Universidade de Aveiro Departamento de Biologia
Ano 2016

Ana Rita da Rocha Alves

Aumento da produção de biossurfactantes por co-cultivo de bactérias produtoras com estirpes formadoras de biofilmes

Increase of biosurfactant production by co-cultivation of producing bacteria with biofilm forming strains

Universidade de Aveiro Departamento de Biologia
Ano 2016

Ana Rita da Rocha Alves

Aumento da produção de biossurfactantes por co-cultivo de bactérias produtoras com estirpes formadoras de biofilmes

Increase of biosurfactant production by co-cultivation of producing bacteria with biofilm forming strains

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia realizada sob a orientação científica da Professora Doutora Maria Ângela Sousa Dias Alves Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus pais

o júri

presidente	Doutora Isabel da Silva Henriques Investigadora auxiliar, Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro
vogal	Doutor Francisco José da Costa Riso Coelho Investigador de pós doutoramento, Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro
vogal	Prof. Doutora Maria Ângela Sousa Dias Alves Cunha Professora auxiliar, Universidade de Aveiro

agradecimentos

Em primeiro lugar, aos meus pais pelo apoio e amor incondicional que recebo diariamente, especialmente nesta fase. Este trabalho não teria sido possível sem vocês. Obrigada por serem os melhores pais do mundo.
À professora Ângela por toda a ajuda, compreensão e disponibilidade durante o meu trabalho no laboratório.
Ao CESAM pelo financiamento indispensável para a realização deste trabalho.
À professora Helena Silva e à Ana Carla Gonçalves pela disponibilização da lupa e do laboratório.
Ao Hungarian National Collection of Medical Bacteria por cederem a estirpe de *C. violaceum* indispensável para este estudo.
Aos meus colegas de laboratório pela ajuda e pelos imprescindíveis momentos de descontração e companheirismo que me proporcionaram ao longo deste ano.
À Ana Marta Sequeira, companheira incansável neste trabalho e sem a qual não o poderia ter realizado.
À minha família, por ser uma fonte incondicional de apoio e carinho em toda a minha vida e em especial à Bia e ao Gil por serem os irmãos que nunca tive.
E finalmente a todos os meus amigos, que estiveram comigo estes anos todos em Aveiro. Obrigada por todos os bons momentos, obrigada pela amizade e pela ajuda, pela compreensão e por todas as memórias que levo comigo. Foram os melhores anos da minha vida, e foram graças a vocês, Diana, Inês, Lídia, Milagros, Arminda, Vanessa, Ana João, Décio, Rafa, Fábio, Tiago, Sá, Basco

palavras-chave *Pseudomonas*; *Bacillus licheniformis*; estirpes indutoras; ramnolipidos; surfactina

resumo

Os biossurfactantes (BSF) são compostos anfipáticos produzidos por uma vasta gama de microorganismos. São capazes de reduzir a tensão superficial e interfacial, bem como emulsificar e transportar substratos hidrofóbicos, regular a aderência de células a superficies e interferir com o desenvolvimento de biofilmes. Os BSF encontram aplicações nas mais diversas áreas, nomeadamente nas indústrias alimentar e do petróleo, no controle de biofilmes microbianos e na prevenção da biocorrosão, biofouling e desenvolvimento de biofilmes. são ainda usados como ingredientes em produtos terapêuticos, de cuidado pessoal e cosméticos.

Os BSF representam uma alternativa vantajosa aos surfactantes químicos porque são menos tóxicos, mais biodegradáveis e estáveis em temperaturas e pH extremos. Contudo, a sua produção e aplicação é limitada devido pelo baixo rendimento do processo produtivo e pelos elevados custos de produção. Considerando que os BSF são metabolitos secundários, a hipótese de que a o co-cultivo com estirpes produtoras de biofilme (indutoras) estimula a síntese de BSF foi testada. Estirpes de *Bacillus licheniformis* e *Pseudomonas* sp produtoras de BSF foram co cultivadas com estirpes indutoras (*Pseudomonas aeruginosa* e *Listeria innocua*) de maneira a estimular a produção de surfactina e ramnolípidos, respetivamente. As culturas axénicas e co-culturas foram testadas quanto ao efeito tensioativo, pelo método do oil spray, cultivo em meio CTAB-azul de metileno e cultivo em meio agar de sangue. O método CPC-BTB foi usado para quantificação de surfatina e ramnolípidos, ambos surfactantes aniónicos, e o método de orcinol foi usado para a quantificação de ramnolípidos. O efeito das culturas e co-culturas na inibição de quorum sensing em *Chromobacterium violaceum* foi também avaliada.

Os resultados do método CPC-BTB indicaram que a estimulação de produção de BSF em *Pseudomonas* #74 foi maior em co-cultura com *L. innocua*. Os resultados do teste do CTAB-azul de metileno indicaram que a produção de BSF em *B. licheniformis* foi mais estimulada em co-cultivo com *P. aeruginosa*. Não se verificaram diferenças significativas no efeito tensioativo avaliado pelos métodos do oil spray, colapso da gota cultivo emagar de sangue, nem pelo método quantitativo do orcinol.

Os resultados indicam que o co-cultivo afecta a concentração de BSF, embora não existam diferenças no efeito tensioactivo, e que um maior rendimento de produção pode ser obtido através da seleção das estirpes indutoras adequadas.

Keywords *Pseudomonas*; *B. licheniformis*; inducer strains; rhamnolipids; surfactin

abstract

Biosurfactants (BSF) are amphipathic compounds, produced by a vast range of microorganisms. They are able to reduce surface and interfacial tensions, as well as to emulsify and transport hydrophobic substrates, to regulate cell adherence to surfaces and to interfere biofilm development.

BSF can have applications in diverse areas, such as petroleum and food industries, control of biofouling and biofilm development, and are also used as ingredients in therapeutic formulations, personal care products, and cosmetics. They represent an advantageous alternative to chemical surfactants because they less toxic, highly biodegradable and stable in extreme temperature and pH. However, mass production and application of BSF is still limited by the low production yield and high production costs.

Considering that BSF are secondary metabolites, the hypothesis that co-cultivation with biofilm-forming strains would induce BSF synthesis was tested. BSF producing strains of *Bacillus licheniformis* and *Pseudomonas* sp. were cultivated with inducing strains (*Pseudomonas aeruginosa* and *Listeria innocua*) as a way to stimulate the production of surfactin and rhamnolipids, respectively.

Axenic cultures and co-cultures were tested as to the tensoactive effect by the oil spray method, cultivation in CTAB-methylene blue medium, cultivation in blood agar, and the drop collapse assay. The CPC-BTB method was used for the quantification of surfactin and rhamnolipids, both anionic surfactants, and the orcinol method for the quantification of rhamnolipids. Effect of the cultures and co-cultures in the quorum sensing inhibition in *Chromobacterium violaceum* culture was also evaluated.

Results of the CPC-BTB test indicate that the stimulation of BSF production by *Pseudomonas* sp. was the highest in co-cultures with *Listeria innocua*. The results of the CTAB-methylene blue test indicate that BSF production in *B. licheniformis* was more stimulated by co-cultivation with *P. aeruginosa*. There were no significant differences in the tensoactive effect of the cell-free extracts as determined by oil spray, the drop-collapse test or by the blood-agar test. The results indicate that co-cultivation affects the concentration of BSF, although it does not have an effect in the tensioactive effect of the BSF, and that higher production yields can be attained by selecting convenient inducer strains.

Table of Contents

List of figures v

List of tables vii

List of Abbreviations ix

1. Introduction 3

1.1. Surfactants and Biosurfactants 3

1.2 Surfactant producing microorganisms 5

1.3. Regulation of biosurfactant production 6

1.4 Methods for detection and quantification of biosurfactants 7

1.5. Classification of bacterial surfactants 9

1.6. Applications of biosurfactants and biosurfactant-producing microorganisms 12

1.7. Co-cultivation as a tool to enhance BSF production 14

1.8. Objectives 16

2. Methods 19

2.1. Bacterial cultures and culture media 19

2.2 Growth time of cultures and co-cultures 20

2.3. Quantification and detection of biosurfactants 20

2.3.1. Oil spray 20

2.3.2. Drop collapse assay 21

2.3.3. Blood agar hemolysis 21

2.3.4. CTAB-methylene blue agar 22

2.3.5. CPC-BTB 22

2.3.6. Orcinol method 22

2.3.7 Quorum sensing inhibition 23

3. Results and Discussion 27

3.1. Growth times of cultures and co-cultures 27

3.2. Oil spray 28
3.3. Drop Collapse assay.....31
3.4. Blood agar hemolysis 33
3.5. CTAB-methylene blue agar 35
3.6. CPC-BTB 38
3.7. Orcinol method 40
3.8 Quorum sensing inhibition 41
3.9 Comparison between the different methods of detection/quantification.....43
4. Conclusion 49

List of figures

Figure 1.Typical biossurfactant structure adapted from Santos et. al(2016)..... 4

Figure 2. Micelles structure adapted from Malik et. al (2011) 4

Figure 3. Structures of a) monorhamnolipid b) di-rhamnolipid adapted from Nguyen and Sabatini 2011..... 10

Figure 4. Structure of surfactin adapted from Nitschke e Pastore (2002) 11

Figure 5. Variation of the optical density of axenic and co-cultures *Pseudomonas* sp. P- *Pseudomonas* #74; P+L – *Pseudomonas* #74 + *Listeria Innocua*; P+P – *Pseudomonas* #74 + *P. aeruginosa*..... 27

Figure 6. Variation of the optical density of the axenic and co-cultures of *B. licheniformis*. B- *B. licheniformis*; B+L – *B. licheniformis* + *Listeria innocua*; B+P – *B. licheniformis* + *P. aeruginosa*. 28

Figure 7. Results of the drop collapse assay performed on axenic and co-cultures . 31

Figure 8.Example of plates corresponding to the blood agar hemolysis method performed on axenic cultures and co-cultures of B. licheniformis or Pseudomonas #74, and controls 33

Figure 9. Example of plates corresponding to the CTAB-methylene blue test performed on axenic cultures and co-cultures of *B. licheniformis* or *Pseudomonas #74*, and controls. 38

Figure 10. Concentration of biosurfactant (CPC-BTB assay) 39

Figure 11. Rhamnolipid concentration (orcinol assay). 40

Figure 12. Example of plates corresponding to quorum sensing inhibition performed on axenic cultures and co-cultures of *B. licheniformis* 43

Figure 13. Example of plates corresponding to quorum sensing inhibition assay performed on axenic cultures and co-cultures of *Pseudomonas #74* 43

List of tables

Table 1. Biossurfactants and their producers adapted from *Nitschke and Pastore (2002)* 6

Table 2. Semi-quantification of the halos produced by axenic cultures and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*) in the oil spray assay 29

Table 3. Results of the blood agar hemolysis assay on axenic and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*)..... 34

Table 4. Semi-quantitative analysis of anionic surfactants in axenic cultures and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua*, B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*) 36

Table 5. Qualitative results of the quorum sensing inhibition assay in axenic cultures and co-cultures of *Pseudomonas #74* and *Bacillus lichenoformis* 42

Table 6. Summary of the different methods used for the detection/ quantification of BSFs in axenic and co-cultures 44

List of Abbreviations

AHL – Acylated homoserine lactones

BSF – Biosurfactants

BTB – Bromotimol blue

CFE – Cell free extracts

CMC – Critical micelle concentration

CPC – Cetylpiridinium chloride

CTAB – Cetyltrimethylammonium Bromide

LB – Luria Bertani

MEOR – Microbial enhanced oil recovery

OD – Optical density

SD – Standard deviation

QS – Quorum-sensing

SDS – Sodium dodecyl sulfate.

TSA – Tryptic soy agar

TSB – Tryptic soy broth

Introduction

1. Introduction

1.1. Surfactants and biosurfactants

Chemical surfactants have had a major role in everyday life for more than a century, although their commercial success has only been increasing over the last few decades. It has become a quickly evolving and profitable field, related with innumerous applications such as detergency and emulsification, biofilm control, health and personal care products and environmental restoration and protection(1–6).

Surfactants, or surface active agents, are amphiphilic molecules with hydrophobic and hydrophilic groups that allows them to interact with different gas, solid and liquid interfaces. They are capable of reducing surface and interfacial tension, as well as to emulsify immiscible fluids (2,3,7). Surface tension refers to the force that is wielded by a liquid solution when interacting with a solid or liquid. Interfacial tension is mainly the intermolecular force exerted by the molecules of liquid, when the interfacial tension of a solution is low, it can be easily emulsified (8,9)

The rapid rise of the surfactant market in recent years, and the fact that 70-75% of the surfactants currently commercialized in developed countries have a petrochemical source, has triggered an eager search for more viable and environmentally friendly alternatives (2,3,10).

Biosurfactants (BSFs) are surfactants produced extracellularly, or as a cell membrane component, by a wide range of microorganisms such as bacteria, yeasts and fungi (11,12). They are usually secondary metabolites, produced at the end of the exponential phase or the beginning of the stationary phase. Structurally, they have similarities with their chemical counterparts, containing hydrophobic and hydrophilic domains. The hydrophobic tail is composed mainly of acids, peptides or polysaccharides, while the hydrophilic moiety comprehends mostly hydrocarbon chains or fatty acids (6,8,13).The typical BSF structure can be observed in figure 1

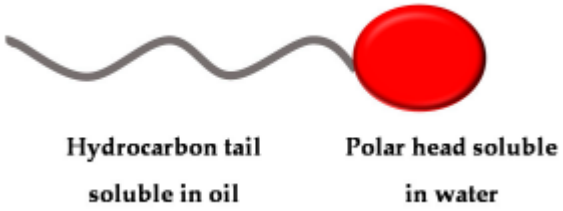

Figure 1 Typical biosurfactant structure. Adapted from Santos *et al. (2016)*

In liquid solutions, BSFs can aggregate and form structures known as micelles, in which the hydrophobic groups are protected from contacting with the aqueous phase. Micelles can have various forms, depending on the BSF, and are affected by temperature, pressure and presence of electrolytes. The concentration value at which micelles are formed is denominated critical micelle concentration (CMC) and it corresponds to the value at which the ability of the BSF to reduce surface tension is considered efficient. Lower CMC values, usually mean a higher efficiency of the BSF (3). The general structure of a micelle structure can be observed in figure 2.

