Universidade de Lisboa

Faculdade de Farmácia

Rhamnolipids: Choosing *Burkholderia thailandesis* for biosurfactant glycolipids synthesis

Igor Alexandre Rodrigues Correia

Mestrado Integrado em Ciências Farmacêuticas

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Resumo

Os biossurfactantes têm sido amplamente estudados nos últimos anos como alternativas naturais aos surfactantes sintéticos. Estas moléculas anfipáticas podem ser sintetizadas por plantas, animais ou uma variedade de microrganismos, incluindo fungos e bactérias. Novas alternativas ecologicamente seguras e biodegradáveis como os biossurfactantes têm potenciais benefícios tais como a capacidade antimicrobiana e antibiofilme. Os ramnolípidos (RLs) são um grupo importante de biossurfactantes que foram descritos pelas suas inúmeras aplicações em muitas indústrias, incluindo a petrolífera, alimentar, biorremediação e agricultura. No entanto, a produção destes compostos ocorre principalmente através da bactéria patogénica *Pseudomonas aeruginosa*, o que se torna um fator limitante.

O objetivo deste projeto é a síntese e identificação de RLs produzidos por *Burkholderia thailandesis,* uma bactéria gram-negativa não patogénica. Primeiramente, os RLs foram biossintetizados por *B. thailandesis* usando diferentes meios de cultura [ex: *Luria-Bertani* (LB) e *Nutrient Broth* (NB)] suplementados com diferentes fontes de carbono hidrofóbico e hidrofílico, tais como glicerol e óleo de coco. Duas abordagens diferentes foram utilizadas para realizar a síntese destes compostos: microescala (método das microplacas) e escala laboratorial (método da agitação de frascos). A produção de RLs ocorreu em ambos os meios de cultura, sendo que a produção foi mais evidente no meio de cultura NB.

Seguidamente, os RLs produzidos foram caracterizados por cromatografia líquida de alta eficiência com espectrometria de massa, e os RLs maioritariamente produzidos foram os mono-rhamnolípidos com uma cadeia longa de ácidos gordos.

Posteriormente, as propriedades antimicrobianas dos RLs foram testadas contra *Staphylococcus aureus* ATCC 25923 através do ensaio da concentração mínima inibitória (CMI), apresentando valores de 0.25 μg L^{-1} e 0.125 μg L^{-1} para os RLs produzidos em meios LB e NB suplementados com óleo de coco, respetivamente. O ensaio do cristal violeta foi realizado para avaliar a formação do biofilme em diferentes condições experimentais. Quando uma concentração de RLs de 3 mg mL^{-1} foi usada para promover a adsorção em silicone de grau médico, a inibição do biofilme de *S. aureus* foi de 20 %.

Em conclusão, os resultados deste estudo demonstraram que novos RLs podem ser isolados e produzidos em microescala por *Burkholderia thailandesis*. Por outro lado, o estudo revelou que estes compostos são capazes de inibir o crescimento bacteriano e apresentaram alguma atividade antibiofilme.

Palavras-Chaves: biosurfactantes; ramnolípidos; método das microplacas; atividade antimicrobiana; inibição do biofilme.

Abstract

Biosurfactants have been widely studied in the last years as natural alternatives to synthetic surfactants. These amphiphilic molecules can be synthesized by plants, animals and a variety of microorganisms, including fungi and bacteria. New environmentally safe and biodegradable alternatives like biosurfactants provides potential benefits such as antimicrobial and antibiofilm efficacy. Rhamnolipids (RLs) are a major group of biosurfactants that have been reported for their applications in many industries including petroleum, food, bioremediation and agriculture. However, the production of these compounds mainly occurs by the opportunistic pathogen bacteria *Pseudomonas aeruginosa*, which is a limiting factor.

The aim of this project is the synthesis and identification of RLs produced by *Burkholderia thailandesis*, a non-pathogenic gram-negative bacteria. Firstly, RLs were biosynthesized by *B. thailandesis* using different culture media [i.e Luria-Bertani (LB) and Nutrient Broth (NB)] supplemented with different hydrophobic and hydrophilic carbon sources such as glycerol and coconut oil. Two different approaches were used to accomplish the synthesis of these compounds: microscale (microtiter plates) and laboratory scale (shake flask). The production of RLs occurred in both culture media, nevertheless it was more evident in the NB culture media.

Secondly, the RLs produced were characterized by high performance liquid chromatography with tandem mass spectrometry, and major RLs produced were mono-rhamnolipids with higher fatty acid chain.

Subsequently, the antimicrobial properties of RLs were tested against *Staphylococcus aureus* ATCC 25923 by the minimum inhibitory concentration (MIC) assay, presenting values of 0.25 µg L^{-1} and 0.125 µg L^{-1} for RLs produced in LB and NB media supplemented with coconut oil, respectively. Moreover, the crystal violet assay was performed to assess biofilm formation under different experimental conditions. When a RLs concentration of 3 mg mL^{-1} was used to promote their adsorption onto medical grade silicone, *S. aureus* biofilm inhibition was 20 %.

In conclusion, the results of this study have demonstrated that new RLs can be isolated and produced by *Burkholderia thailandesis* on a microscale approach. On the other hand, the study revealed that these compounds were capable of inhibit bacterial growth and showed a modest antibiofilm activity.

Keywords: biosurfactants; rhamnolipids; microtiter plate-approach; antimicrobial; biofilm inhibition.

