 64.6 % (Figure 26). However, no activity was observed against *P. aeruginosa* (Figure 28). The anti-adhesive assays with biosurfactants produced in CWM, on the contrary presented no activity against *S. aureus* but a good anti-adhesive activity against *P. aeruginosa* (46.2 %), for the concentration 1 mg/ml. Considering these results, both biosurfactants were mixed aiming at producing a mixture with a wider spectrum of action, i.e. effective against the two pathogenic microorganisms. The mixture at 1:1 proportion (individual concentration of biosurfactants 10 mg/ml) proved to be effective against the two strains tested. Specifically, it was observed a reduction of cell adhesion of 47.4 % for *P. aeruginosa* (Figure 29) and 22.7 % for *S. aureus* (Figure 27). In the case of *P. aeruginosa* strain, the mixture showed an increase of activity, 47.4 %, when compared with the individual biosurfactants, 0 %, for the ones produced in MRS medium; and 35.2% for the ones produced in CMW (Figure 28). Therefore, it is possible to conclude that the mixture showed a positive interaction, resulting in a synergistic anti-adhesive effect. On the other hand, for the strain *S. aureus* the anti-adhesive effect observed, 22.7 %, was reduced as compared with the individual effects of the biosurfactants produced in MRS medium, 64.0 % (Figure 26). In this case, the results suggest a negative interaction between the biosurfactants, i.e. an antagonistic effect.

The remarkable anti-adhesive activity observed for some of the biosurfactants and mixtures, tested in this work, point them as potential agents for diverse applications.

Bacteria growing as a biofilm remain a significant challenge in the biomedical field, growing on abiotic material such as catheters and prosthesis, as they tend to be more tolerant to antimicrobial treatments. Frequent replacement of the prosthesis is uncomfortable, costly, time consuming, and may lead to damage of the patients cellular tissues [136]. Therefore, the application of these biosurfactants with anti-adhesive activity against pathogenic microorganisms could limit biofilm infection by preventing the microbial adhesion to the surfaces of medical devices, and consequently preventing biofilm formation and leading to a reduction of a large number of hospital infections without the use of synthetic drugs and chemicals [101].

For example, as a potential application, these biosurfactants could be used to pre-coat the surfaces of prosthesis, implants and surgical instruments, where no microbial populations are desirable [168]. These applications were already explored in other studies, for example PTFE (Polytetrafluoroethylene) surfaces pre-conditioned with rhamnolipids were able to reduce *L. monocytogenes* attachment [176]. Pre-coating of vinyl urethral catheters with surfactin from *B. subtilis* caused a reduction in the amount of biofilm formed by several microorganisms, such as *A. faecalis*, *Klebsiella aerogenes*, *S. typhimurium* [94, 168]. *L. lactis* and *S. thermophilus* A produce biosurfactants that are able to decrease the amount of bacteria in a multi-species biofilm on voice prosthesis [101, 117]. Surfactin and rhamnolipids are able to prevent *E. coli* and *P. mirabilis* biofilm attachment. *S. enterica* biofilm adhesion on vinyl urethral catheters (14Fr) was inhibited by pre-coating the surface of the device with 100 µg of surfactin and the same effect was observed for *E. coli*. On the other hand, the treatment was completely ineffective against *P. aeruginosa* [94].

The anti-adhesive activity observed for the biosurfactants tested, highlighting the ones produced by *L. agilis* CCUG31450 and *B. subtilis* PX573 in CSL, against the pathogens evaluated indicate their potential usefulness for biomedical applications in the protection of biomaterials, but also to prevent food contamination [177, 178]. Nitschke et al [179] tested biosurfactants with anti-adhesive activity and observed a reduction in the adhesion of food pathogens *L. monocytogenes*, *Enterobacter sakasakii* and *Salmonella enteritidis* on stainless steel and polypropylene surfaces.

3.4.3. Oil recovery

Two chemical surfactants and three biosurfactants were selected to perform oil recovery assays. From all the individual surfactants studied, the best results of oil recovery from contaminated sand were obtained with the chemical surfactant Enordet (54.4 %) and the biosurfactants from *P. aeruginosa* PX112 (produced in CSLM medium) (55.0 %) (Figure 30). On the other hand, at the same concentration, the biosurfactants produced in CSL medium by *B. subtilis* PX573 showed a poorer activity in oil recovery, only 25.1 %, corresponding to half the percentage observed for the most efficient surfactants. The biosurfactants from *L. agilis* CCUG31450 in CWM were also tested, although no oil recovery was observed.