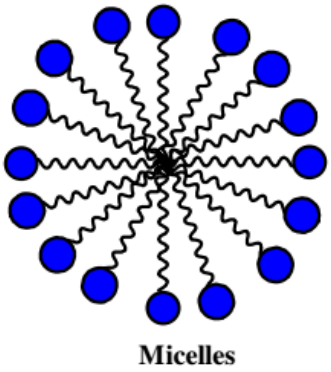

Figure 2. Micelles structure adapted from Malik *et. al* (2011)

BSFs present several advantages over chemical surfactants, such as low toxicity, higher biodegradability and higher stability in extreme conditions of temperature, pH and salinity. They can also be synthesized from renewable sources such as ethanol, glycerol, vegetable oils and wastewater (1,7,11).

However, the cost of their production at an industrial scale is still very high, and so, there has been an increasing search for optimization in the cultivation processes of BSF producing microorganisms, in an effort to increasing their production yield and lower their production cost (12,14).

1.2. Surfactant producing microorganisms

Bacteria are known to exhibit physiological and chemical adaptations when presented with extreme conditions or stress. In some of those cases, they produce secondary metabolites, such as BSFs (15). BSFs can reduce surface and interfacial tension and solubilize hydrocarbons, which facilitates the microorganism growth in those substrates. BSFs can also interfere in the regulation of bacterial population density and cell to cell communication (2,11,16). BSF producing bacteria are able to inhabit the most diverse habitats, whether terrestrial, aquatic or extreme conditions. They are most likely found in areas with high levels of hydrocarbons or organic residues, such as industrial zones, oil reservoirs, petroleum exploration areas and oil contaminated site (17–22)

Some *Bacillus, Enterobacter and Pseudomona*s strains have been shown to grow and produce BSF successfully in extreme environmental conditions, with temperatures ranging from 55-70 ºC, as well as high levels of salinity, from 10-15 % (23,24). Recently, some *Pseudomonas* strains originating from floral nectar, a sugar-rich matrix, were observed to thrive and produce BSF, that further demonstrates the ability of BS-producing microorganisms to inhabit the most unusual microniches (25).

While bacterial production of BSFs is already an extensively studied field, there are still few fungi reported to produce BSFs. Some *Candida* strains, as well as *Aspergillus ustrus, Ustilago maydis and Rhodococcus erythropolis* are some of the few reported to produce (26–29). Table 1 presents some of the most common BSF producing microorganisms and their respective BSF.

Table 1 – Biossurfactants and their producers. Adapted from Nitschke and Pastore (2002) (6).

Surfactant type	Microorganism (example)
Glycolipids • **Rhamnolipids** • **Shoforolipids** • **Trehalolipids**	 *Pseudomonas aeruginosa* *Torulopsis bombicola* *Rhodococcus erythropolis*
Lipopeptides e Lipoproteins • **Surfactin** • **Viscosin** • **Subtilisin**	 *Bacillus subtilis* *Pseudomonas fluorescens* *Bacillus subtilis*
Fatty acids and phospholipids	*Corynebacterium lepus; Thiobacillus thiooxidans*
Polymeric surfactants • **Emulsan** • **Liposan**	 *Acinetobacter calcoaceticus* *Candida lipolytica*
Particulate surfactants	*Acinetobacter calcoaceticus*

1.3. Regulation of biosurfactant production

The production of BSF is, to some extent, depending on cell-to-cell communication mediated by chemical signals, a process designated by quorum sensing (QS). QS is the regulation of gene expression, as a result of changes in the

cell population density and it was first described in the marine Gram negative bacteria bioluminescent bacteria *Vibrio fischeri* and *Vibrio harveyi* (30). Bacteria produce and release chemical molecules, known as autoinducers, that increase in concentration as the cell population density also increases. When a minimal threshold concentration of autoinducers is reached, bacterial gene expression is altered and a change in phenotype occurs. QS communication is known to control some physiological activities such as symbiosis, virulence, competence, antibiotic production and biofilm formation (31). Both Gram positive and gram negative bacteria are capable of QS, although using different systems to do so. Generally, Gram positive bacteria use oligopeptides as autoinducers and gram negative bacteria use acylated homoserine lactones (32,33)

Some molecules, known as quorum quenchers, are known to interfere in the QS process and can, therefore, be used to inhibit this intercellular communication between bacteria. An example of this quorum quenching effect, is the synthesis of the purple pigment violacein in *Chromobacterium violaceum* that is regulated by QS. As a result of the inhibition of QS, the violet phenotype of the colonies is lost (34).

1.4. Methods for detection and quantification of biosurfactants

There are a number of different methodologies to detect and quantify the production of BSF and they can be divided into indirect methods, colorimetric methods and molecular methods.

The indirect methods rely mostly in the physical properties of the BSFs and may or may not be quantitative.

- Drop collapse assay: an easily performed method where a drop of cell suspension or surfactant sample is placed on an oil/water surface. The drops containing BSF will collapse, while the BSF free ones will continue to be stable (35). It is a semi-qualitative method and the diameter of the final drop allows,

indirectly, to calculate the BSF concentration. Nevertheless, it can lead to false negatives, if the producing strain produces a low amount of BSF (35,36).

- Oil spray method: an expedite assay involving atomized oil, that allows for the detection of BSF produced by colonies developing on solid culture medium. Using an airbrush, the colonies are sprayed with a fine layer of oil and BSF can immediately be detected by the formation of halos around producing colonies. This method may be performed on multiple colonies at the same time, and permits the detection of lower BSF concentrations than the drop collapse assay (35,37).
- Hemolysis: In a solid medium containing 5% blood, BSF production can be detected by the partial or total hemolysis of red blood cells. This hemolytic activity is due to the ability of BSF to disrupt the cell membrane (38). However, this method is considered unreliable, as hemolysis may be the result of enzymes, such as lysozyme and, on the other hand, not all biosurfactants display hemolytic activity (39,40).
- Emulsifcation index: This method is performed by adding hydrocarbons to the BSF producing bacteria culture. The culture is then centrifuged and laid to rest for 24h. If there is presence of BSF, a layer between the hydrocarbon and the liquid culture will appear. Then, the emulsification index is calculated by dividing the height of the emulsification layer by the total height (41).

Colorimetric methods are based on the binding of a dye to the BSF or a specific part of the BSF molecule. They are specific for different BSF groups.

- CTAB-methylene blue: A test that is mostly used for the detection of rhamnolipids, it is based on the binding of the rhamnolipids (anionic surfactants) to Cetyl trimethylammonium bromide CTAB (cationic surfactant), in the presence of methylene blue, as a color indicator (42,43). The appearance of dark halos around in the cultures, in TSA containing CTAB and methylene blue, confirms the presence of the BSF and the halos diameter can be measured to give a relative concentration of BSF. However, the halos can

be affected by various factors like incubation time, temperature, rhamnolipids migration form the cell and the cellular growth phase (44).

- CPC-BTB: This is an innovative highly precise method, that is used to quantify surfactin in high production yield cultures (surfactin concentration around 100-500mg/L). A green complex, formed by CPC (cetilpiridinium chloride), as a mediator, and BTB (bromotimol blue), as color indicator is used and in the presence of surfactin, CPC is removed from the CPC-BTB complex, creating a chromatic shift from light green to dark green and blue. This is due to the free BTB molecules, after release from the CPC-BTB complex. The optical density may also be observed (600 nm) for quantification of BSF (45).
- Orcinol method: This is the standard method to specifically quantify rhamnolipids. It is based on the extraction of the BSF and consequently acidic hydrolysis with a orcinol reagent (3-5 dihydroxitoluene and sulfuric acid), in order to release rhamnose, which is later quantified by spectroscopy (OD 420 nm) (39,46).

Molecular methods are usually based on chromatography and spectrometry. They allow the quantification, purification and identification of the BSF structure. Although they are highthroughput methods, they present high costs (9,46,47).

Chromatography methods rely on chromatic separation of the BSF and are usually coupled with mass spectrometry. The most used chromatography in BSF identification is the high performance liquid chromatography (HPLC), which is the most efficient method for BSF identification and quantification (39).

Spectrometry methods are usually mass spectrometry, that can be used without a previous chromatography (46).

1.5. Classification of bacterial surfactants

Bacterial surfactants are classified accordingly to their chemical structure, microbial origin and molecular weight (2,7). Based on their chemical composition,

they are distributed in 4 classes: glycolipids; lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric and particulate surfactants (2,11,48). According to their molecular weight, they can divided in biosurfactants and bioemulsifiers. Biosurfactants generally have low molecular weight and are capable of lowering surface and interfacial tension. Bioemulsifiers refer mainly to high molecular weight BSFs, and have a major role in the binding and detachment of bacteria to surfaces as well as emulsification of hydrocarbons (8,9,49). Biossurfactants are usually able of emulsification as well. Bioemulsifiers, however, do not typically reduce surface and interfacial tension (50).

Glycolipids (rhamnolipids) and lipopeptides (surfactin and liquenisin) are two of the most important and well characterized classes of bacterial biossurfantants. Rhamnolipids are BSFs of the glycolipid family. Their production was first described in *P. aeruginosa* and they are comprised by one or two molecules of L-rhamnose linked to one or two monomers of β-hydroxy fatty acid, as shown in figure 1 (2,10,51). There are at least 28 rhamnolipid homologues, with four of them (R1, R2, R3 e R4) being the most predominant. A typical structure of a mono and di-rhamnolipid can be observed in figure 4. The homologues differ mainly in the concentration of rhamnose molecules (one or two) and in the length of fatty acid chain (between 8 and 12 carbons) (52).

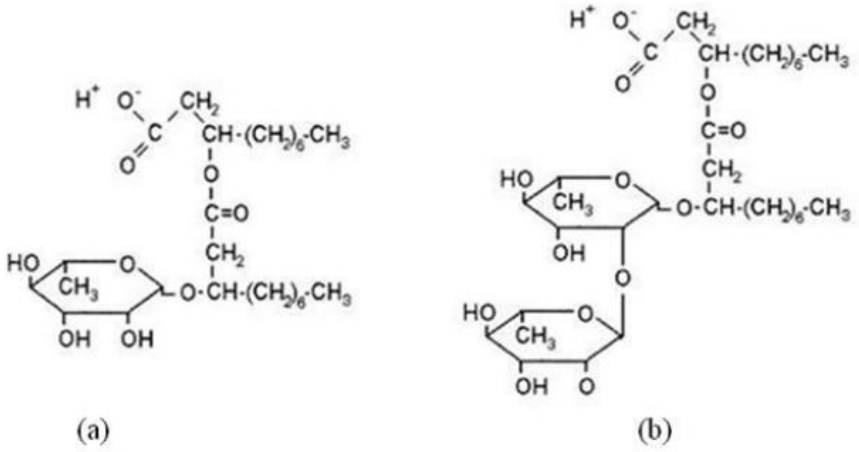

Figure 3 Structures of a) monorhamnolipid b) di-rhamnolipid adapted from Nguyen *et. al* (53)

Rhamnolipids play various roles in the physiology of the producing microorganism. Their main functions appear to be the solubilization and the uptake of hydrocarbons, antimicrobial activity, as to compete with neighboring microorganisms and mediation in cell attachment and detachment to surfaces (10,20,54). Rhamnolipid production is dependent on quorum sensing signaling and in extreme conditions, such as lack of nutrients, there is an increasing production of rhamnolipid, which can then be used as nutrients themselves (55,56). One of the main concerns in the rhamnolipid production remains the fact that the one of the most prominent producers, *P. aeruginosa*, includes highly pathogenic strains. However, recent studies have demonstrated that different *Pseudomonas* species, such as *P. putida* and *P. chlororaphis* are also able to produce rhamnolipids (20,56).

Surfactin and lichenisin are BSFs from the lipopeptide family and are mainly produced by *Bacillus* species. They are known to interfere in the attachment/detachment of the microorganism to surfaces, due to their ability to alter the hydrophobicity of the bacterial membrane (57,58). Surfactin is a cyclic lipopeptide, containing seven aminoacids linked to a β-hydroxy fatty acid chain by a lactone bridge, as shown in figure 4. Surfactin exhibits remarkable surficial activity, and it is capable of lowering the water surface tension from 72 to 27 Mn/m in a 20 µM concentration (59,60). There are different surfactin homologues, depending on the number of carbons on the fatty acid chain (13 to 15), as well as on the composition of aminoacids in the peptide sequence. These modifications may alter the BSF activity (58,61).

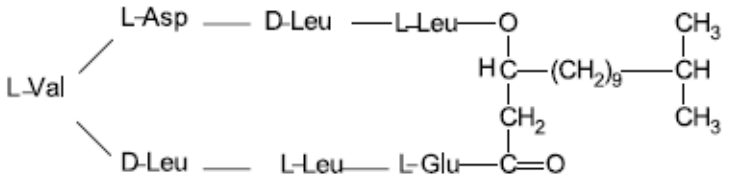

Figure 4 Structure of surfactin. Adapted from Nitschke and Pastore (2002)

Lichenisin is a cyclic lipopeptide with seven aminoacids structurally similar to surfactin. The main difference between the two BSFs resides in the presence of a residue, glutaminyl, instead of a glutamic acid in the first position. This difference makes it possible for lichenisin to have a lower CMC and higher surfactant power and hemolytic activity (62).

1.6. Applications of biosurfactants and biosurfactant-producing microorganisms

BSFs have a myriad of applications in various areas, like petroleum recovery, food industry, cosmetics, detergents, pharmaceutics and paints (14). Their capability to emulsify, and dissolve hydrocarbons and oils, makes them a remarkable resource in bioremediation in oil contaminated areas(16,17,63,64).