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List of Abbreviations

ATCC- American Type Culture Collection

CFU – Colony-forming Unit

ECDC – European Center for Disease Control and Prevention

HAI – Healthcare-associated infection

HPLC-MS/MS – High Performance Liquid Chromatography with tandem mass spectrometry

LB – Luria-Bertani

MHB – Müeller-Hinton Broth

MIC – Minimum Inhibitory Concentration

MTP – Microtiter Plate

NB – Nutrient Broth

OD – Optical Density

PPS – Point of Prevalence

RL – Rhamnolipid

SL – Sophorolipid

TLC – Thin-layer Chromatography

TSA – Tryptic Soy Agar

TSB – Trypto-casein Broth

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1. Introduction

Biosurfactants, also named microbial surfactants, represents a new emerging and diverse class of surface-active molecules that are synthesised non-ribosomally by a wide range of different microorganisms including bacteria, yeast and filamentous fungi. [1 – 2]. Biosurfactants are amphipathic molecules with both the hydrophilic and hydrophobic domains with partition preferentially at the interface between fluid phases with different polarities [3]. One of the advantages derives from the fact of being produced by microorganisms and their positive biodegradability and eco-friendly attributes [4].

Referring to the chemistry of these biomolecules, the polar (hydrophilic) moiety consists of mono-, oligo- or polysaccharides, peptides or proteins, while the non-polar (hydrophobic) moiety contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols [5]. In addition, biosurfactants can be classified by their molecular weight, chemical nature, their microbial origin and as extracellular or attached to the wall [6]. Accordingly to their molecular structure, biosurfactants can be classified into glycolipids [e.g. rhamnolipids (RLs) and sophorolipids (SLs)], lipopeptides (e.g. surfactin), polymeric biosurfactants (e.g. emulsan and alasan), fatty acids and phospholipids [7].

Rhamnolipids (RLs) were first described in 1946 and during the early stage of discovery, these compounds were thought to be produced only by the opportunist pathogen *Pseudomonas aeruginosa* [8]. However, recent research projects concluded that this type of biosurfactants are produced by a variety of microorganisms (e.g. bacteria, fungi, yeast), and the main producing species are the gram-negative strains of *P. aeruginosa* isolated from various habitats such as water, soil and plants [9]. Due to this reason, *P. aeruginosa* has been used as a model to highly understand the RLs biosynthesis, as well as the genes that are critical in this process [10].

These RLs are glycolipidic biosurfactants that have been studied along the time due to the fact of being compounds highly biocompatible, biodegradable, with low toxicity and stable in a wide range of temperatures and pH, when compared with chemical surfactants [11 -13]. Likewise, RLs are a crystalline acid composed of rhamnose moieties (glycon part) and lipid moieties (aglycon part) linked to each other via an O-glycosidic linkage (Figure 1). On the one hand, the glycon part, hydrophilic component, is composed of one (mono-RLs) or two (di-RLs) rhamnose moieties linked to each other through a α-1,2-glycosidic linkage. Furthermore, the aglycon part (hydrophobic component) is mainly one or two (or in some cases three) β-hydroxy fatty acid chains (saturated, mono-, or polyunsaturated with a chain length varying from C8 to C16) linked to each other through an ester bond formed between the β-hydroxyl group of the

distal (relative to the glycosidic bond) chain with the carboxyl group of the proximal chain [14 - 15].

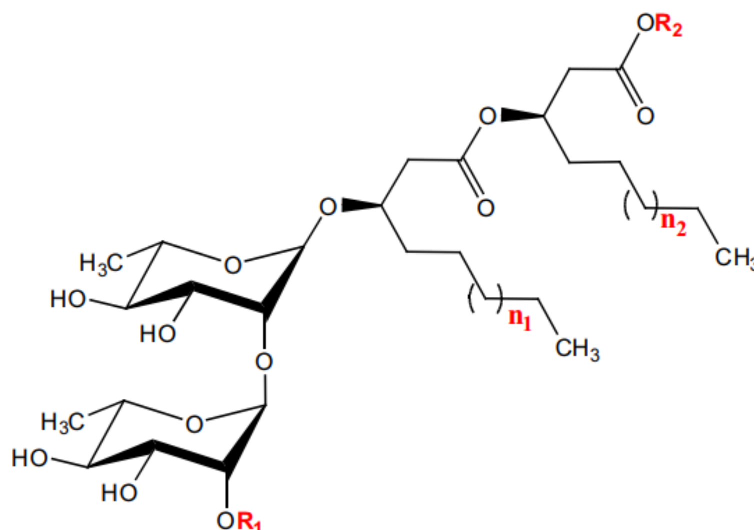


Figure 1. Example of the chemical structure of a rhamnolipid. Adapted from Abdel-Mawgoud *et al.* (2010) [14].

Addedly, the production of this compounds in *P. aeruginosa* implies different steps, as shown in Figure 2, starting with the synthetization of the sugar moiety (dTDP-l-rhamnose) from d-glucose, and the synthetization of the hydrophobic moiety [(3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA)] through the fatty acid synthesis pathway [10] [16]. In addition, RLs synthesis occurs by the actions of three enzymes: RhlA, RhlB, and RhlC [16]. The HAA is synthesized by RhlA enzyme, which is converted to mono-rhamnolipid by RhlB enzyme [17]. At last, the mono-rhamnolipids are converted to di-rhamnolipids by the RhlC enzyme [18]. However, the enzymes that are required to the synthesis of the precursors are present in most of bacteria, excluding the enzymes for the synthesis of HAA, mono- and di-rhamnolipids, which are mostly found in *Pseudomonas* sp. and *Burkholderia* sp. [10].