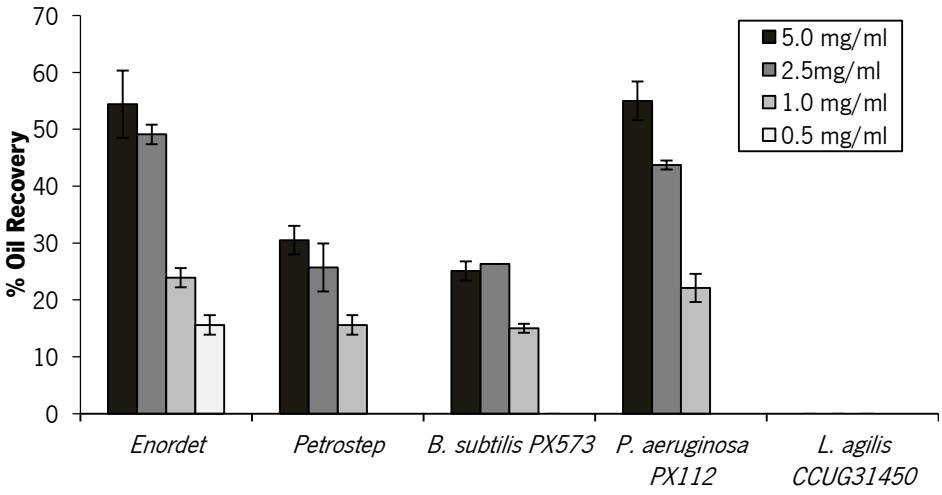

Figure 30. Removal of crude oil from contaminated sand by different biosurfactants and chemical surfactants. Percentages of oil recovered were obtained with freeze-dried biosurfactants isolated from several bacterial strains and surfactants dissolved in demineralized water at different concentrations. Only Enordet and the biosurfactants recovered from *B. subtilis* PX573 were tested at a concentration of 0.5 mg/ml. Results represent the average of two independent experiments and error bars represent SD. In the control conditions (demineralized water) no oil recovery was observed.

Several mixtures with chemical surfactants and biosurfactants were prepared at different proportions and concentrations to study oil recovery. The analysis of the results in Table 28 showed that the mixture yielding the best percentage of oil recovery, 55.0 %, was the one containing biosurfactants produced in alternative media by *B. subtilis* PX573 and *P. aeruginosa* PX112 at an individual concentration of 5 mg/ml. However, when comparing this mixture with the results

obtained for the individual biosurfactants, it was not observed any improvement on the oil recovery. The values obtained for all the concentrations tested for this mixture were very similar to the values observed for the individual biosurfactants from *P. aeruginosa* PX112.

Another mixture that yielded a good oil recovery, 52.0 %, was the one containing the biosurfactant from *B. subtilis* PX573 (1 mg/ml) and Enordet (2.5 mg/ml). However, this mixture did not revealed an enhanced activity when compared with the chemical surfactant alone (49.1 %).On the other hand, analyzing the results obtained for the mixture with the same surfactants, but at different concentrations (*B. subtilis* PX573 (0.5 mg/ml) and Enordet (1 mg/ml)), an interesting and different effect was observed. The effect of this mixture in oil recovery was enhanced (31.1 %) when compared with the effect of Enordet alone (23.9%), although the individual biosurfactants from *B. subtilis* PX573 at this concentration (0.5 mg/ml) did not show any ability to recover oil from the contaminated sand. Another example of a positive interaction between biosurfactants was observed for the mixture of biosurfactants from *B. subtilis* PX573 (2.5 mg/ml) and Enordet (1 mg/ml). As can be seen, the mixture showed an enhanced activity, 42.5 %, when compared with the individual activity of the biosurfactants from *B. subtilis* PX573, 26.3 %, and Enordet, 23.9 %. The other two combinations of *B. subtilis* PX573 and Enordet tested also resulted in an increased percentage of oil recovered.