Bioremediation relies on the natural capacity of plants and microorganisms to degrade contaminants, turning them into less toxic or completely convert them to nontoxic substances, like water and carbon dioxide (14). A natural and inexpensive way to increase bioremediation of contamnated areas relies on biostimulation. This may be achieved through the increase of nutrients and oxygen. However, it is crucial to know beforehand the population density as well as the concentration of oil, and the correct pH in order to have a successful bioremediation effect (17,64). Another way to increase the bioremediation success is bioaugmentation, in which the concentration of natural microorganisms inhabiting the contaminated area is increased to accelerate the process (14).

BSFs can also be useful in the washing of oil reservoirs and in the microbial enhanced oil recovery technology (MEOR). The oil residue that is trapped in the small pores of rocks, is thought to represent 50-65% of the oil and it is kept there by elevated capillary forces and interfacial tension between the oil and the aqueous phase. MEOR employs microorganisms and their respective BSF to reduce the interfacial tension between the rock and oil, allowing the oil to move freely through the rock pores and reach the surface with ease (64–66).

Due to their ionic nature, BSF can also perform a major role in the removal of heavy metals from soil and sediments. Solutions of surfactants can be used for the solubilisation and dispersion of the contaminants, and also allow the reuse of the soil (67). By adding the BSF solution to the soil, the heavy metals are trapped in BSFs micelles by electrostatic interactions and are easily retrieved by precipitation or membrane separation (14). Recent studies put surfactin, rhamnolipids and sophorolipids as the most promising candidates to heavy metal removal by BSFs (68,69). There has also been some recent studies with *Candida* strains, mainly *C. lipolytica,* that demonstrate that the produced BSF can remove up to 96% of Zn and Cu, in groundwater (70).

Although it is known that BSFs play a major role in the development and sustainability of biofilms, it has recently come to light that they may be important in the disruption of already established ones (3). A biofilm is a community of microorganisms that adhere to a solid or fluid interface. They are usually organized in microcolonies and surrounded by a self-produced matrix of water and polymeric substances (71). They present a severe problem in healthcare contexts, as they are related to pathogenic and chronic diseases, in the food industry, by attaching to equipment and work surfaces, as well as the environment, by triggering biocorrosion (72–74). The emulsifying properties of BSFs give them an advantage in the control of biofilms. However, this efficiency is dependent on a number of conditionings such as the composition of the medium, temperature, BSF concentration and time of exposure (5). An example of this biofilm-control effect has been demonstrated in the lipopeptide putisolvin, produced by *Pseudomonas putida*, that was able to arrest the growth of biofilms in other *Pseudomonas* strains (75). Another interesting example was shown when cirugic catheters were covered in surfactin solution and later inoculated with cell bacterial suspensions. The catheters with surfactin exhibited lower level of colonization by *Salmonella, E.coli and Proteus mirabilis* (48).

BSFs possess antibacterial, antifungal and antiviral properties, having been suggested as alternative antibiotics and antimicrobials (49). They have been reported to inhibit bacterial growth, stimulating the immune system of the host, and even

capable of cell lysis since their structure allows them to affect the permeability of the cell membrane with the same properties as a detergent (48). However, real BSF applications on pharmaceutics and health areas is still limited (76). Surfactin has proved to be one of the most resourceful and useful biosurfactants in this area. It has the ability to inhibit coagulation processes and even forming ionic channels within the membrane, as well as reducing apoptosis in breast cancer cells (49). Surfactin also displays effective antiviral activity, being able to inactivate herpes virus, retrovirus and some other RNA and DNA viruses (14). Iturin, another lipopetide produced by *B. subtilis* exhibits remarkable antifungal activity and is known to alter both morphology and structure of cell membranes in yeasts. Daptomicyn, a lipopeptide produced by *Streptomyces roseosporus* was incorporated in a commercial antibiotic, as a drug named cubicin. It is used in the treatment of methicillin resistant Staphylococcus aureus and other Gram positive bacteria (77,78). In the glycolipids family, it has been demonstrated that some rhamnolipids, produced by *P. aeruginosa* cultures, exhibit high antifungal activity against some fungi strains (79).

BSFs can also have an important role in the food industry. Emulsification is a major determinant of the consistency and texture of foods (80,81). The stability of the emulsification complex can be improved by addition of a BSF to the system. Processed foods, like butter and mayonnaise, are some examples of emulsions. A recent study has proved the utility of a BSF produced by *Candida utilis* in the formulation of mayonnaise, used as a salad dressing (82), while some rhamnolipids are used to enhance the properties of frozen desserts and butter (83).

1.7. Co-cultivation as a tool to enhance BSF production

In order to increase the yield of BSF production, and, therefore minimize its production costs in an industrial scale, there has been a search for new ways to cultivate BSF-producing microorganisms (8). The search for BSF producing bacteria able to do so under extreme conditions or in uncommon habitats is a recent and still limited field, but it has shown great promise. Darvish et al (24) reported the discovery

of microbial consortium of *Enterobacter cloacae* and *Pseudomonas* in extreme conditions of temperature and pH. Some *Pseudomonas* strains are able to grow from orchids and forest herb floral nectar (84). A strain of *Bacillus licheniformis* was observed to growth in anaerobic conditions and producing a BSF capable of reducing surface tension to 28 m N/m (25).

Another strategy to minimize the BSF production costs is the cultivation in low-cost and raw materials, like industrial waste and vegetable oil (6). There has been a number of waste products that have been used in the cultivation of BSF producing bacteria, such as oily effluents, animal and vegetable fat, whey, corn, glycerol, ethanol, soapstock, etc (14). Recent studies in molecular biology of microorganisms have reported that often some microbial compounds are not produced under laboratory conditions, but rather remain unexpressed, resulting in only a fraction of the microbial compounds potential being obtained (85,86).

A study by Mearns-Spragg (87) showed an increase in the production of antimicrobial compounds in marine bacteria when cultured with terrestrial bacterial strains, such as *S. aureus, E. coli* and *P. aeruginosa.* Co-cultivation, then, appears as an emerging strategy to induce the expression of secondary metabolites. This approach attempts to recreate the natural environment of the complex microbial communities, where they establish biotic relations. The competition for nutrients and antagonism are examples of situations that may trigger the production of secondary metabolites, such as BSF (85,86).

A study by Kanagasabhapathy and Nagata (88) reported that four antibiotic producing bacteria isolated from the marine sponge *Pseudoceratina purpurea* increased their antimicrobial activity when exposed to two strains of human pathogenic bacteria. In 2011, Dusane *et. al* (85) showed that there was an increase in BSF production between bacteria and fungi, when co-cultured together. It also showed an increase of antimicrobial activity in *Bacillus* strains, especially when co-cultivated with *P. aeruginosa* and *B. pumilis*, both biofilm forming strains.

This cultivation strategy can be performed on either solid or liquid media, and has shown promising results, although much remains to be studied (89).

1.8. Objectives

The aim of this work was to test the hypothesis that co-cultivation of BSF the producing strains, *B. licheniformis* and *Pseudomonas* sp., with biofilm forming strains, *Pseudomonas aeruginosa* and *Listeria innocua*, could stimulate the production of surfactin and rhamnolipids, respectively. The tensioactive effect was tested by four methods: the oil spray test, the drop collapse assay and cultivation in blood agar. Quantification of BSFs was performed by cultivation in CTAB-methylene blue medium; the CPC-BTB method, and the specific quantification of rhamnolipids was conducted by the orcinol method. Quorum sensing inhibition effect of cultures and co-cultures in *Chromobacterium violaceum* was also assessed.

Methods

2. Methods

2.1. Bacterial cultures and culture media

As BSF-producing strains, *Bacillus licheniformis*, a surfactin producer isolated in volcano sediments of the Cadis golf, provided by António Louvado (personal contact) and *Pseudomonas* #74, a rhamnolipid producer, isolated in Aveiro Ria by Patrícia Domingues, (90), were used. As inducing strains (pathogenic/biofilm forming) *Pseudomonas aeruginosa* ATCC® 27853 and *Listeria innocua* were used. *L. innocua* was obtained from Escola Superior de Biotecnologia (Universidade Católica Portuguesa, Portugal). *Listeria innocua* is a gram positive bacteria, that forms biofilms and although non pathogenic, it is genetically close to the food pathogen *Listeria monocytogenes* (91,92). *P. aeruginosa* is a gram-negative, biofilm forming bacteria, that is highly pathogenic. It can grow within a wide range of environmental condtitiond, although optimmaly at 37°C and in aerobic conditions (93).

All cultures were stored at -80°C, with glycerol as a crio-protector. In order to obtain active cultures, the frozen culture was inoculated in Tryptic Soy Broth (TSB, Liofilchem) and incubated at 37°C, with agitation (150 rpm) for 24h. The cultures were successively re-inoculated and incubated in the same conditions. Plate streakings were also performed in Tryptic Soy Agar (TSA, Liofilchem) between every liquid re-inoculation, in order to confirm the purity of the cultures. The plates were incubated at 37°C for 24 h and stored at 4 ºC.

For the experiments of cultivation and co-cultivation fresh cultures of *B. licheniformis* and *Pseudomonas* #74 were transferred from TSB to optimized media, by co-inoculating 1ml of the producing strain with 100 µl of either of the inducing strains (85). The *B. licheniformis* axenic culture and the co-cultures *B. licheniformis* + *Listeria innocua* and *B. licheniformis* + *P. aeruginosa* were cultured in Luria Bertani medium (LB, Liofilchem). The *Pseudomonas* axenic culture and and the co-cultures (*Pseudomonas* + *Listeria innocua* and *Pseudomonas* + *P. aeruginosa*) were

cultivated in minimal salt medium, adapted from Rikalovic *et. al* (54), with 1.07 g/L NH_4CL, 1.49 g/L KCL, 14.54 g/L TrisHCL, 0.2 g/L $MgS0_4$, 10 g/L triptone and 0.7 ml/L olive oil.

The incubation time corresponding to early stationary phase was determined and used in the experiments of biosurfactant production.

2.2. Growth time of cultures and co-cultures

In order to monitor and determine the optimal time for the production of biosurfactants (late exponential phase, early stationary phase), growth curves of every axenic culture and co-cultures were constructed using the media described above. The cultures were incubated a 37°C, with agitation (120 rpm), for 10 h. During the incubation period, aliquots were collected hourly to determine the optical density (OD 600 nm) of the cultures, using non-inoculated media as blanks. 3 independent assays, with 1 replica each were conducted.

2.3. Quantification and detection of biosurfactants

The presence and activity of biosurfactants in early stationary phase cultures and co-cultures was evaluated by six different methods. *Escherichia coli* DH5α was used as a negative control. The chemical surfactants Tween 80 (12 mM), CTAB (0.2 mM) and SDS (5%) were used as positive controls.

2.3.1. Oil spray

The oil spray method was conducted as described by Burch et. al (2010) (37). Using a needle, TSA plates were inoculated with axenic and co-cultures and incubated at 37°C for 24 h. Paraffin oil was sprayed (Merck) on the surface of the TSA plates using an airbrush (Thomas 50 Hz) and the formation of halos surrounding the producing colonies were the interpreted as an indication of BSF production.

Each assay had 3 replicas, in a total of 3 independent assays.

2.3.2. Drop collapse assay

For a quantitative analysis, an adapted method of drop collapse assay from Bodour and Miller-Maier, 1998 (94) was performed on axenic cultures and co-cultures cell free extracts (CFE). The CFEs were obtained by centrifugation (6000 rpm, 3 min) (Themo scientific, motor radius: 8 cm) and filtration with a serynge and filtration membrane (Frilabo, 0.22 µm)

A drop of destilled water (5 µl) was placed on a plastic petri dish, previously cleaned with distilled water and ethanol (97%). Using a micropipette, 1 µl of sample or controls (SDS 5% and CTAB 0.2 mM as positive controls, water as negative control) were then placed at the center of the water drop. The drop was left to rest for 1 minute and later examined using a magnifying glass (Olympus cover-015) and the diameter was measured. If the drop collapsed after BSF addition, in comparison with the negative control, the results was interpreted as positive (94).

4 independent assays, with 5 replicas each, were conducted.

2.3.3. Bood agar hemolysis

Blood Agar plates (Merck) were inoculated with axenic cultures and co-cultures and incubated at 37°C for 48 h.The presence of grey halos, considered as α-hemolysis (or partial hemolysis), or transparent halos, interpreted as β-hemolysis (or total hemolysis) around the colonies confirmed both the presence and activity of the BSF. The absence of halos was considered γ-hemolysis (lack of hemolysis) (95).

3 independent assays were conducted, with 1 replica of each test.

2.3.4. CTAB-methylene blue agar

Plates containing the CTAB-methylene blue medium (0.2 g/L CTAB Sigma Aldrich, 0.005 g/L methylene blue; 15 g/L agar Liofilchem, 1.38 g/L Triptone Oxoid, 4g/L $MgSO_4.7H_20$ Applichem, 1 g/L $Na_2HPO_4.2H_2O$ Applichem; 0.005 g/L $CaCl_2.2H_2O$, 25 ml/L glycerol Sigma Aldrich (96) were inoculated with axenic and co-cultures. The plates were incubated at 37°C for 48 h (44) and subsequently stored in the refrigerator at 4°C for 7 days (97). The presence of dark blue halos was considered a positive result for the presence and activity of anionic BSF.

2.3.5. CPC-BTB

For this colorimetric assay, an adapted method from Yang et. al, 2015 (45) was used. Cetilpiridinium chloride (CPC) (Sigma Aldrich) and bromotimol blue (BTB) (Alfa Aesar) were dissolved separately in 0.1M PBS (NaH_2PO_4/Na_2HPO_4, pH 8.0) to obtain 0.2 mM solutions. Subsequently, 20 mL of each solution were mixed together in order to obtain a green reagent. 800 µl of the reagent was added to 100µl of the CFE of axenic cultures and co-cultures (previously incubated in optimized medium for 24h) and left to rest for 5 minutes, at room temperature. The chromatic shift from green to blue, created by addition of the reagent to the cultures, confirmed the presence and activity of BSFs. The absorbance was read at 590 nm. For each individual test, a standard curve was prepared, using SDS solutions with the concentrations of 0, 50, 100, 200 and 300 mg/L (45).