In addition, the biosynthesis of RLs by *P. aeruginosa* requires water soluble carbon sources, such as glycerol, glucose, mannitol or ethanol, and water insoluble substrates [19]. As a consequence, glucose can be used as a strategy to reduce costs of the production of RLs as it can be converted into the precursors needed for the process [20]. Glucose can be converted into the sugar moiety-deoxythymidine diphosphate (dTDP)-l-rhamnose by catalytic enzymes present in most bacteria [20]. Additionally, the highest yield of RLs production was obtained from oil-type carbon sources as they can be degraded through the β oxidation pathway [21].

Over and above that, there are two major groups of RLs, mono-RLs and di-RLs, also referred to as Rha-C10-C10 and Rha-Rha-C10-C10, respectively [22]. *Pseudomonas aeruginosa* is capable of synthesise a big variety of congeners. However, di-rhamnolipids are

the most abundant compounds produced by this bacterium [23]. Furthermore, it is estimated that around 60 RLs congeners and homologues can be present in the fermentation broth [14].

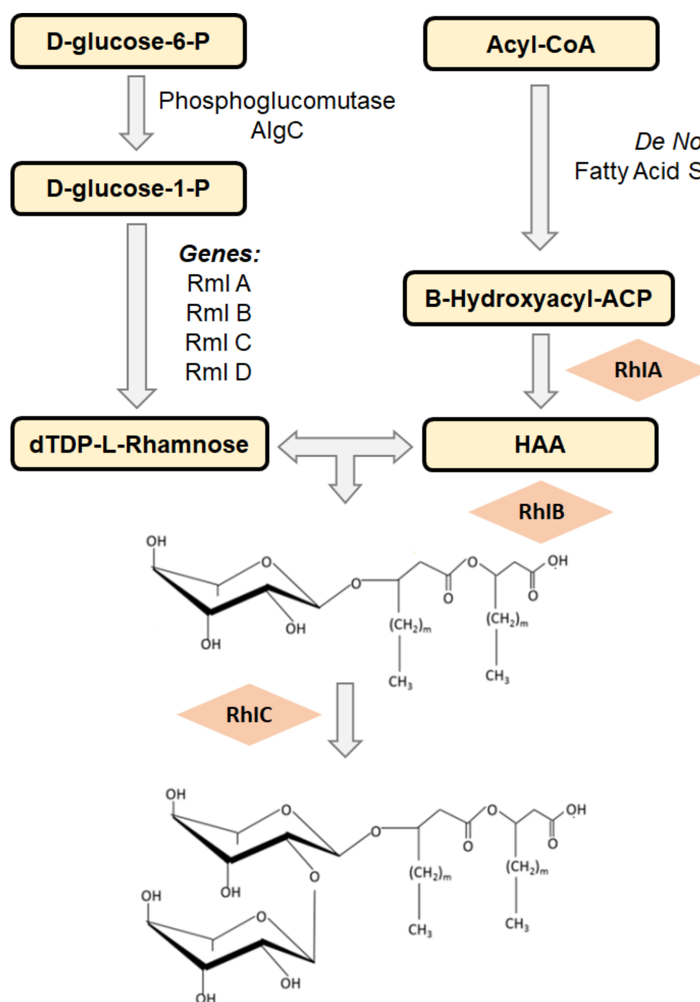


Figure 2. Process of RLs biosynthesis in *Pseudomonas aeruginosa.* Adapted from Chong. H. *et al.* 2017 [10].

Nevertheless, one of the disadvantages of the RLs is the high production cost of these compounds due to the expensive raw materials used in bacterial fermentation and the complex purification process [24]. As a consequence, many strategies have been carried out in order to increase yield and reduce costs [24], such as the optimization of the production conditions and the application of different processes (e.g. cultivation of *P. aeruginosa* in medium containing inexpensive soybean oil refinery [25]; production of RLs and surfactin from olive oil mill waste as carbon source [26]), the use of genetic engineering techniques (e.g. introduction of different key genes for RLs biosynthesis in E. *coli* [27]) and the screening of new natural producing strains (e.g. production of RLs from *B. thailandesis* [28]). Apart from this, the development and implementation of bioconversions and bioprocess optimization in small-scale approach as shaken flasks and microtiter plates have been extensively used. Microtiter plates

approach has appealing features such as the possibility of performing simultaneously a large number of experiments under similar conditions and the possibility of cost reduction because of the small volumes used and time reduction. Additionally, a rapid translation to other process scales is possible with this approach [29].

Moreover, RLs have shown potential applications in many industrial fields such as bioremediation of petroleum at contaminated sites, bioremediation of heavy metal at contaminated lands and pesticides [30 - 32]. Furthermore, these compounds have also shown applications in cosmetics, food processing, and pharmaceuticals because of their excellent surface activities and biological activities [30] [33]. Due to this fact, RLs have been studied for the synthesis of nanoparticles [34]. Lastly, RLs antitumor activity has recently been studied. It was reported that RLs have shown considerable sensitive toxicity on human breast cancer cells (e.g. cytotoxic activities of Rha-Rha-C10-C10 and Rha-Rha-C10-C12 isolated from *P. aeruginosa* B189, against human breast cancer cell lines [35]).