Each mixture has an interaction between their biosurfactants that can be very distinct and unique. A positive interaction could also be observed (23.9 % oil recovery) for the biosurfactants from *B. subtilis* PX573 (1 mg/ml, 15.0 %) and the surfactant Petrostep (1 mg/ml, 15.6 %). On the other hand, the other combinations tested with these surface active compounds did not resulted in a positive effect. For the three different mixtures of biosurfactants from *B. subtilis* PX573 and from *P. aeruginosa* PX112, no increase in the oil recovery could be observed. Moreover, the mixtures of biosurfactants from *P. aeruginosa* PX112 and the chemical surfactants resulted in similar and low amounts of oil recovered.

Table 28. Removal of crude oil from contaminated sand by different biosurfactants and chemical surfactants mixtures. Percentages of oil recovered were obtained with freeze-dried biosurfactants isolated from several bacterial strains and surfactants mixtures dissolved in demineralized water at different proportions. In the control assays (demineralized water) no oil recovery was observed. Results represent the average of two independent experiments ± SD.

Mixtures	Surfactant concentration (mg/ml) A : B	% Oil Recovery (A)	% Oil Recovery (B)	% Oil Recovery (Mixture)
***B. subtilis* PX573 : *P. aeruginosa* PX112**	5.0 : 5.0	25.1 ± 1.7	55.0 ± 3.4	55.0 ± 6.8
	2.5 : 2.5	26.3 ± 0.0	43.7 ± 0.8	43.7 ± 4.2
	1.0 : 1.0	15.0 ± 0.8	22.1 ± 2.5	21.5 ± 3.4
***B. subtilis* PX573 : Enordet**	1.0 : 1.0	15.0 ± 0.8	23.9 ± 1.7	32.9 ± 2.5
	1.0 : 2.5	15.0 ± 0.8	49.1 ± 1.7	52.0 ± 2.5
	2.5 : 1.0	26.3 ± 0.0	23.9 ± 1.7	41.9 ± 5.6
	0.5 : 1.0	0.0 ± 0.0	23.9 ± 1.7	31.1 ± 3.4
	1.0 : 0.5	15.0 ± 0.8	15.6 ± 1.7	28.1 ± 2.5
***P. aeruginosa* PX112 : Enordet**	1.0 : 1.0	22.1 ± 2.5	23.9 ± 1.7	21.5 ± 0.0
***B. subtilis* PX573 : Petrostep**	1.0 : 1.0	15.0 ± 0.8	15.6 ± 1.7	23.9 ± 3.4
***P. aeruginosa* PX112 : Petrostep**	1.0 : 1.0	22.1 ± 2.5	15.6 ± 1.7	15.0 ± 2.5

The study of the efficiency in oil recovery by chemical surfactants and biosurfactants has also been reported by other researchers. Pornsunthorntawee et al. [99] showed that biosurfactants from *B. subtilis* PT2 had a greater efficiency in oil recovery than those produced by *P. aeruginosa* SP4, suggesting that the two excreted biosurfactants should have different characteristics, which are probably governed by the sources of the bacteria. On the contrary, the results obtained in this work showed that the biosurfactants from *P. aeruginosa* PX112 were more efficient than the ones from *B. subtilis* PX573. The results suggested that probably the *P. aeruginosa* PX112 biosurfactants could be more compatible with the Arabian Light oil tested. As previously discussed, the emulsifying activity of biosurfactants from *P. aeruginosa* PX112 (Figure 12) was higher compared with the values obtained for the biosurfactants from *B. subtilis* PX573 (Figure 7), suggesting that the *P. aeruginosa* PX112 biosurfactants are more effective in removing oil from the sand and promoting oil solubilization.

Pornsunthorntawee et al. [99] showed that biosurfactants could recover oil more effectively than three synthetic surfactants (Tween80, SDBS, Alfoterra 145-5PO). In this study, we saw that the *P. aeruginosa* PX112 biosurfactants were equally effective as Enordet and more effective than Petrostep (Figure 30). The higher ability of these biosurfactants to solubilize oil could be explained by their hydrophobicity. Moreover, being an anionic biosurfactant, its negative charge on the head group is able to aid the repulsion between the attached oil droplets and the sand surface, leading to the detachment of the oil from the sand surface. Briefly, on one hand the hydrophobicity of biosurfactants improves the solubilization of the oil, and on the other hand their anionic nature promotes the detachment of oil from sand.

The two biosurfactants tested gave interesting values of oil recovery, either individually or in combination with chemical surfactants, suggesting that they have a great potential for application in MEOR, as well as in oil removal from contaminated places.