3 independent assays, with 3 replicas each, were conducted.

2.3.6. Orcinol method

For specific detection and quantification of rhamnolipids, the colorimetric orcinol method adapted from Tahzibi et al., 2003 (98) was used. The orcinol reagent was prepared by mixing 0.19 g of orcinol (3-5 dihydroxitoluene, Sigma Aldrich) with

54.6 mL of sulfuric acid (97%) (Merck) and adding distilled water until final volume of 100 ml. For each independent assay, a calibration curve using different rhamnose concentrations (0mg/L (blank); 1.563 mg/L; 3,125 mg/L; 6.25 mg/L; 12.5 mg/L; 25mg/L; 50 mg/L) was constructed. The CFEs were obtained by centrifugation (6000 rpm, 3min) (Thermo Scientifc; motor radius: 8 cm) and filtration membrane (Frilabo, 0.22µm)

For the determination of BSF in the CFE, 100µl of CFE was added to 900 µl of orcinol reagent, in 2 ml microtubes. The samples were heated up to 100°C for 20 min., in a heating plate, and later cooled down to room temperature. The optical density at 421 nm was measured (96).

2.3.7. Quorum sensing Inhibition

The method for evaluating quorum sensing inhibition was adapted from McLean et al., 2004 (99). Axenic cultures and co-cultures were inoculated in LB agar plates and incubated overnight at 37°C. The indicator strain, *Chromobacterium violaceum* , was grown in LB broth, overnight at 30°C. Aliquots of 5µl of *C. violaceum* culture were mixed with 5 mL of LB soft agar (5%), previously heated and cooled to 45°C. The test plates were covered with the overlay of inoculated LB soft agar and incubated overnight at 30°C. Garlic slices were used as positive controls (100) and a colony of *C. violaceum* was used as negative control. The depigmented zones surrounding the colonies were interpreted as quorum sensing inhibition

Results and Discussion

3. Results and Discussion

3.1. Growth time of cultures and co-cultures

Growth time graphics are represented in figures 5 (*Pseudomonas* #74 axenic and co-cultures) and 6 (*Bacillus licheniformis* axenic and co-cultures). The experimental conditions correspond to *Pseudomonas #74, Pseudomonas #74 + P. aeruginosa; Pseudomonas #74 + Listeria innocua, Bacillus licheniformis, B. licheniformis + P. aeruginosa, B. licheniformis + Listeria innocua*.

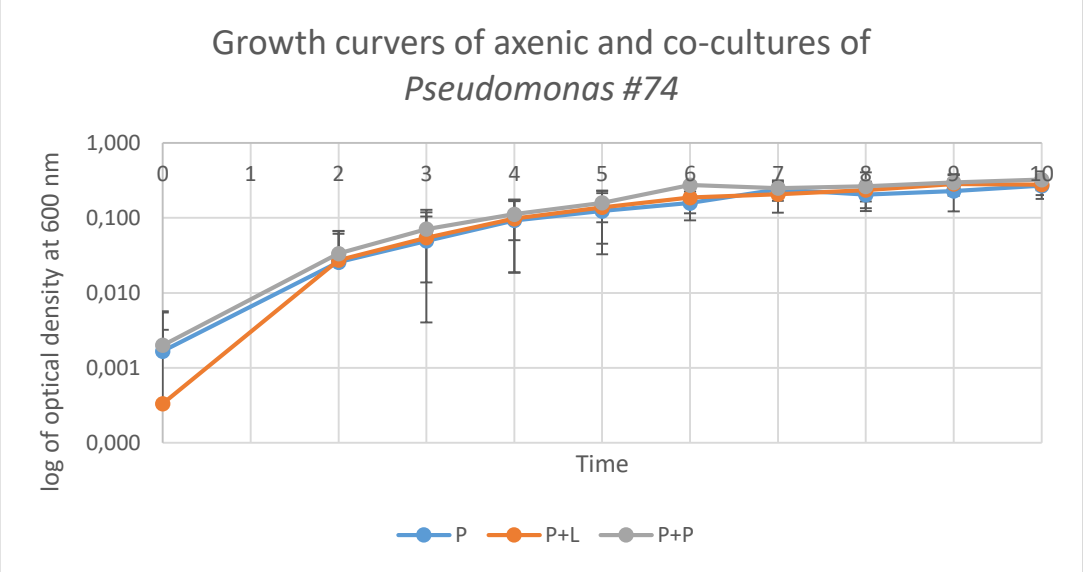

Figure 5 Variation of the optical density of axenic cultures and co-cultures *Pseudomonas #74*. P- *Pseudomonas #74*; P+L – *Pseudomonas #74 + Listeria Innocua*; P+P – *Pseudomonas #74 + P. aeruginosa*. The presented values represent the average of 3 independent assays. Error bars represent the standard deviation.

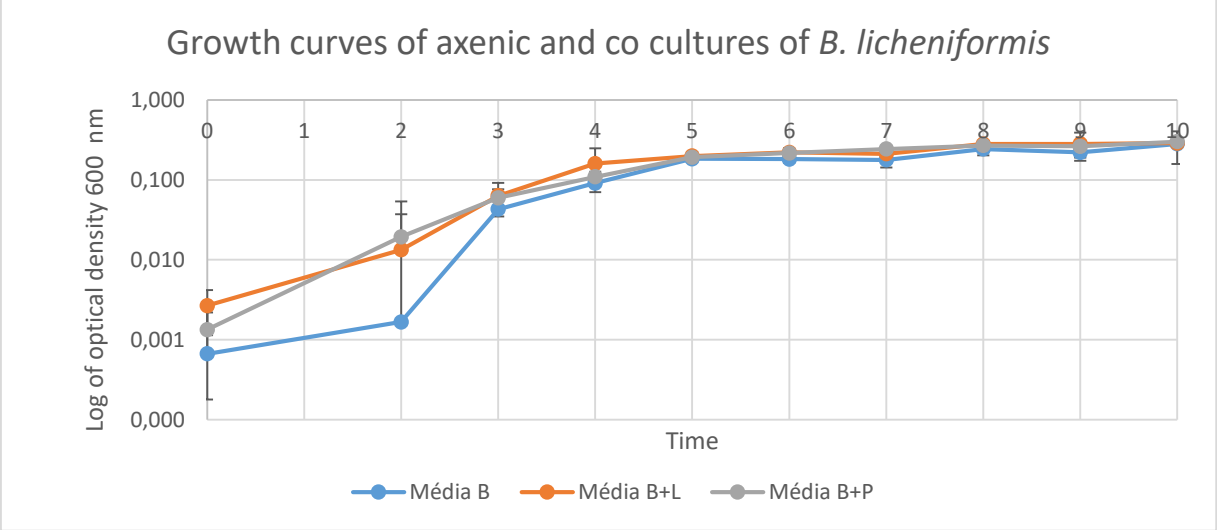

Figure 6 Variation of the optical density of the axenic and co-cultures *of Bacillus licheniformis*. B- *Bacillus licheniformis*; B+L – *Bacillus licheniformis* + *Listeria innocua*; B+P – Bacillus licheniformis + *P. aeruginosa*. The presented values represent the medium of the 3 independent assays. The bars represent the deviations.

The growth time of the axenic cultures and co-cultures of *Pseudomonas #74* stationary phase was reached after approximately 6 h, and slightly earlier (4 h) in the cultures with *B. licheniformis*. Axenic cultures and co-cultures of each of the BSF producing strains reached similar maximum optical density at stationary phase (0.2 nm for *Pseudomonas #74* cultures and 0.21 nm *B. licheniformis).* This is in accordance with existing literature that does not present significant differences between axenic and co-cultures growth curves (101)

Since BSF are considered secondary metabolites, produced in the stationary phase, for the following tasks a period of incubation of 48 h was established. after stationary phase proceeds, BSF are released to the medium but they can be later incorporated and used as carbon sources, as other substrates in the medium become exhausted (8)

3.2. Oil spray

The oil spray assay is a qualitative method for the detection for BSF in solid media based on the appearance of halos around the producing colonies, after being

pulverized with a fine layer of paraffin oil. The appearance of halos is due to the tensioactive reaction of the BSF with the paraffin oil (35). The diameter of the produced halo is directly correlated with the concentration and activity of BSFs (35,37).

Table 2 – Semi-quantification of the halos produced by axenic cultures and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74 + P. aeruginosa*; P+L *Pseudomonas #74 + L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis + P. aeruginosa*; B+L – *B. licheniformis + L. innocua*) in the oil spray assay. CTAB 0.2 mM was used as positive control and *E.coli* DH5α was used as negative control. Values are expressed in cm.

		Result	Diameter (average in cm + SD)	Qualitative classification
Cultures	P	+	2.37± 0.3	++
	P+P	+	1.83± 0.2	++
	P+L	+	2.78±0.2	++
	B	+	0.49±0.3	+
	B+P	+	0.69±0.3	+
	B+L	+	0.62±0.2	+
Controls	CTAB (0.2Mm)	+	0.81±0.1	+
	E. coli DH5α	-	0.00	-

Table 2 summarizes the results of the oil spray assay conducted on colonies originating from axenic cultures or co-cultures, as well as controls. This results were further classified qualitatively as weakly positive (+; diameter <1 cm), positive (++; diameter ≥ 1 cm) or negative (-; no halo). As expected, halos were formed around all the axenic cultures and co-cultures and the positive control (CTAB 0.2mM) which is in accordance with literature (37,102).

The halos of the Pseudomonas cultures P – *Pseudomonas #74*, P+P – *Pseudomonas #74* + *P. aeruginosa*, and P+L – *Pseudomonas #74* + *L. innocua* were significantly larger than the B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua* cultures, with *Pseudomonas* cultures displaying an average diameter of 2.33 cm and *Bacillus* cultures an average of 0.62 cm.The surfactin CMC is estimated to be 9.4 × 10−6 M (~9.7 mg/L) (103) and the rhamnolipid CMC is estimated to be 18 x 10-6 M.This could mean that, even though rhamnolipid displays less tensioactive ability than surfactin, it is liberated to the medium in higher quantities and produces bigger halos diameters. However, the diameter of the halos produced by co-cultures was not significantly different from those produced by the axenic cultures of the producer species (ANOVA, $p>0.05$). Considering that this method in an expedite tool for the screening of BSF producing strains but it suffers from low accuracy, in comparison to other quantitative methods (104) the results may interpreted as an indication that co-cultivation did not induce a change in BSF production in either of the producing bacterial species or that shifts in productivity were too subtle to be detected by the diameter of the halos. This assay does, however, display some major advantages in relation to some more accurate methods, such as the drop collapse assay because it detects low concentrations of BSF, making it possible to identify some BSF producing strains that would not be overlooked if tested by other methods (35,37).

3.3. Drop collapse assay

When a drop of water is applied to a hydrophobic surface, it will form a bead, due to the polar water molecules that are repelled from the surface. However, if the droplet contains a surfactant, the interfacial tension between drop and surface is reduced, which results in the collapse and spreading of the water drop (94). This is the principle of the drop collapse assay that is performed on an oil/water droplet, where a small amount of cell suspension or surfactant sample is placed at the center. If the cell suspension contains BSF, the water/oil droplet will collapse and spread (94,105).

After placing a water drop at the center of petri dish, samples (1 µL) of water, CTAB (0.2 mM) and SDS (5%) and axenic and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*) were added to the water droplet. Positive results were obtained for the positive controls (SDS 5% and CTAB 0.2mM) as well as for all the axenic cultures and co-cultures (fig. 7).

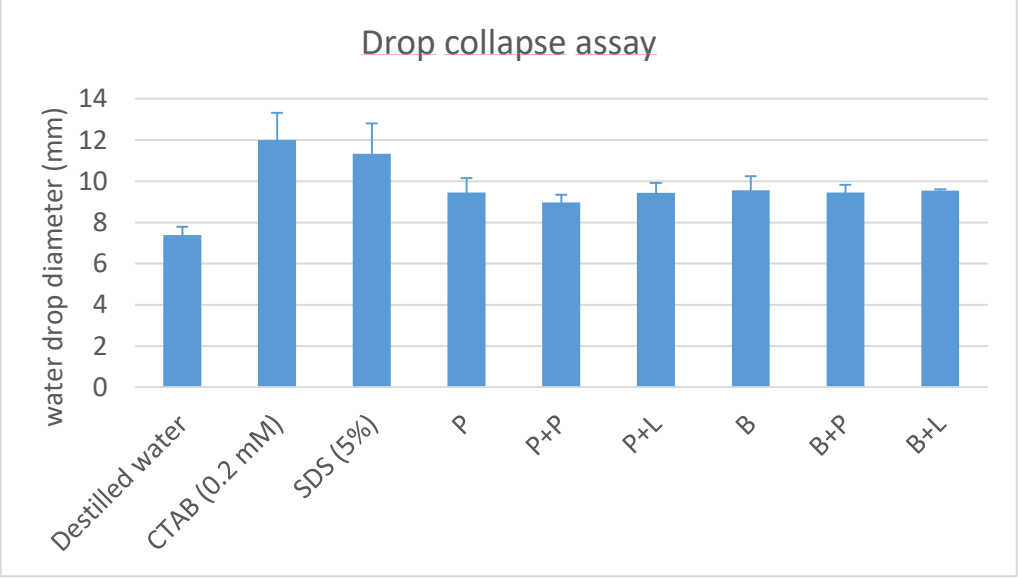

Figure 7. Results of the drop collapse assay performed on axenic and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L.*

***innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua).* Distilled water was used as a negative control. CTAB (0.2 mM) and SDS (5%) were used as positive controls. The values are presented mm. The bars represent the standard deviation.**

As expected, drops with CFE of axenic and co-cultures presented a significantly larger diameter than drops with negative control, water, as is extensively reported in literature (94,106–109) Positive controls, SDS (5%) and CTAB (0.2 mM) presented the largest diameters.