As mentioned before, a variety of studies have shown that other gram-negative bacteria, as *Burkholderia* spp., are also capable of RLs production (*B. thailandensis*, *B. plantarii* and *B. kururiensis*) [36 - 38]. First isolated from the soils and stagnant waters of central and north-eastern Thailand [39], *Burkholderia thailandesis* has been vastly studied because of the safety and non-infectious capacities [36]. This bacterium does not require biosafety level 2 conditions as well as there is no restriction of the use of antibiotic-resistance markers for its genetic manipulation [36]. The production of RLs is possible to occurs by *B. thailandesis* E264, once it contains the gene orthologs RhlA, RhlB and RhlC, which specifically are responsible for their biosynthesis [40].

Apart from that, microbial biofilms have become prevalent in medical, industrial and environmental settings, causing undesirable effects due to their pathogenicity and resistance to antibiotics [41]. By this reason, medical devices predispose patients to device-related infections due to microbial biofilms [42]. Healthcare-associated infections (HAIs), also designated as nosocomial infections, occurs during the process of care in a health care facility which was not present or incubating at the time of admission. [43]. These infections first appear 48 hours or more after hospitalization or within 30 days after having received health care services [44].

According to the report of the point of prevalence survey (PPS) of healthcare-associated infection and antimicrobial use in European acute care hospitals elaborated by the European Centre for Disease Control and Prevention (ECDC) in 2011-2012, it is estimated that the total annual number of patients with a HAI in European acute care hospitals was 3.2 million [45].

The contamination of medical devices may be due to gram-positive or gram-negative bacteria or yeasts. *Staphylococcus aureus*, *Staphylococcus epidermis*, *Pseudomonas*

aeruginosa, *Escherichia coli*, *Clostridium difficile and Klebsiella* spp. are the most common bacteria isolated from medical devices and these microorganisms can be transferred to the devices throw the skin of patients or healthcare workers, contaminated water, as well as other sources in the environment [45 - 46].

Additionally, the most common HAI types were urinary tract infections, pneumonia, surgical site infections, bloodstream infections and gastro-intestinal infections as mentioned in the ECDC PPS [45]. The occurrence of these HAI is due to urinary catheters, intravenous infusion devices, hemodialysis and respiratory therapy equipment since these devices are commonly used in clinical practice [42].

As a consequence, a wide variety of approaches have been studied along the years and one of the most promising strategy for bacterial inhibition is the use of biosurfactants, such as RLs, due to their anti-adhesive and disruptors properties [47 - 48].

This project aims to determine the differences in RLs production by *B. thailandensis* E264 grown in different culture medium supplemented with different carbon sources (i.e. coconut oil and glycerol). The evaluation of these parameters was done by assessing the growth of microorganism and the production of RLs under the microscale approach. Furthermore, other purpose of this study is the isolation and characterization of the RLs produced by *B. thailandesis,* using the high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) to determine the major congeners produced by this microorganism. Lastly, this project aims to determine if the compounds that are produced can have antimicrobial properties against *S. aureus.* This capacity was evaluated by the determination of the minimum inhibitory concentration. In addition, it was investigated the capacity of the biosurfactants RLs to prevent biofilms formation on silicone rubber by the crystal violet assay.

2. Materials and Methods

2.1. Chemicals/Reagents

The following chemicals were used: ethanol absolute, ethyl acetate, hydrochloric acid and chloroform from Carlo Erba Reagents (Rodano, Italy); sodium chloride, sodium sulphate, crystal violet and resazurin sodium salt from Sigma Aldrich (St. Louis, USA); anthrone, methanol and sulfuric acid 95–97 % from Merck (Darmstadt, Germany); acetic acid glacial from Fisher Chemical (Leicestershire, United Kingdom); coconut oil from local supermarket; glycerol from Uniquema (Lisbon, Portugal); agar bacteriological, peptone, trypto-casein soy broth (TSB), tryptone, yeast extract and Müeller-Hinton broth (MHB) from Biokar Diagnostics (Beauvais, France); meat extract from Oxoid (Hampshire, United Kingdom); Furthermore, ultrapurified sterile water from a Mili-Q system was used to perform the assays.

2.2. Microorganisms and culture conditions

Burkholderia thailandesis E264 DSMZ 13276 was obtained from Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen DSMZ and stocks were stored at -80 ºC. Microorganisms were cultured in tryptic soy agar (TSA) and were incubated (Incubator REVCO ultima) for 24 h at 25 ºC. Working stocks were preserved at 4 ºC on TSA cultures for a maximum of one month.

All culture media used were sterilized in a vertical autoclave, 121 ºC for 15 min (Mediline Italia) and aseptically prepared (PBI mini flow chamber).

In the pre-cultivation, eight colonies of *B. thailandensis* were inoculated in 20 mL of Luria-Bertani (LB) culture medium (10 g L^{-1} of tryptone, 5 g L^{-1} of yeast extract, 5 g L^{-1} of sodium chloride) or Nutrient Broth (NB) culture medium (10 g L^{-1} of peptone, 5 g L^{-1} of meat extract and 5 g L^{-1} of sodium chloride) and incubated for 48 h at 25 ºC on an orbital shaker (200 rpm).

For RLs production, a volume of the 48 h culture was adjusted to an optical density of 0.6 in culture medium at 600 nm (OD600) using a Microplate Multimode Detector (Anthos, Zenyth 3100) and used as inoculum for RLs production. The final inoculum charge in the production medium (e.g LB or NB) was 10 % (v/v) and different hydrophobic and hydrophilic carbon sources were added, namely, coconut oil and glycerolin a final concentration of 2 % (v/v).

Growth and RLs production were studied in a microscale approach and for that 24-well microtiter plates (MTPs) were used. Each well contained 900 µL of medium and 100 µL of inoculum. Non-inoculated culture media supplemented of each lipid source was used as blank. For RLs production, the plates were maintained at 25 ºC on an orbital shaker at 150 rpm for 169 h. All assays were carried out in triplicate.