4. GENERAL CONCLUSIONS AND RECOMMENDATIONS

4.1. Conclusions

The aim of the present work was to prepare mixtures of biosurfactants produced by different microorganisms in order to evaluate their main characteristics (surface tension, emulsification ability, antimicrobial and anti-adhesive activities) for several applications, namely in the oil recovery, health care and biomedical fields. To reach these purposes several subjects were studied and different applications were positively tested. The main conclusions drawn from this work are the following:

- From the several bacteria tested, *L. agilis* CCUG31450, *P. aeruginosa* PX112 and *B. subtilis* PX573 were found to be the best biosurfactants producers.

- The optimization of environmental factors showed that an increase on the agitation up to 200 rpm improved the biosurfactants production by *B. subtilis* PX573 and *P. aeruginosa* PX112, probably due to a higher oxygen transfer.

- Low-cost media, prepared with molasses and CSL, proved to be suitable alternatives to synthetic media for the production of biosurfactants by *B. subtilis* PX573 and *P. aeruginosa* PX112, leading to a remarkable increase in the biosurfactant yields of 2.8 and 2.5 times, respectively.

- The supplementation of the low-cost media with trace elements, Fe^{2+}, Mn^{2+} and Mg^{2+}, showed to be a beneficial for the production of biosurfactants by *B. subtilis* PX573, most probably because these trace elements play a key role as co-factors in the enzymes that are involved in the biosurfactants synthesis.

- The conjugation of acid precipitation with a second recovery methodology, mainly ammonium sulfate precipitation, led to an improvement of the biosurfactants recovery from the culture broth supernatants.

- Growth of *L. agilis* CCUG31450 under aerobic conditions demonstrated to be the most favorable condition for the synthesis of this type of biosurfactants.

- The low-cost medium prepared with cheese whey for biosurfactant production by *L. agilis* CCUG31450, although conducting to an increase of the biosurfactants yield, did not led to a further decrease of the surface tension as compared to the biosurfactants being produced in MRS medium.

- The biosurfactants herein studied showed, in general, a capacity to withstand extreme conditions of pH, salinity and temperature without losing their physical and chemical properties. Therefore, they are promising molecules to be used in processes as bioremediation of hydrocarbon-contaminated sites or in the petroleum industry (MEOR).

- The evaluation of the antimicrobial activity of the individual biosurfactants revealed that the lactic acid bacteria, *L. animalis* ATCC35046, *L. paracasei* A20 and *L. agilis* CCUG31450, exhibit a common positive effect against *S. aureus*. Besides, *L. agilis* CCUG31450 was the lactic acid bacteria with the greatest range of antimicrobial activity, being effective against three out of five pathogenic strains tested. The biosurfactants produced by *B. subtilis* PX573 and *P. aeruginosa* PX112 in low-cost media, demonstrated to be, in general, more effective against the microorganisms tested, supporting the positive replacement of synthetic media by the alternative media suggested.

- In summary, the biosurfactants with a more pronounced activity evidenced a stronger antimicrobial activity against Gram-positive bacteria than against the Gram-negative ones, probably due to their easier interaction with Gram-positive cell membrane leading to the loss of cell integrity. Moreover, biosurfactants produced by *B. subtilis* PX573 and *P. aeruginosa* PX112, besides their notable activity against Gram-positive bacteria; were also found to be active against the yeast strain used. Therefore, these biosurfactants can be used as antimicrobial agents or can be conjugated with antibiotics to enhance their activity and reduce the resistance of pathogenic microorganisms. Nevertheless, some rhamnolipids have been reported as virulence factors, thus further work should be conducted in order to understand if these rhamnolipids are adequate for given applications.

- Biosurfactants are pointed as an alternative to antibiotic treatments, being effective against multi-resistant microorganisms, shush as *S. aureus*. The mixture of several biosurfactants, depending on the biosurfactants and pathogenic organism evaluated, showed either synergistic or antagonistic effects. The most interesting synergistic effect observed was for the mixture of *L. paracasei* A20 with *L. animalis* ATCC35046 biosurfactants.