Experimental conditions P – *Pseudomonas #74*, P+P – *Pseudomonas #74* + *P. aeruginosa* and P+L – *Pseudomonas #74* + *L. innocua* presented medium diameter values of 9.4 mm, 8.9 mm and 9.4 mm, respectively. B – *B. licheniformis*, B+P – *B. licheniformis* + *P. aeruginosa* and B+L – *B. licheniformis* + *L. innocua* displayed medium diameters of 9.5 mm, 9.4 mm and 9.5 mm, respectively. SDS (5%) and CTAB (0.2 mM) increased the diameter of the water drops to medium values of 11.3 mm and 12.0 mm, respectively. Differences between the results corresponding to axenic cultures and co-cultures were not significant (ANOVA $p>0.05$), which means there is no influence in the tensioactive effect of the BSF when its producing strains are co-cultured with the biofilm forming strains. In 2011, Dusane et al, described a positive result, in which the zone diameter of the drop collapse assay performed on CFE of *B. pumilus* co-cultured with *P.aeruginosa* and *B. licheniformis* co-cultured with *B. pumilus* increased significantly (85). This indicates that the outcome of the production efficiency is highly dependent on the producing strain but also on the inducer strain elected for co-cultivation.

The cultures and co-cultures also exhibit a lower tensioactive effect that the commercialized surfactants, which might be related to the low sensitivity of this method, as a significant concentration of the BSF has to be present in order to cause a bigger collapse of the drop (110).

3.4. Blood agar hemolysis

Agar plates supplemented with 5% blood were used to detect BSF production through the appearance of transparent or yellow/green halos around colonies, corresponding to hemolysis of red blood cells by the BSF produced (96).

SDS (5%) was used as positive control and presents β-hemolysis (total hemolysis) and *E.coli* SH5α was used as negative control, as shown in fig. 6. The plates inoculated with P – *Pseudomonas #74*, P+P – *Pseudomonas #74* + *P. aeruginosa* and P+L – *Pseudomonas #74* + *L. innocua* cultures showed yellow/green halos (fig. 6) indicating the occurrence of partial hemolysis (α-hemolysis) and oxidation of hemoglobin. *B. licheniformis* axenic cultures and co-cultures, B – *B. licheniformis*, B+P – *B. licheniformis* + *P. aeruginosa* and B+L – *B. licheniformis* + *L. innocua,* did not display hemolytic activity, as seen in fig 8.

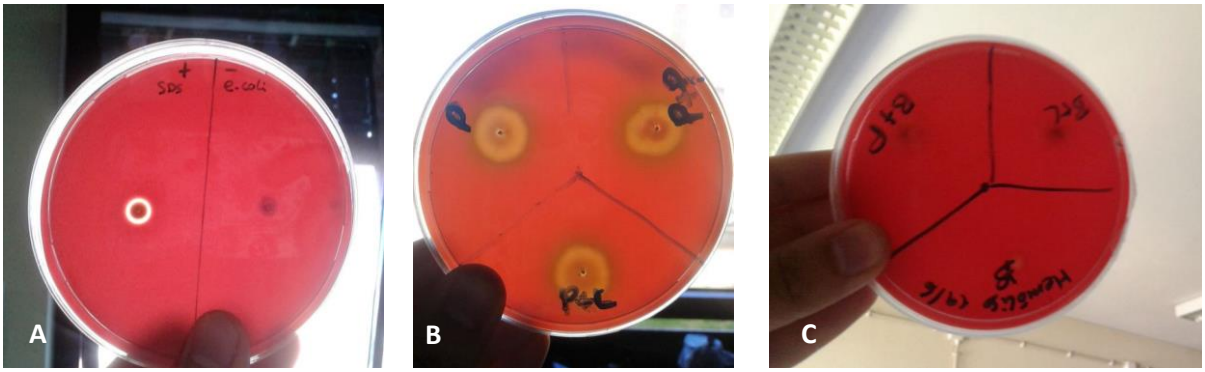

Figure 8. Example of plates corresponding to the blood agar hemolysis method performed on axenic cultures and co-cultures of *B. licheniformis* or *Pseudomonas* #74, and controls. A solution of SDS 5% was used as positive control and *E. coli* DH5 was used as negative control. A- Positive control, SDS, and negative control, *E. coli* DH5α. B- Axenic and co-cultures of *Pseudomonas #74*. C – Axenic and co-cultures of *B.licheniformis.*

The diameters of the hemolysis halos are presented in table 4. Differences between cultures are not significant (ANOVA, p>0.5). It is important to refer that, this method is performed on a very specific medium that is not the optimal medium determined for the axenic and co-cultures, and it's difficult to determine if the productivity would be different in other mediums (44)

Table 3- Results of the blood agar hemolysis assay on axenic and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*). An SDS solution (5%) was used as positive control and *E. coli* DH5α was used as negative control. Diameters average are presented in mm

		Results	Diameter (average in mm±SD)
Cultures	P	+	17±0.5
	P+P	+	15±0.4
	P+L	+	14±0.5
	B	-	0
	B+P	-	0
	B+L	-	0
Controls	SDS 5% (positive)	+	6±0.1
	E. coli DH5α (negative)	-	0

Pseudomonas strains typically exhibit hemolytic activity (β-hemolysis), that may be caused by toxins, enzimes or rhamnolipids, although the hemolytic ability is mostly related to rhamnolipid production (111). However, due to the multiple factors that may originate hemolysis, this assay is considered neither reliable nor accurate as the sole approach to BSF as it may deliver false positive or false negative results (112) and it should always be interpreted in the light of other sources of evidence. Although the *B. licheniformis* cultures did not exhibit hemolysis, that does not mean

surfactin was not produced. To detect the surfactin produced by *Bacillus* strains, methods based in cell suspensions are more usually applied (113,114), as concentrations tend to build up in suspensions where hemolytic activity is more likely to be detected (112).

3.5. CTAB-methylene blue agar

This is a specific test based on the appearance of dark blue halos due to the binding of anionic surfactants to CTAB (cationic surfactant), in the presence of methylene blue (42,43,96). This method is mainly used for the detection and quantification of rhamnolipids, but it was also used here for detection of surfactin production, as it is also an anionic surfactant. The diameter of the halo is dependent on the concentration of the BSF which makes it possible to interpret the results on a semi-quantitative way (44). The production of BSF was classified as weakly positive (+, diameter < 5 mm), positive (++, diameter ≥ 5 mm) or negative (-, no halo).

After a 48h incubation period, only the cultures containing the inducer strain *P. aeruginosa* (P+P, B+P) displayed positive results (Table 3). However, when the plates were refrigerated for 72 hours more, all the axenic cultures and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*), except for the *B. licheniformis* axenic culture, developed dark blue halos considered to be positive results. The low temperatures enhance the precipitation of the dye and intensify the color of the halos that were undistinguishable immediately after the incubation (43).

Table 4. Semi-quantitative analysis of anionic surfactants in axenic cultures and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua*, B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*) inoculated in CTAB-methylene blue agar. SDS (5%) was used a positive control and *E.coli* DH5α was used as negative control. The development of a dark blue halo around the colonies was interpreted as a positive result. Diameters are expressed in mm

		Results (after 48h)	Results after refrigerating (72h)	Diameter (average in mm + SD)
Cultures	P	-	+	4.7±0.6
	P+P	+	++	11.7±1
	P+L	-	+	3.3±0.5
	B	-	-	0
	B+P	+	++	12.7±1
	B+L	+	+	3.6±0.6
Controls	SDS 5%	++	++	17±0.1
	E. coli DH5α	-	-	0

There was a significant increase (ANOVA, $p<0.05$) in BSF production in co-cultures with *P. aeruginosa* (P+P; B+P), in relation to the corresponding axenic cultures, as illustrated in 5. When the axenic cultures were co-cultured with the inducing strain, *P. aeruginosa*, their halo diameter increased from 4,7mm to 11,7mm

(P<P+P) and from 0mm to 12,7mm. This method is more rigorous and reliable than the oil spray and blood agar test, as it considers only the anionic properties of the BSF, and other metabolites do not interfere(98).

One possible explanation to the fact that the co-cultures with *P. aeruginosa* displayed a significant increase over axenic and co-culture with *L. innocua* may be due to the fact that *P. aeruginosa* was acting not only as inducing, biofilm forming strain but also as a rhamnolipids producing strain. *P. aeruginosa* is one of the most prominent rhamnolipids producer, and it is well described in literature that rhamnolipids from *P. aeruginosa* produce positive results in this method.(56,96,115) It is also to be noted that this method, contrary to the oil spray and the blood agar method, used the minimal salt medium (supplemented with CTAB and methylene blue) considered optimal for the *Pseudomonas* culture, which could have helped increase BSF production. To further investigate the *P. aeruginosa* role in these results, cultivation in CTAB-methylene blue agar of an axenic culture of *P. aeruginosa* should be performed.

The cultures P – *Pseudomonas #74*, P+L – *Pseudomonas #74* + *L. innocua* and B+L – *B. licheniformis* + *L. innocua* showed positive results for the production of anionic BSF production, which is in accordance with the literature (43,115). *B licheniformis* was the only culture in which BSF production could not be detected by this method. A similar result was obtained by Lin *et. al*, 1998, in which this screening method did not work for a *B. subtilis* strain, because its growth was inhibited by CTAB. CTAB has been shown to inhibit some bacteria activity, by binding to cell substrate and inhibiting bacteria to adhere to substrates(44,116,117).

According to the results of the CTAB-methylene blue assay, co-cultivation with *Pseudomonas aeruginosa* showed a clear increase in the concentration of BSF molecules in the cultures.. The best combination for the co-cultivation of *Pseudomonas* #74 was P+P – *Pseudomonas #74* + *P. aeruginosa* and the best co-culture for *Bacillus licheniformis* was B+P – *B. licheniformis* + *P. aeruginosa*.

A B C

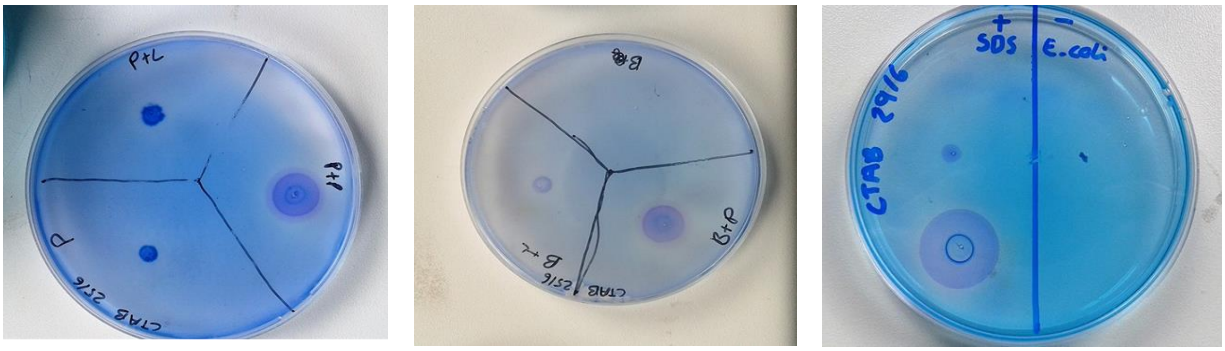

Figure 9. Example of plates corresponding to the CTAB-methylene blue test performed on axenic cultures and co-cultures of *B. licheniformis* or *Pseudomonas* #74, and controls. A solution of SDS 5% was used as positive control and E. coli DH5 was used as negative control. A- Axenic and co-cultures of *Pseudomonas* #74. B – Axenic and co-cultures of *B. licheniformis*. C – Positive control, SDS, and negative control, *E. coli* DH5α.

3.6. CPC-BTB method

This method allows a qualitative detection and quantitative analysis of anionic surfactants and it is based on the formation of a complex between BTB-bromotimol blue, (negative charge), a color indicator, and CPC-cetylpiridinium chloride (positive charge), a mediator. BTB displays a blue color (pH 8), but when CPC is added, a green complex is formed. Adding BSF will bind to CPC, freeing the BTB that goes back to displaying the blue color (45).

This chromatic shift is a qualitative result of the BSF production and by reading the optical density a quantitative result of BSF activity can be determined (45). For each assay, a calibration curve was constructed with different SDS concentrations. The BSF concentration was estimated as SDS equivalents and the results are presented in table 5.

All the cultures showed BSF production, with the exception of axenic culture of *Pseudomonas* #74. The values obtained range from 6 mg/L in the axenic culture of *B. licheniformis*, to 41 mg/L in the co-culture of *Pseudomonas #74* with *L. innocua*. There was an enhancement of BSF production in co-cultures, in relation to axenic cultures (B – *B. licheniformis* <B+P – *B. licheniformis* + *P. aeruginosa* <B+L – *B.*

licheniformis + *L. innocua* and P – *Pseudomonas #74* <P+P – *Pseudomonas #74* + *P. aeruginosa* <P+L – *Pseudomonas #74* + *L. innocua*).