RLs production was also performed in erlenmeyer flasks (200 mL) with 50 mL of LB or NB media supplemented with different carbon sources in a percentage of 2 % (v/v). All assays were carried out in triplicate.

2.3. *Burkolderia thailandesis* growth

2.3.1. Optical density measurement and colony-forming unit

Optical density (OD) of the samples was measured through time during 168 h. The OD was measured at 600 nm wavelength (OD600) in a Microplate Multimode Detector (Anthos, Zenyth 3100). After each reading, samples were collected to 2 mL tubes and stored at -18 ºC for further studies. An equivalent volume of sterile distilled water was used to replace each sample.

2.3.2. pH measurements

The pH was measured using a Metrohm pH-LL Biotrode electrode in Methrom 744 pH meter. Initially, the calibration of the equipment was made using pH 4 and pH 7 standards. Afterwards, the pH of the samples supplemented with different carbon sources was measured.

2.4. RLs production

2.4.1. Production media contact angle

Reduction of the contact angle, as an indicator of biosurfactant production, was measured by using the sessile drop technique according to Biria D. *et al.* (2013) [49]. The samples of LB and NB culture media supplemented with hydrophobic and hydrophilic carbon sources were previously centrifuged at 5000 rpm for 5 min at room temperature, with the objective of removing the remaining cells in the media. The images were obtained using a digital microscope, RoHS HD Color CMOS sensor, and AMCap software used to capture an image of a 2 µL liquid droplets placed in parafilm (at 0 and 10 min). Images were captured as soon as the drops were placed in the parafilm at room temperature. The contact angle was determined by using the ImageJ software.

2.4.2. RLs extraction and characterization

In the microtiter plates (MTPs) approach the combination of three wells content (3 mL) was made to assure an appropriate amount for RLs detection and quantification. The medium was extracted twice with ethyl acetate (1:1).

Samples were taken from the MTP at each time point and added with 3 mL of ethyl acetate. Then, the samples were shaken in vortex (Vortex mixer RSLab-6Pro) for 1 min and

centrifuged (Allegra 6R Centrifuge) at 4000 rpm for 5 min at room temperature. Two phases appear and the upper phase would be the cell free supernatant. Then the upper phase was removed and transferred to a new empty tube. Subsequently, sodium sulphate is added to adsorb the remaining water in the upper phase and the samples were filtered. Lastly, the extracts were evaporated to dryness on a rotatory evaporateur at 40 ºC followed by nitrogen stream.

In the shake flask approach, the culture medium supplemented with different hydrophobic carbon sources was extracted twice with ethyl acetate (1:1) and centrifuge (Allegra 6R Centrifuge) at 4000 rpm for 10 min at room temperature.

After the extraction, the upper phases were removed and transferred to a new Erlenmeyer. Subsequently, sodium sulphate is added to adsorb the remaining water and the samples were filtered. Lastly, the extracts were evaporated to dryness on a rotatory evaporateur at 40 ºC followed by nitrogen stream.

2.4.2.1 Thin-Layer Chromatography

RLs mixtures obtained were first characterized and separated by Thin-Layer Chromatography (TLC). The TLC analysis of RLs production was performed by using silica gel plates F_{254} from Merck (Darmstadt, Germany) as stationary phase and a mixture of chloroform/methanol/water (65:15:2) as mobile phase [50]. The application volume of the extraction samples was 20 µL (5 µL x 4). A RLs mixture produced by *P. aeruginosa* was used as a standard.

The visualisation of the compound bands was performed by spraying the TLC plate with *p*-anisaldehyde reagent (50 mL of acetic acid, 1 mL of sulfuric acid and 0.5 mL of 4-methoxybenzaldhyde) and heating at 110 ºC for 10 min [50].

2.4.2.2 High performance liquid chromatography with tandem mass spectrometry

Biosurfactant mixtures composition were characterized by high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS), according to Ribeiro I. *et al.* (2013) [50]. A reverse phase Waters Atlantis dC18 column (150 mm ´ 2.1 mm id, and 5 mm particle size) was used at 35 ºC in a Waters Alliance 2695 High Performance Liquid Chromatographer (HPLC) coupled to a Waters Micromass Quatromicro API Tandem Mass Quadrupole Mass Spectrometer equipped with a Waters ESI probe. The mobile phase consisted of a formic acid aqueous solution 0.5 % (v/v) as eluent A and acetonitrile as eluent B. With a flow rate of 0.25 mL min^{-1} the following elution program was used: isocratic 5 min at 50 % of eluent B; gradient for 20 min until 60% of eluent B; gradient for 20 min until 100 % of eluent B; isocratic 10 min at 100% of eluent B and 10 min of equilibrium time. ESI capillary voltage was 4 kV, cone voltage was set at 10 V and source and desolvation temperatures were

adjusted to 120 ºC and 360 ºC respectively. Data acquisition was performed with MassLynx Software [51].

2.5. Silicone-functionalization with RLs

2.5.1. Preparation of silicone rubber samples

FDA approved medical grade silicone rubber from Sove (Lisbon, Portugal) with 0.5 mm thickness was cut into 0.8 x 0.8 cm segments. The silicone squares were washed with deionized water and autoclaved at 121 ºC for 15 min in a vertical autoclave (Mediline Italia) and aseptically prepared in a PBI mini flow chamber.