- Biosurfactants usefulness as anti-adhesive agents was also assessed against two pathogenic strains. In the case of *S. aureus*, the most efficient individual biosurfactant was the one produced by *L. agilis* CCUG31450 (MRS), and the best results of anti-adhesive activity (68.4 %) were detected for the mixture (1:1 proportion) prepared with biosurfactants from *L. agilis* CCUG31450 and *B. subtilis* (CSL). For the pathogenic strain *P. aeruginosa*, the higher adhesion inhibition was achieved with the individual biosurfactants of *L. agilis* CCUG31450 (CWM), and the mixture (1:2 proportion) of biosurfactants from *P. aeruginosa* PX112 (CSLM) and *L. agilis* CCUG31450 (MRS), 62.5 %. In conclusion, each mixture prepared showed a unique effect on the adhesion of pathogenic microorganisms that was dependent on the type of biosurfactants, the proportion of biosurfactants in the mixture and the pathogenic organism.

- The anti-adhesive activity observed, mostly for probiotic biosurfactants, suggests their application as bio-detergent solutions for cleaning surgical material, and for coating agents to increase prosthesis and catheters lifetime, thus reducing health costs.

- The good surface and emulsifying activity of biosurfactants from *B. subtilis* PX573 and *P. aeruginosa* PX112 indicated their potential to be applied in oil recovery. The results from the oil recovery assays showed that these biosurfactants were efficient, and in some case were even more active than the two chemical surfactants (Enordert and Petrostep) tested. From all the mixtures prepared with biosurfactants and chemical ones, the higher oil recovery percentage (55 %) was obtained for the mixture containing biosurfactants produced in alternative media by *B. subtilis* PX573 and *P. aeruginosa* PX112, at an individual concentration of 5 mg/ml. An interesting result was detected for the mixture of *B. subtilis* PX573 biosurfactants (0.5 mg/ml) and Enordet (1 mg/ml), The effect of

this mixture in oil recovery was enhanced (31.1%) when compared with the effect of Enordet alone (23.9%), although the individual biosurfactants from *B. subtilis* PX573 at this concentration (0.5 mg/ml) did not show any ability to recover oil from the contaminated sand. Therefore, biosurfactants could be applied in MEOR or could be mixed with chemical surfactants already used in oil recovery in order to reduce the concentrations required of these surfactants, and consequently reduce their negative environmental impacts.

4.2. Recommendations

The results achieved in this work showed the successful implementation of some strategies to improve biosurfactants yields and reduce production costs. Moreover, it was given an interesting perspective of the properties and activities of distinct biosurfactants, as well as their potential to be used in the therapeutic, biomedical and oil recovery field. However, further research should be done as suggested bellow:

- Since the results achieved in the production of biosurfactants by *B. subtilis* PX573 and *P. aeruginosa* PX112, using low-cost substrates as medium for fermentation, were very promising, it would be interesting to study the fermentation scale-up, reactor design and mode of operation.

- In the case of *L. agilis* CCUG31450 growth in CWM, the results were not as favorable as predicted, i.e. the biosurfactants produced in this medium showed a poor activity. Therefore, more studies should be conducted in order to optimize the cheese whey concentration in the alternative medium, the requirement of trace elements, or even test different low-cost substrates.

- The strategies applied to reduce the production costs, namely the use of low-cost substrates, provided encouraging results. Nevertheless, other strategies involving different variables of the production process can also be explored, as for example the assessment of overproducing strains.

- The effect of environmental factors in the biosurfactants activity was tested distinctly, without considering the effects of any interactions between them. In future research, the effect of extreme conditions of pH, temperature and salinity should be evaluated also for the mixtures.

- Another point of interest is the biochemical composition of biosurfactants. In general, they have been characterized as multi-components biosurfactants. Therefore, further purification and characterization steps are required, mainly in the case of biosurfactants produced by the lactic acid bacteria, for which information on their structures is still scarce. This characterization of biosurfactants, in terms of structure, would help to better understand their interaction in the mixtures, thus facilitating the prediction of the mixture effect and activity.

- In the biomedical and therapeutic applications, most of the time it is desirable an agent that is active against more than one pathogenic microorganism. Therefore, it would be worth to test the antimicrobial and anti-adhesive activity of biosurfactants against microbial consortia. Moreover, several pathogenic strains are biofilm forming-microorganisms which could interfere with the antimicrobial effect of biosurfactants. The analysis of anti-biofilm activity of the biosurfactants will provide a new overview of the biosurfactants effectiveness.

- Biosurfactants showed interesting antimicrobial and anti-adhesive activities indicating their potential application in the medical field. Nevertheless, the therapeutic application of these biosurfactants, produced by different bacteria, requires an evaluation of their biocompatibility before they can be considered for use in health-related areas.

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