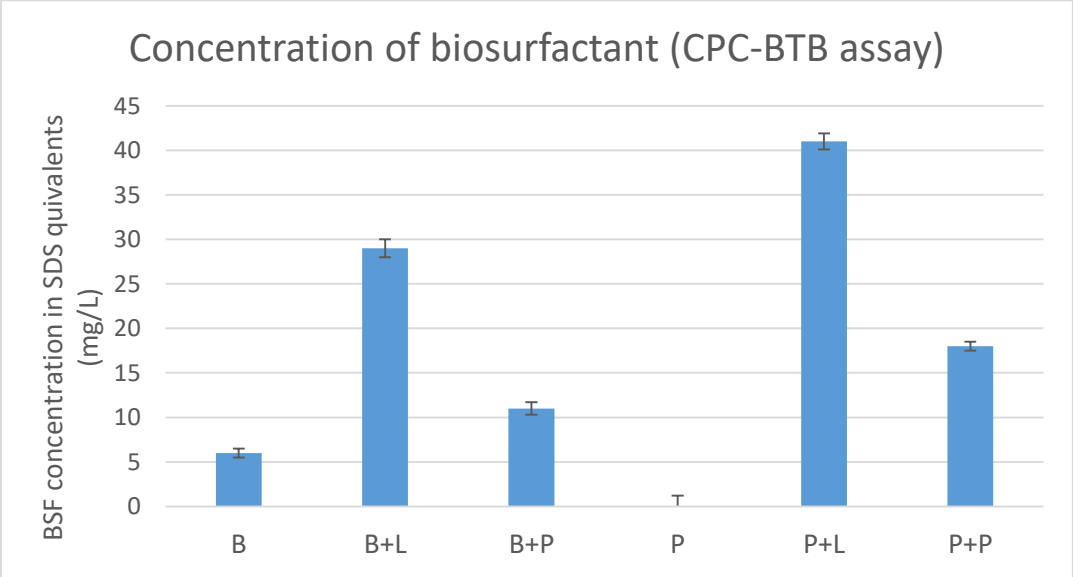

Figure 10. BSF concentration in axenic cultures and co-cultures (B – *B. licheniformis* B+P – *B. licheniformis* + *P. aeruginosa* B+L – *B. licheniformis* + *L. innocua* and P – *Pseudomonas #74* <P+P – *Pseudomonas #74* + *P. aeruginosa* P+L – *Pseudomonas #74* + *L. innocua*) estimated in mg/L SDS equivalents by the CPC-BTB assay.

Since it is a new method, the literature data for the concentration of biosurfactants in *Pseudomonas* cultures is still not available. The values are within the range reported in literature for different *Bacillus* strains (45,118). However, these values should be interpreted in a comparative context, as they are expressed in SDS equivalents, and not in surfactin or rhamnolipid concentrations. SDS displays a chromatic shift to blue around 300 mg/L and surfactin only at 600 mg/L (45). This might mean that SDS has a higher binding power to CPC, and the values estimated here may represent an underestimation. Rhamnolipid and surfactin standards must be used in future to obtain exact concentration estimates in the samples.

3.7. Orcinol method

The orcinol method is the most widely used for the quantification of rhamnolipids. The rhamnose groups in the rhamnolipids react with the orcinol reagent and produce different colours, depending on rhamnolipid concentration (Chandrasekaran and BeMiller 1980; Koch *et al*. 1991). For each assay a standard curve is prepared with rhamnose, for quantification, but a correction factor has to be taken into account, in order to compensate the extra mass in the lipid portion of the rhamnolipids (119). Déziel *et al* (46) proposed a correction factor of 2.25. One problem with this approach is that the results will vary with the proportion of mono- to di-RLs in the culture to be analyzed.

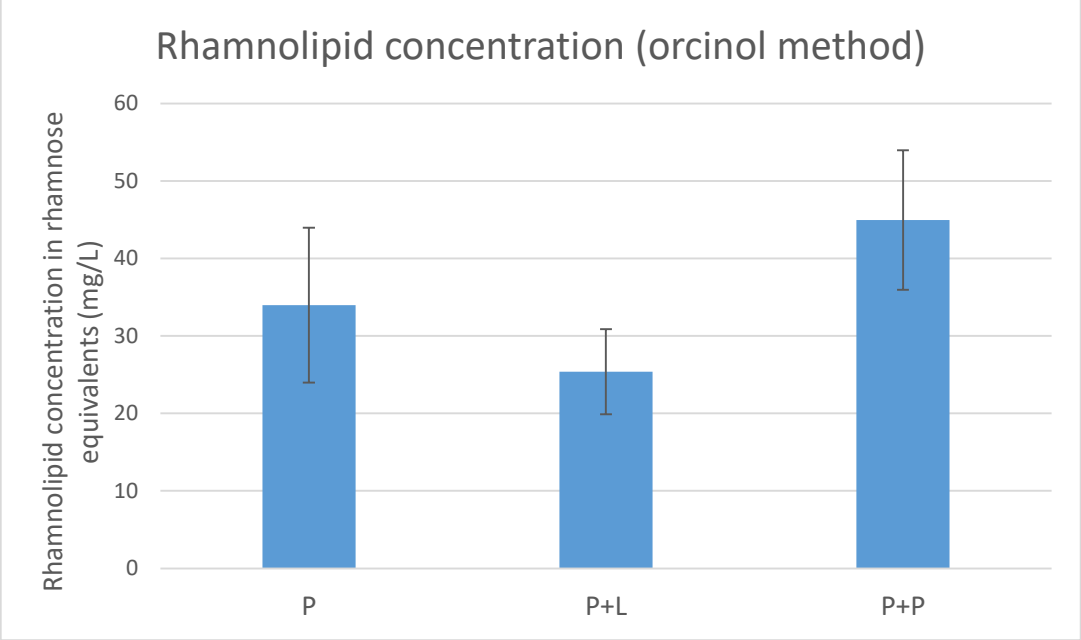

Figure 11. Rhamnolipid concentration by the orcinol assay. Results are expressed in rhamnose equivalents (mg/L). The bars represent the deviations.

As expected, there was production of rhamnolipids by the *Pseudomonas* cultures, detected by the orcinol method, as it is extensively reported in literature (20,115,120,121).

The average rhamnolipid concentration was 33.9 mg/L for axenic cultures of *Pseudomonas*, 25.3 mg/L for P+L – *Pseudomonas #74* + *L. innocua* and 44.9 mg/L for P+P – *Pseudomonas #74* + *P. aeruginosa*. Differences between the rhamnolipid concentrations in axenic cultures and co-cultures were not significant (ANOVA > 0.05).

3.8. Quorum-sensing inhibition

The main role of quorum-sensing is thought to be the control of bacterial population density. In habitats where bacterial populations compete for resources or in cases where bacteria infect a determined host, the ability to interfere with this bacterial cell-cell communication is both an advantage and a defense mechanism. This process is known as quorum-quenching (30,33)

In this qualitative assay, the effect of axenic cultures and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*) was tested on the inhibition of the quorum sensing mechanism of *Chromobacterium violaceum*. A positive result of quorum-quenching was interpreted as a depigmentation around the *C. violaceum* culture and corresponds to the inhibition of the production of the purple pigmentation, the results are summarized in Table 5.

Positive results corresponding to a quorum quenching effect were obtained for P – *Pseudomonas #74*, P+P – *Pseudomonas #74* + *P. aeruginosa*, P+L – *Pseudomonas #74* + *L. innocua*, as seen in figure 13. However, it is not possible, through this method to conclude if these positive results were due to the co-cultivation or even due to the BSFs present in the cultures, as these results may be caused by other molecular mechanisms. Existing literature has shown that *C. violaceum* regulates his pigmentation production by the N-hexanoyl HSL (C6-HSL), a AHL auto-inducer (30,32), while some studies have shown that *Pseudomonas* strains use two signal molecules, 3-oxo-dodecanoyl homoserine lactione (3-oxo-C12 HSL) and N-

butanoyl homoserine lactone (C4-HSL) as their inducers. These molecules will competitively bind and, subsequently, inhibit the receptor for the cognate signal C6-HSL *C. violaceum*, readily inhibiting its QS process (32). It is possible that this inhibition effect could be caused by *Pseudomonas* metabolites other than AHLs. A mutated strain of *P. aeruginosa* incapable of producing both 3-oxo-dodecanoyl homoserine lactione (3-oxo-C12 HSL) and N-butanoyl homoserine lactone (C4-HSL), retained significant QS inhibition activity, suggesting this activity to be regulated by other QS products (99) namely BSFs. In general, although a quorum sensing inhibition effect was demonstrated in *Pseudomonas #74* cultures and co-cultures, the effect cannot be unequivocally related to the BSF, as other secondary metabolites may be involved.

Table 5 - Qualitative results of the quorum sensing inhibition assay in axenic cultures and co-cultures (P- *Pseudomonas #74*; P+P – *Pseudomonas #74* + *P. aeruginosa*; P+L *Pseudomonas #74* + *L. innocua* ; B – *B. licheniformis*; B+P – *B. licheniformis* + *P. aeruginosa*; B+L – *B. licheniformis* + *L. innocua*). Garlic was used as positive control and *C. violaceum* was used as negative control.

Axenic and co-cultures-	Qualitative result
B	-
B+L	-
B+P	+
P	+
P+L	+
P+P	+
Garlic	+
C. violaceum	-

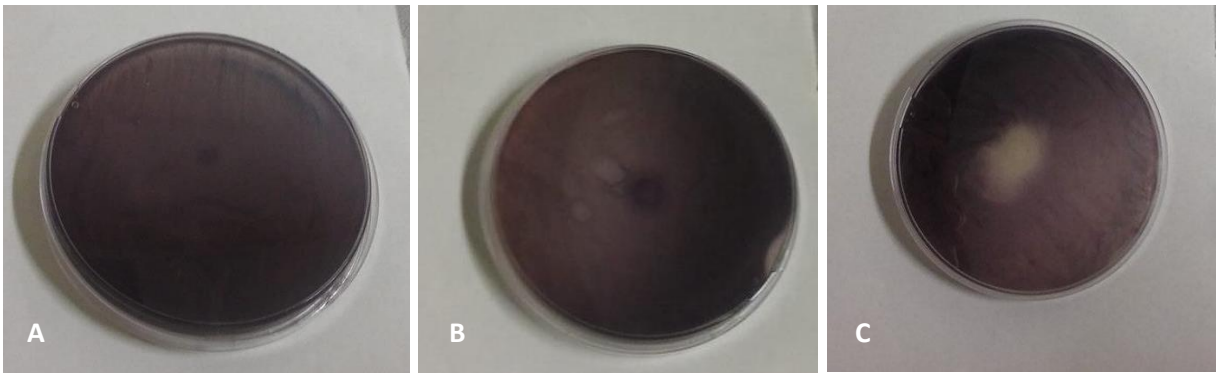

Figure 12.. Example of plates corresponding to quorum sensing inhibition performed on axenic cultures and co-cultures of *B. licheniformis*. A – Axenic culture of *B. licheniformis*. B – Co-culture of B – *B.licheniformis* +L – *L. innocua* C- Co-culture of B – *B. licheniformis* +P – *P-aeruginosa.*

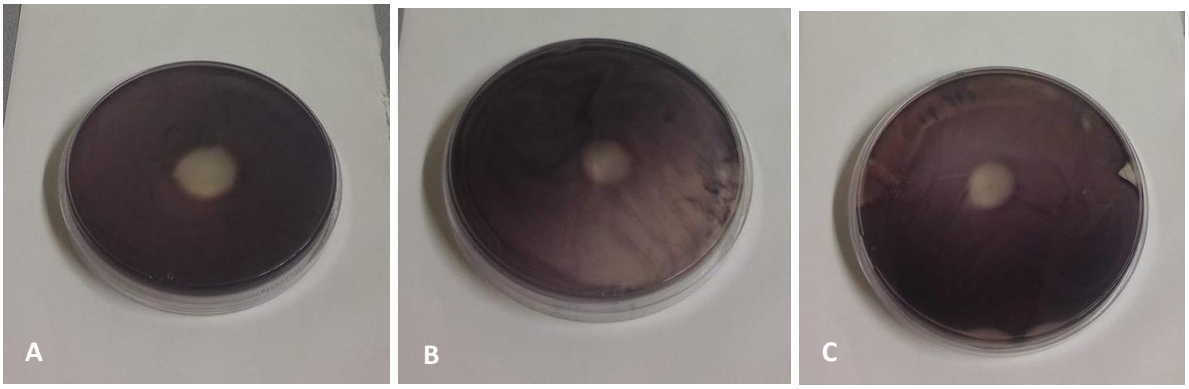

Figure 13. Example of plates corresponding to quorum sensing inhibition assay performed on axenic cultures and co-cultures of *Pseudomonas* #74. A – Axenic culture of *Pseudomonas* #74; B - Co-culture of *Pseudomonas* #74 and *Listeria innocua*; C - Co-culture of *Pseudomonas* #74 and *Pseudomonas aeruginosa*.

The co-culture B+P – *B. licheniformis* + *P. aeruginosa* showed a positive result, but the B – *B. licheniformis* and B+L – *B. licheniformis* + *L. innocua* cultures displayed negative results, as shown in fig 12.

3.9 Comparison between the different methods of detection/quantification

A summary of the results obtained by different methodological approaches is presented in Table 7. CTAB methylene blue agar method and CPC-BTB assay

indicate a positive effect of co-cultivation on the increase of concentration of BSF molecules produced by *Pseudomonas* #74 and *Bacillus licheniformis*

Table 6 – Summary of the different methods used for the detection/ quantification of BSFs in axenic and co-cultures

	Oil Spray	Drop Collapse Assay (mm)	Blood agar hemolysis	CTAB-methylene blue agar	CPC-BTB (SDS equivalents)	Orcinol (mg/L rhamnose equivalents)	Quorum sensing inhibition
P	++	9,4	+	+	-	33,9	+
P+P	++	8,9	+	++	18	25,3	+
P+L	++	9,4	+	+	41	44,9	+
B	+	9,5	-	-	6	-	-
B+P	+	9,4	-	++	11	-	+
B+L	+	9,5	-	+	29	-	-

The results weren't always coherent between each other. This might be due to the fact that each method analyses different properties of the BSFs. Oil spray, for example, is only used for BSF detection. CTAB-methylene blue relies on the anionic properties of the BSF. Blood agar hemolysis analyses the haemolytic properties of the BSF. The orcinol method is used for rhamnolipid concentration assessment and the drop collapse assay is an indicator of the BSF tensioactive activity that depends on the size and ramification level of the lipid(122,123).

A more detailed chemical characterization of the BSF present in the extracts would be necessary for a better understanding of the results and of the mechanisms of biological interaction between the producer and inducer strains

Even so, it is clear, through the CTAB-methylene blue agar and CPC-BTB assays the most intense reactions were displayed by the co-cultures of the producing bacteria.