Adsorption assays were performed in MTPs containing silicone segments. Different RLs mixture solutions with a concentration ranging from 0.1 to 3 mg mL^{-1} (0.1 mg mL^{-1}; 0.375 mg mL^{-1}; 0.75 mg mL^{-1}; 1 mg mL^{-1}; 1.5 mg mL^{-1}; 3 mg mL^{-1}) were used to promote the biosurfactant adsorption onto silicone surface under low agitation (120 rpm) on an orbital shaker at 25 ºC, overnight. On the next day, RLs solutions were removed and the silicone specimens with adsorbed RLs were dried under vacuum for 24 h, using a Vacuum Line Huber TC100.

2.5.2. Silicone specimens characterization

2.5.2.1. Quantification of adsorbed biosurfactant onto silicone surface

RLs incorporation onto silicones was measured by the colorimetric anthrone method previously described by Pontes C. *et al.* (2016) [51]. The method measures the concentration of RLs present in the supernatant, allowing to indirectly assess the concentration that was incorporated in the silicone segments.

Firstly, the anthrone reagent (0.2 %) was prepared by weighting 0.2 g of anthrone that was dissolved in an aqueous solution of sulfuric acid 75 % (v/v).

To obtain a calibration curve, different standards concentrations were prepared (0.05 mg mL^{-1}; 0.1 mg mL^{-1}; 0.2 mg mL^{-1}; 0.4 mg mL^{-1}; 0.6 mg mL^{-1}; 0.8 mg mL^{-1}) by dilution of a RLs standard aqueous solution of 1 mg mL^{-1}.

Afterwards, 1 mL of anthrone reagent was added to each test tube, containing 200 µL of each sample. Each tube test was vortexed (Vortex Mixer RSLab-6Pro) for 1 min and then heated in a water bath at 100 ºC for 9 min. Subsequently, all the test tubes were taken out of the water bath and cooled. From each tube, 150 µL were transferred into a 96-well MTP from Sarstedt and the absorbance was read at 620 nm in a Microplate Multimode Detector (Anthos, Zenyth 3100).

The concentration of RLs in the supernatant was assessed by the least-squares

method, by observing linearity between the concentration of the standard solutions and the absorbance in a calibration curve (that presented an equation $y = mx + b$ and a regression coefficient (r^2)). Assays were performed in triplicate.

2.5.2.2. Contact angle measurements

Contact angle measurements were performed with the sessile drop technique according to Pontes C. *et al.* (2016) [51]. This method was performed by evaluating Milli-Q water drops contact angle on the silicone surface of each functionalized sample. The images were obtained using a digital microscope (RoHS HD Color CMOS sensor) and AMCap software to capture an image of each Milli-Q water drop. Images were captured at time 0 and after 10 min of drop deposition at room temperature. The contact angle was determined by using the ImageJ software.

2.6. RLs antimicrobial activity

2.6.1. Microorganisms and culture conditions

Staphylococcus aureus ATCC 25923 was obtained from the American Type Culture Collection (ATCC) and stocks were stored at -80 ºC. Microorganisms were cultured in tryptic soy agar (TSA) and were incubated (Incubator REVCO ultima) for 24 h at 37 ºC. Working stocks were maintained at -4 ºC on TSA slants for a maximum of one month.

2.6.2. RLs antimicrobial activity towards planktonic bacteria

Firstly, a bacterial suspension was prepared by resuspending 3 colonies in 3 mL of Müeller-Hinton broth and adjusting the optical density to 0.5 McFarland units at 600 nm (OD600) using a UV-vis Spectrophotometer (Ultrospec® 10 Cell Density Meter, Biochrom). A final microorganism's concentration of 5 x 10^5 CFU/mL was used in this assay as described by Pontes C. *et al.* (2016) [51].

Subsequently, to determine the susceptibility of *S. aureus* to RLs produced by *B. thailandesis* (in modified LB and NB media with coconut oil) and to determine the minimal inhibitory concentration of RLs that would inhibit the visible growth of this bacteria, the Microdilution Method in a 96-well microtiter plate was selected. RLs concentrations ranging from 8 mg mL^{-1} to 0.06 mg mL^{-1} were used. In addition, all the assays were performed with negative controls (not inoculated media), positive controls (inoculated media) and media with antibiotic (levofloxacin) ranging from 8 mg mL^{-1} to 0.06 mg mL^{-1} used as assay control. Plates were incubated for 24 h at 37 ºC (Incubator REVCO ultima), Lastly, the resazurin assay (AlamarBlue assay) was performed by adding 20 µL of AlamarBlue (resazurin sodium salt)

and the results were obtained by measuring the absorbance at 600 nm (OD600) in a Microplate Multimode Detector (Anthos, Zenyth 3100).

2.6.3. RLs antibiofilm activity

Firstly, according to Pontes C. *et al.* (2016) [51], inocula were prepared from direct colony suspension of selected strains (24 h slants), adjusted to 1.0 McFarland units and further diluted in BHI medium with glucose at 1% (w/v). In each well of the 24-well MTPs, containing silicone-surfactant specimens, the final bacteria concentration was 3 x 10^6 CFU mL^{-1} and the assayed volume was 1 mL. The 24-well MTPs were incubated at 37 ºC for 24 h under static conditions. Silicone specimens were used with and without inoculated medium to assess assay response to biofilm formation in the absence of an inhibitor and the response to culture media, respectively [51].

Therefore, for the antibiofilm quantification, the crystal violet assay was performed. This is a classical colorimetric assay for biofilm quantification, which allows the bind of crystal violet (a basic dye) to negatively charged surface molecules and polysaccharides in the extracellular matrix.

After the biofilm formation in the silicone specimens, the content of the wells was removed from the 24-well MTP. Subsequently, the wells were washed twice with 1 mL of PBS (phosphate buffered saline). These washes were performed to remove all the non-adherent cells in the wells. Afterwards, 0.5 mL of ethanol 96 % was added for biofilm fixation. Supernatants were removed, after 1 h, and 0.5 mL of crystal violet 0.1 % (v/v) was added for 10 min. Excess of crystal violet was removed by washing the wells three times with 1 mL of water and then the silicone segments were removed to sterile eppendorf´s. The adherent crystal violet on biofilm was solubilized with 500 µL of acetic acid 1% in ethanol under agitation and the absorbance was measured at 595 nm in a Microplate Multimode Detector (Anthos, Zenyth 3100).

3. Results

3.1. *Burkolderia thailandesis* growth

Firstly, *Burkholderia thailandesis* E264 was grown for a period of 168 h under an incubation temperature of 25 ºC with 150 rpm rotatory shaking, using the microtiter plate (MTP) approach. The evaluation of microorganism growth on culture media supplemented with glycerol or coconut oil was performed by inferring *B. thailandesis* colony-forming units (CFU/mL) and pH measurement (Fig. 3 and Fig. 4).

The assessment of CFU/mL was inferred by a mathematic equation ($y = mx + b$) that correlates the absorbance with CFU. This correlation is represented by $y = 10^{-10}x + 0.041$ where y represents the absorbance and x represents the colony forming units (CFU).

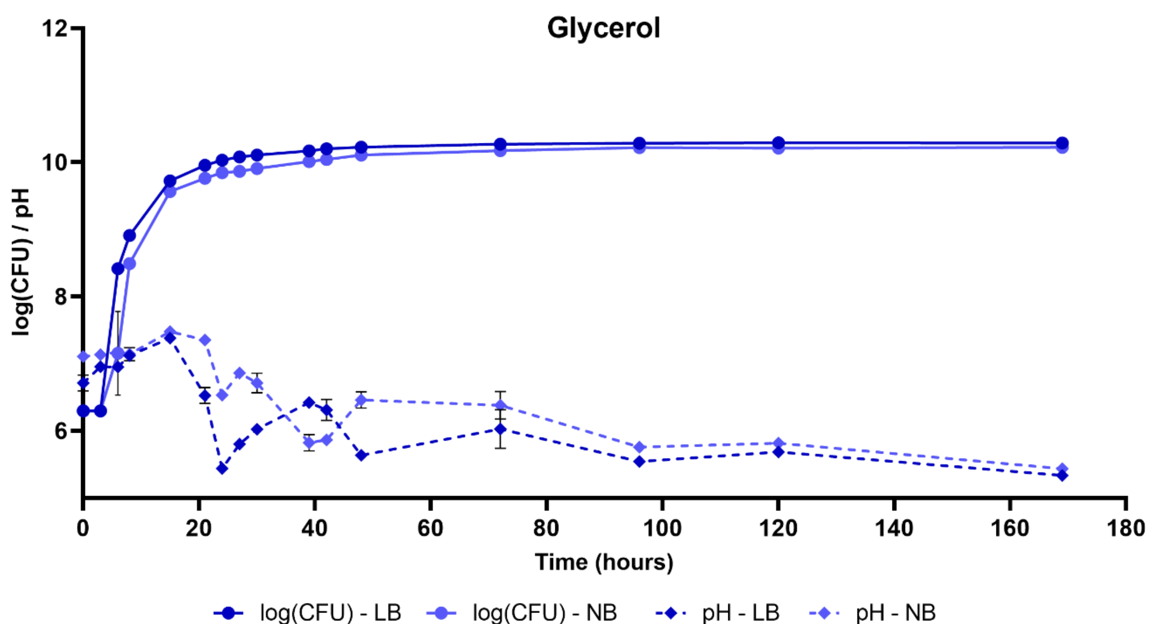


Figure 3. Time course profile of *B. thailandesis* growth (CFU) and pH measurement in LB and NB media supplemented with glycerol.

When comparing the different curves, the growing started increasing rapidly until 24 h and the stationary phase started since then. In addition, the growth with both culture medium shares a similar profile. Moreover, the highest value for the *B. thailandesis* growth in culture media supplemented with glycerol were 10.29 ± 0,003 log units in LB culture media and 10.22 ± 0.01 log units in NB culture media. On the other hand, the highest values obtained in culture

media supplemented with coconut oil were 10.31 ± 0.01 log units in LB culture media and 10.20 ± 0.31 log units in NB culture media.

Additionally, both cultures followed the same growth pattern with the exponential phase occurring within the first 24 h. The stationary phase was observed between the 24 h and 168 h where growth of *B. thailandesis* continued at a slower rate.