Conclusion

In conclusion, the CPC-BTB method and the CTAB-methylene blue test showed a clear increase in the concentration of BSF molecules produced which sustains the hypothesis that co-cultivation of BSF-producer strains with inducers strains may cause a shift the production of BSFs. However, the oil spray, blood hemolysis, drop collapse and orcinol methods did not show significant differences between axenic cultures and co-cultures. Different methods describe different properties of the BSF (e.g. tenso-active effect, concentration) and they must be interpreted as a whole. However, considering that BSF are most often released as mixtures of molecules, it is imperative to obtain information on the chemical characterization of the BSF pool produced in each experimental condition so that the effect of the interaction between producers and inducers can be understood at the molecular level and key for further biotechnological applications are provided.

References

4. References

1. Gautam, K.K., Tyagi, V.K. Microbial Surfactants: A Review. Journal of Oleo Science. 2006;55(4) p. 155-166.
2. Banat IM, Desai JD. Microbial Production of Surfactants and Their Commercial Potential. Microbiology and Molecular Biology Journal. 1997;61(1) p. 47-64.
3. Marchant R, Banat I. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends in Biotechnology. 2012;30(11) p. 558 -565.
4. Malik, M.A., Hashim, M.A., Nabi, F., AL-Thabaiti, S.A., Khan Z.. Anti-corrosion ability of surfactants: A review. Int J Electrochem Sci. 2011;6(6) p. 1927-1948.
5. Banat, I.M., De Rienzo, M.D., Quinn, G.. Microbial biofilms: biosurfactants as antibiofilm agents. Applied Microbiology and Biotechnology. 2014 (24):9915–29.
6. Nitschke, M., Pastore, M.. Biossurfactantes: Propriedades e Aplicações. Quimica Nova. 2002;25(5) p. 772-776.
7. Saharan, B.S., Sahu, R.K., Sharma, D.. A Review on Biosurfactants : Fermentation , Current Developments and. Genetic Engeenering Biotechnology Journal. 2011 (GEBJ-29) .
8. Ron, E.Z., Rosenberg, E. Natural roles of biosurfactants. Environmental Microbiology. 2001;3(4) p. 229-236.
9. Satpute, S.K., Banpurkar, A.G., Dhakephalkar, P.K., Banat, I.M., Chopade, B.. Methods for investigating biosurfactants and bioemulsifiers: a review. Critical Review Biotechnology. 2010;30(2) 127-144 .
10. Henkel, M., Müller, M.M., Kügler, J.H., Lovaglio, R.B., Contiero, J., Syldatk, C., Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. Process Biochemistry. 2012;47(8) p. 1207-1209.
11. Geys, R., Soetaert, W., Van Bogaert, I.. Biotechnological opportunities in biosurfactant production. Current Opinions in Biotechnology. 2014;30 p. 66-72.
12. Rahman, P.K.S.M., Gakpe, E.. Production, characterisation and applications of biosurfactants - Review in Biotechnology. 2008;7 p. 360-370.
13. Banat, I.M. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review. Bioresourses in Technology. 1995;51(1) p. 1-12.
14. Santos, D., Rufino, R., Luna, J., Santos, V., Sarubbo, L. Biosurfactants: Multifunctional Biomolecules of the 21st Century. International Journal Molecular Science. 2016;17(3).

15. De Carvalho, C.C.C.R., Fernandes, P.. Production of metabolites as bacterial responses to the marine environment. Marine Drugs. 2010;8(3) p. 705-727.

16. Araújo. L.V., Freire, D.M.G. Biossurfactantes: Propriedades Anticorrosivas, Antibiofilmes e Antimicrobianas. Quimica Nova. 2013;36(6) p. 848-858.

17. Christofi, N., Ivshina, I.B.. Microbial surfactants and their use in field studies of soil remediation. Journal of Applied Microbiology. 2002;93(6) p 915-929.

18. Bento, F.M., Camargo. F.A.O., Okeke, B.C., Frankenberger, W.T. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresourses inTechnology. 2005;96(9) p. 1049-1055.

19. Saravanan, V., Vijayakumar, S. Isolation and screening of biosurfactant producing microorganisms from oil contaminated soil. Journal of Academia Industrial Research. 2015;15.

20. Wei, Y.H., Chou, C.L., Chang, J.S.. Rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater. Biochemistry and Engineering Journal. 2005;27(2) p. 146-154.

21. Wan Nawawi, W.M.F., Jamal, P., Alam, M.Z. Utilization of sludge palm oil as a novel substrate for biosurfactant production. Bioresourses in Technology. 2010;101(23) p. 9241-9247.

22. Batista, S.B., Mounteer, A.H., Amorim, F.R., Tótola, M.R. Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. Bioresourses in Technology. 2006;97(6) p. 868-875.

23. Ghojavand, H., Vahabzadeh, F., Mehranian, M., Radmehr, M., Shahraki, K.A., Zolfagharian, F. Isolation of thermotolerant, halotolerant, facultative biosurfactant- producing bacteria. Applied Microbiology and Biotechnology. 2008;80(6) p. 1073-1085.

24. Darvishi, P., Ayatollahi, S., Mowla, D., Niazi, A. Biosurfactant production under extreme environmental conditions by an efficient microbial consortium, ERCPPI-2. Colloids Surfaces Biointerfaces. 2011;84(2) p. 292-300.

25. Ben Belgacem, Z., Bijttebier, S., Verreth, C., Voorspoels, S., Van de Voorde I, Aerts G. Biosurfactant production by *Pseudomonas* strains isolated from floral nectar. Journal of Applied Microbiology. 2015;118(6) p. 1370-1384.

26. Kiran, G.S., Hema, T.A., Gandhimathi, R., Selvin, J., Thomas, T.A., Rajeetha Ravji, T. Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3. Colloids Surfaces Biointerfaces. 2009;73(2) p. 250-256.

27. Bhardwaj, G., Cameotra, S.S., Chopra, H.K.. Biosurfactants from Fungi: A

Review. Journal of Petroleoum Environmental Biotechnology. 2013;4(6) p. 1-6.

28. Cortes-sánchez, A., Hernández-sánchez, H., Jaramillo-flores, M. Production of glycolipids with antimicrobial activity by *Ustilago maydis* FBD12 in submerged culture. African Journal of Microbiol Research. 2011;5(17) p. 2512-2523.

29. Cavalero, D.A., Cooper, D.G. The effect of medium composition on the structure and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214. Journal of Biotechnology. 2003;103(1) p. 31-41.

30. Waters, C.M., Bassler, B.L. Quorum sensing: Cell-to-Cell Communication in Bacteria. Annual Review in Cell Development Biology. 2005;21(1) p. 319-346.

31. Miller, M.B., Bassler, B.L. Quorum sensing in Bacteria. Annual Reviews in Microbiology. 2001;55.

32. Fuqua, C., Parsek, M.R., Greenberg, E.P. Regulation of gene expression by cell to cell communication. 2001;35(1) p. 439-468.

33. Bassler, B.L.. How bacteria talk to each other: Regulation of gene expression by quorum sensing. Current Opinions in Microbiology. 1999;2(6) p. 582-587.

34. Bjarnsholt, T., van Gennip, M., Jakobsen, T.H., Christensen, L.D., Jensen, P., In vitro screens for quorum sensing inhibitors and in vivo confirmation of their effect. Nature Protocols. 2010;5(2) p. 282-293.

35. Burch, A.Y., Browne, P.J., Dunlap, C.A., Price, N.P., Lindow, S.E. Comparison of biosurfactant detection methods reveals hydrophobic surfactants and contact-regulated production. Environmental Microbiology. 2011;13(10) p. 2681-2691.

36. Zhu, L., Xu, Q., Jiang, L., Huang, H., Li, S. Polydiacetylene-based high-throughput screen for surfactin producing strains of *Bacillus subtilis*. PLoS One. 2014;9(2) p. 1-9.

37. Burch, A.Y., Shimada, B.K., Browne, P.J., Lindow, S.E.. Novel high-throughput detection method to assess bacterial surfactant production. Applied Environmental Microbiology. 2010,76(16) p. 5363-5372.

38. Carrillo, P.G., Mardaraz, C., Pitta-Alvarez, S.I., Giulietti, A.M.. Isolation and selection of biosurfactant-producing bacteria. World Journal of Microbiology and Biotechnology. 1996;12(1) p. 82-84.

39. Heyd, M., Kohnert, A., Tan, T.H., Nusser, M., Kirschhöfer, F., Brenner-Weiss, G. Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. Analytical and Bioanalytical Chemistry. 2008;391(5) p. 1579-1590.

40. Youssef, N.H., Duncan, K.E., Nagle, D.P., Savage, K.N., Knapp, R.M., McInerney, M.J. Comparison of methods to detect biosurfactant production by

diverse microorganisms. Journal of Microbiology Methods. 2004;56(3) p. 339-347.

41. Cooper, D.G., Goldenberg, B.G. Surface-Active Agents from Two *Bacilllus* Species. Microbiology. 1987;53(2) p. 224-229.

42. Pinzon, N.M., Ju, L.. Analysis of rhamnolipid biosurfactants by methylene blue complexation. Applied Microbiology and Biotechnology. 2009;82(5) p. 975-981.

43. Pinzon, N.M., Ju, L.K. Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. Biotechnology Letters. 2009;31(10) p. 1583-1588.

44. Siegmund, I., Wagner, F.. New method for detecting rhamnolipids excreted byPseudomonas species during growth on mineral agar. Biotechnology Technology. 1991;5(4) p. 265-268.

45. Yang, H., Yu, H., Shen, Z.. A novel high-throughput and quantitative method based on visible color shifts for screening *Bacillus subtilis* THY-15 for surfactin production. Journal of Industrial Microbiology and Biotechnology. 2015;42(8)p. 1139-1147.

46. Déziel, E., Lépine, F., Milot, S., Villemur, R.. Mass spectrometry monitoring of rhamnolipids from a growing culture of *Pseudomonas aeruginosa* strain 57RP. Biochemistry, Biophysics Acta - Mollecular and Cell Biology of Lipids. 2000;1485(2–3) p. 145-152.

47. Mubarak, M,Q., Hassan, A.R., Hamid, A.A.S., Isa, M.H.M. A simple and effective isocratic HPLC method for fast identification and quantification of surfactin. Sains Malaysiana. 2015;44(1) p. 115-120.

48. Gharaei-Fathabad, E.. Biosurfactants in Pharmaceutical Industry (A Mini-Review). American Journal of Drug Discovery Development. 2010;

49. Gudiña, E.J., Rangarajan, V., Sen, R., Rodrigues, L.R.. Potential therapeutic applications of biosurfactants. Trends in Pharmacology Science. 2013;34(12) p. 667-675.

50. Karant, N.G.K., Deo, P.G., Veenanadig, N.K.. Microbial production of biosurfactant and their importance. Current Science. 1999;77 p. 116-126.

51. Randhawa, K.K.S., Rahman, P.K.S.M.. Rhamnolipid biosurfactants-past, present, and future scenario of global market. Frontiers in Microbiology. 2014.

52. Lang, S., Wullbrandt, D.. Rhamnose lipids--biosynthesis, microbial production and application potential. Applied Microbiology and Biotechnology. 1999;51(1) p. 22-32.

53. Nguyen, T.T., Sabatini, D.A.. Characterization and emulsification properties of rhamnolipid and sophorolipid biosurfactants and their applications. International

Journal of Molecular Science. 2011;12(2) p. 1232-1244.

54. Rikalovic, M., Gojgic, Cvijovic. G, Vrvic. M, Karadzic. Production and characterization of rhamnolipids from *Pseudomonas aeruginosa* san ai. Journal of Serbian Chemistry 2012;77(1) p. 27-42.

55. Reis, R.S., Pereira, A.G., Neves, B.C., Freire, D.M.G. Gene regulation of rhamnolipid production in *Pseudomonas aeruginosa*- A review. Bioresourses in Technology. 2011.102(11):6377–84.

56. Soberón-Chávez, G., Lépine, F., Déziel, E.. Production of rhamnolipids by *Pseudomonas aeruginosa*. Applied Microbiology and Biotechnology. 2005;68(6) p. 718-725.

57. Plaza, G., Chojniak, J., Rudnicka, K., Paraszkiewicz, K., Bernat, P.. Detection of biosurfactants in *Bacillus* species: Genes and products identification. Journal of Applied Microbiology. 2015;119(4) p. 1023-1234.

58. Pecci, Y., Rivardo, F., Martinotti, M.G., Allegrone, G.. Characterisation of lipopeptide biosurfactants produced by the *Bacillus licheniformis* V9T14 strain. Journal of Mass Spectrometry. 2010;45(7) p. 772-778.

59. Barros, F.F.C., De Quadros, C.P., Marstica, M.R., Pastore, G.M. Surfactina: Propriedades químicas, tecnológicas e funcionais para aplicações em alimentos. Quimica Nova. 2007;30(2) p. 409-414.

60. Navpreet, K.W., Cameotra, S.S.. Lipopeptides: Biosynthesis and Applications. J Microbiology and Biochemical Technology. 2015;7(2):103–7.

61. Liu, Q., Lin, J., Wang, W., Huang, H., Li. S. Production of surfactin isoforms by *Bacillus subtilis* BS-37 and its applicability to enhanced oil recovery under laboratory conditions. Biochemistry Engeneering Journal. 2014;93(3) p. 31-37.

62. Grangemard, I., Wallach, J., Maget-Dana, R., Peypoux, F. Lichenysin: a more efficient cation chelator than surfactin. Applied Biochemistry and Biotechnology. 2001;90(3) p. 199-210.

63. Mulligan, C.N. Environmental applications for biosurfactants. Environmental Pollution. 2005;133(2) p. 183-198.

64. Mao, X., Jiang, R., Xiao, W., Yu, J. Use of surfactants for the remediation of contaminated soils: A review. Journal of Hazardous Materials. 2015;285 p. 419-435.

65. Pacwa-Płociniczak, M., Płaza, G.A., Piotrowska-Seget, Z., Cameotra, S.S. Environmental applications of biosurfactants: Recent advances. International Journal of Molecular Science. 2011;12(1) p. 633-654.

66. Silva, R.C.F.S., Almeida, D.G., Rufino, R.D., Luna, J.M., Santos, V.A., Sarubbo, L.A. Applications of biosurfactants in the petroleum industry and the

remediation of oil spills. International Journal of Molecular Science. 2014;15(7) p. 12523-12542.

67. Hashim, M.A., Mukhopadhyay, S., Sahu, J.N., Sengupta, B.. Remediation technologies for heavy metal contaminated groundwater. Journal of Environmental Management. 2011;92(10) p. 2355-2388.

68. Mulligan, C.N., Yong, R.N., Gibbs, B.F. Removal of Heavy Metals from Contaminated Soil and Sediments Using the Biosurfactant Surfactin. Journal of soil Contamination. 1999;8(2) p. 231-254.

69. Herman, D.C., Artiola, J.F, Miller, R.M.. Removal of cadmium, lead, and zinc from soil by a rhamnolipid biosurfactant. Environment Science Technology. 1995;29(9) p. 2280-2285.

70. Rufino, R.D., Rodrigues, G.I.B., Campos-Takaki, G.M., Sarubbo, L., Ferreira S.R.M.. Application of a Yeast Biosurfactant in the Removal of Heavy Metals and Hydrophobic Contaminant in a Soil Used as Slurry Barrier. Applied Environmental Soil Science. 2011 p. 1-7.

71. Alhede, M., Qvortrup, K., Liebrechts, R., Høiby, N., Givskov, M., Bjarnsholt, T. Combination of microscopic techniques reveals a comprehensive visual impression of biofilm structure and composition. FEMS Immunological Medical Microbiology. 2012;65(2) p. 335-342.

72. Wingender, J., Flemming, H.C. Biofilms in drinking water and their role as reservoir for pathogens. International Journal of Hyg Environmental Health. 2011;214(6) p. 417-423.

73. Flemming, H., Meier, M., Schild, T.. Mini-review: microbial problems in paper production. Biofouling. 2013;29(6) p. 683-696.

74. Srey, S., Jahid, I.K., Ha, S.D. Biofilm formation in food industries: A food safety concern. Food Control. 2013;31(2) p. 572-585.

75. Marchant, R., Banat, I.M. Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnology Letters. 2012. 34(9) p. 1597-1605.

76. Fracchia, L.J., Banat, J., Cavallo, M., Ceresa. C., Banat, I.M. Potential therapeutic applications of microbial surface-active compounds. AIMS Bioengeneering. 2015;2(3) p. 144-162.

77. Robbel, L., Marahiel, M.A.. Daptomycin, a bacterial lipopeptide synthesized by a nonribosomal machinery. Journal of Biological Chemistry. 2010;285(36) p. 27501 - 27508.

78. Tally, F.P., Zeckel, M., Wasilewski, M.M., Carini, C., Berman, C.L., Drusano, G.L. Daptomycin: a novel agent for Gram-positive infections. Expert Opinions Investigating Drugs. 1999;8(8) p. 1223-1238.

79. Abalos, A., Pinazo, A., Infante, M.R., Casals, M., García, F., Manresa, A.. Physicochemical and Antimicrobial Properties of New Rhamnolipids Produced by *Pseudomonas aeruginosa* AT10 from Soybean Oil Refinery Wastes. Langmuir. 2001;17(5) p. 1367-1371.

80. Campos, J.M., Montenegro Stamford, T.L., Sarubbo, L.A., de Luna, J.M., Rufino, R.D., Banat, I.M. Microbial biosurfactants as additives for food industries. Biotechnology Progress. 2013;29(5) p. 1097-1108.

81. Campos, J.M., Stamford, T.L.M., Sarubbo, L.A. Production of a bioemulsifier with potential application in the food industry. Applied Biochemistry Biotechnology. 2014;172(6) p. 3234-3252.

82. Campos, J.M., Stamford, T.L.M., Rufino, R.D., Luna, J.M., Stamford, T.C.M., Sarubbo, L.A. Formulation of mayonnaise with the addition of a bioemulsifier isolated from Candida utilis. Toxicology Reports; 2015;2 p. 1164-1170.

83. Winterburn, J.B., Russell, A.B., Martin, P.J.. Integrated recirculating foam fractionation for the continuous recovery of biosurfactant from fermenters. Biochemistry Engeneering Journal; 2011;54(2) p. 132-139.

84. Javaheri, M., Jenneman, G.E., Mcinerney, M.J., Knapp, R.M.. Anaerobic Production of a Biosurfactant by *Bacillus-licheniformis* Jf-2. Applied Environmental Microbiology. 1985;50(3) p. 698-700.

85. Dusane, D.H., Matkar, P., Venugopalan, V.P., Kumar, A.R., Zinjarde, S.S.. Cross-species induction of antimicrobial compounds, biosurfactants and quorum-sensing inhibitors in tropical marine epibiotic bacteria by pathogens and biofouling microorganisms. Current Microbiology. 2011;62(3) p. 974-980.

86. Marmann, A., Aly, A.H., Lin, W., Wang, B., Proksch, P.. Co-cultivation - A powerful emerging tool for enhancing the chemical diversity of microorganisms. Marine Drugs. 2014;12(2):1043–65.

87. Mearns-Spragg, A., Bregu, M., Boyd, K.G., Burgess, J.G.. Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. Letters in Applied Microbiology. 1998;27(3) p. 142-146.

88. Kanagasabhapathy, M., Nagata, S. Cross-species induction of antibacterial activity produced by epibiotic bacteria isolated from Indian marine sponge *Pseudoceratina purpurea*. World Journal of Microbiology and Biotechnology. 2008;24(5) p. 687-691.

89. Bertrand, S., Bohni, N., Schnee, S., Schumpp, O., Gindro, K., Wolfender, J. Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. Biotechnology Adv. 2014;32(6) p. 1180-1204.

90. Coelho, F.J.R.C., Domingues, P., Santos, A.L., Gomes, N.C.M., Almeida, A., Cunha, Â.. Isolation of Surfactant-Resistant *Pseudomonads* from the Estuarine Surface Microlayer. 2012, p. 283–91.

91. Zhou, Q., Feng, F., Wang, L., Feng, X., Yin, X., Luo, Q.. Virulence regulator PrfA is essential for biofilm formation in *Listeria monocytogenes* but not in listeria innocua. Current Microbiology. 2011;63(2):186–92 p. 186-192.

92. Buchrieser, C., Rusniok, C., Kunst, F., Cossart, P., Glaser, P., Frangeul, L. Comparison of the genome sequences of *Listeria monocytogenes* and *Listeria innocua*: Clues for evolution and pathogenicity. FEMS Immunological Medical Microbiology. 2003;35(3) p. 207–13 .

93. Rasamiravaka, T., Labtani, Q., Duez, P. Jaziri, M. The formation of biofilms by *Pseudomonas aeruginosa*: A review of the natural and synthetic compounds interfering with control mechanisms. Biomedical Research Institute. 2015.

94. Bodour, A., Miller-Maier, R.M. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. Journal of Microbiology Methods. 1998.32(3) p. 273-280.

95. Kenneth, J. R., Ray, C.G. Sherris Medical Microbiology - an introduction to infectious diseases. 2003.

96. Rahman, P.K.S.M., Pasirayi, G., Auger, V., Ali, Z.. Production of rhamnolipid biosurfactants by *Pseudomonas aeruginosa* DS10-129 in a microfluidic bioreactor. Biotechnology Applied Biochemistry. 2010;55, p. 45-52.

97. Gunther, I.V., Nereus, W., Nuñez, A., Fett, W., Solaiman, D.K.Y. Production of Rhamnolipids by *Pseudomonas chlororaphis*, a Nonpathogenic Bacterium. Applied Environmental Microbiol. 2004; p. 2288-2293.

98. Tahzibi, A., Kamal, F., Assadi, M.M.. Improved Production of Rhamnolipids by a *Pseudomonas aeruginosa* mutant. Iranian Biomedical Journal. 2004;8, p. 25-31.

99. McLean, R.J.C., Pierson, L.S., Fuqua, C.. A simple screening protocol for the identification of quorum signal antagonists. Journal of Microbiological Methods. 2004;58(3) p. 351-360.

100. Rasmussen, T.B., Bjarnsholt, T., Skindersoe, E., Hentzer, M., Kristoffersen, P., Köte, M.,Screening for Quorum-Sensing Inhibitors (QSI) by Use of a Novel Genetic System. 2005;187(5), p. 1799-1814

101. Xu, F., Xudong, L., Kangmei, Z., Qinghua, L. Process of Mixed Culture of Functional Strains. Chinese J Applied Environmental Biology. 2009

102. Raaijmakers, J.M., de Bruijn, I., Nybroe, O., Ongena, M.. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: More than surfactants and antibiotics. FEMS Microbiology Reviews. 2010;34(6) p. 1037-1062.

103. Carrillo, C., Teruel, J.A., Aranda, F.J., Ortiz, A.. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochemistry Biophysics Acta - Biomembranes. 2003;1611(1–2) p. 91-97.

104. Costa, S.G.V. A.O., Nitschke, M., Lépine, F., Déziel, E., Contiero, J. Structure, properties and applications of rhamnolipids produced by *Pseudomonas aeruginosa* L2-1 from cassava wastewater. Process Biochemistry. 2010;45(9), p. 1511-1516.

105. Płaza, G.A., Zjawiony, I., Banat, I.M.. Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated and bioremediated soils. Journal of Petroleoum and Science Engeneering. 2006;50(1).

106. Caiazza, N.C., Shanks, R.M.Q., O'Toole, G.A.. Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. Journal of Bacteriology. 2005;187(21) p. 7351-7361.

107. Pemmaraju, S.C., Sharma, D., Singh, N., Panwar, R., Cameotra, S.S., Pruthi, V. Production of microbial surfactants from oily sludge-contaminated soil by *Bacillus subtilis* DSVP23. Applied Biochemistry and Biotechnology. 2012;167(5) p. 1119-1131.

108. Mohammadipour, M., Mousivand, M., Jouzani, G.S., Abbasalizadeh, S.. Molecular and biochemical characterization of Iranian surfactin-producing *Bacillus subtilis* isolates and evaluation of their biocontrol potential against Aspergillus flavus and Colletotrichum gloeosporioides. Canadian Journal of Microbiology. 2009;55(4) p. 395-404.

109. Jain, D.K., Collins-Thompson, D.L., Lee, H., Trevors, J.T.. A drop-collapsing test for screening surfactant-producing microorganisms. Journal of Microbiological Methods. 1991;13(4) p. 271–9.

110. Walter, V., Syldatk, C., Hausmann, R.. Screening concepts for the isolation of biosurfactant producing microorganisms. Advances in Experimental Medicine and Biology. 2010;672 p. 1-13.

111. Vallet-Gely, I., Novikov, A., Augusto, L., Liehl, P., Bolbach, G., Péchy-Tarr, M., Association of hemolytic activity of *Pseudomonas entomophila*, a versatile soil bacterium, with cyclic lipopeptide production. Applied Environmental Microbiology. 2010;76(3) p. 910 - 921.

112. Kuznetsova, M. V., Karpunina, T.I.. Cell-mediated hemolytic activity of nosocomial *Pseudomonas aeruginosa* strains. Bulletin of Experimental Biology and Medicine. 2015;159(2) p. 258 - 261.

113. Morán, A.C., Martínez, M.A., Siñeriz, F. Quantification of surfactin in culture supernatants by hemolytic activity. Biotechnology Letters. 2002;

114. Shalel, S., Streichman, S., Marmur, A. Monitoring Surfactant-Induced Hemolysis by Surface Tension Measurement. Journal of Colloid Interface Science. 2002;255(2) p. 265 - 269.

115. Nakhate, P.H., Pathaka, A. Optimisation of Rhamnolipid: A New Age Biosurfactant from *Pseudomonas aeruginosa* MTCC 1688 and its Application in Oil Recovery, Heavy and Toxic Metals Recovery. Journal of Bioprocessing and Biotechenology. 2015;5(5).

116. Lin, S., Lin, K., Lo, C., Lin, Y. Enhanced biosurfactant production by a *Bacillus licheniformis* mutant. Applied Microbiology and Biotechnology. 1998;23(3) p. 267 - 273.

117. Rodrigues, A., Nogueira, R., Melo, L.F., Brito, A.G.. Effect of low concentrations of synthetic surfactants on polycyclic aromatic hydrocarbons (PAH) biodegradation. Int Biodeterior Biodegradation

118. Jiao, S., Li, X., Yu, H., Yang, H., Li, X., Shen, Z. In situ enhancement of surfactin biosynthesis in *Bacillus subtilis* using novel artificial inducible promoters. Biotechnology Bioengeneering. 2016.

119. Abdel-mawgoud, A.M., Hausmann, R., Lépine, F., Muller, M.M., Déziel, E.. Rhamnolipids: Detection, Analysis, Biosynthesis, Genetic Regulation, and Bioengineering of Production. 2011 p. 13 . 56

120. Jeong, H., Lim, D., Hwang, S., Ha, S., Kong, J. Rhamnolipid production by *Pseudomonas aeruginosa* immobilised in polyvinyl alcohol beads. Biotechnology Letters. 2004. 26(1) p. 35–9.

121. Ballot F. Bacterial Production of Antimicrobial Biosurfactants. 2009.

122. Zhang, Y., Miller, R.M.. Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Applied Environmental Microbiology. 1994;60(6) p. 2101 - 2106

123. Perfumo, A., Banat, I.M., Canganella, F., Marchant, R. Rhamnolipid production by a novel thermophilic hydrocarbon-degrading *Pseudomonas aeruginosa* AP02-1. Applied Microbiology Biotechnology. 2006;72(1) p. 132 - 138.