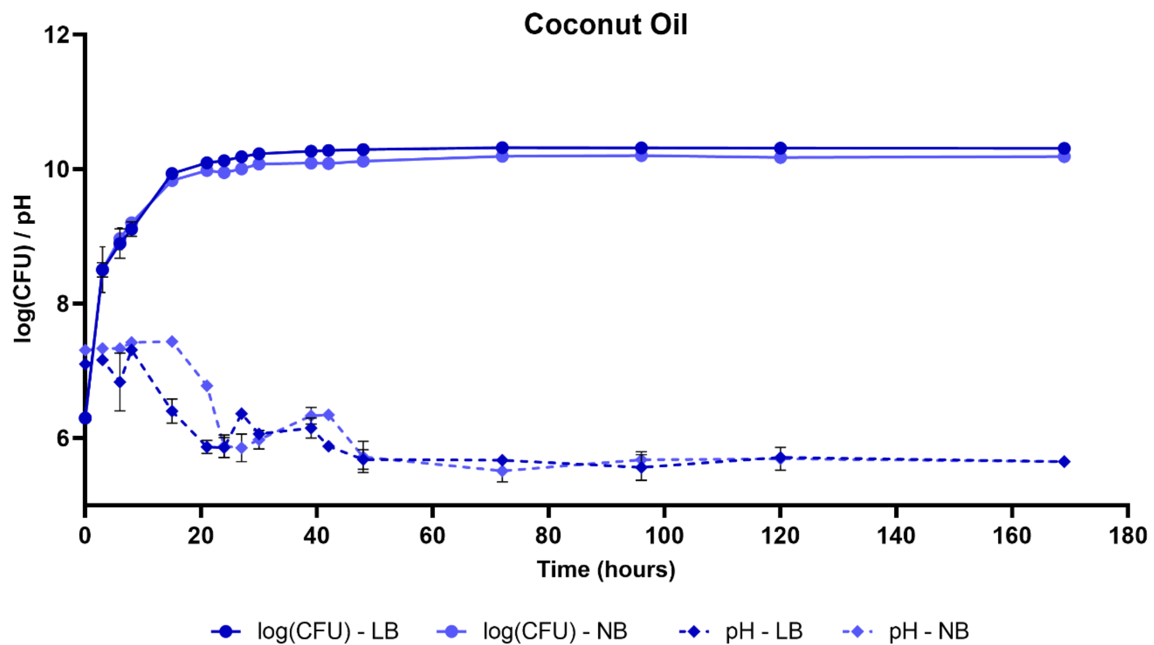


Figure 4. Time course profile of *B. thailandensis* growth (CFU) and pH measurement in LB and NB media supplemented with coconut oil.

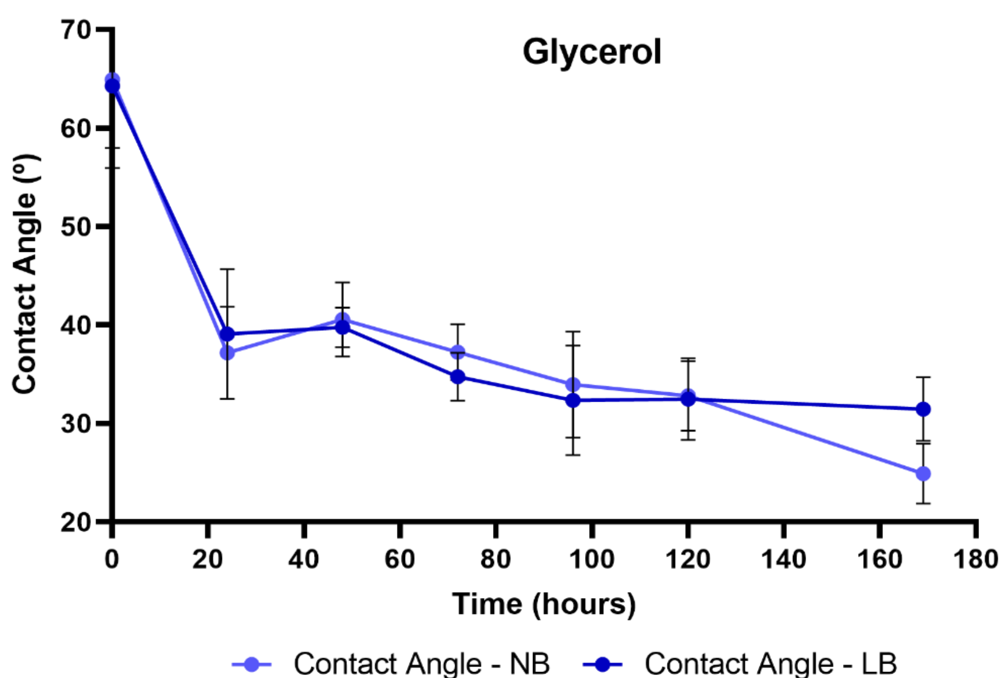
Furthermore, in Fig. 3 and Fig. 4 it is shown the pH profiles from samples obtained from LB and NB media supplemented with glycerol and coconut oil, respectively. This is a parameter used to evaluate the growth of microorganisms since it is influenced by the RLs produced in samples supplemented with hydrophobic and hydrophilic carbon sources by *B. thailandesis* during growth.

Accordingly to the graphics, the pH profile is very similar in both media supplemented with different carbon sources. Firstly, the pH values started around pH 7 and a slightly increase of the parameter occurred up to 8 h in the media supplemented with coconut oil and up to 15 h in the media supplemented with glycerol. Between the 15 h and 21 h a sudden decrease took place in all samples reaching values of 5.8. The decrease of pH values that occurred around 15 h could probably be because of the rapid production of the RLs that are natural sugar fatty acid compounds [52].

3.2. RLs Production

The evaluation of RLs production in culture media supplemented with different carbon sources was made by inferring the contact angle measurement and TLC analysis. The effectiveness of a surfactant can be determined by its ability to lower the contact angle. Due to this fact, this parameter was used as an indirect measure of the RLs production from culture medium supplemented with different hydrophobic carbon sources by *B. thailandesis*.

As shown in Fig. 5 and Fig. 6, the contact angle decreased throughout the 169 h. Additionally, in samples of LB and NB media supplemented with coconut oil, a gradual decreased of the contact angle was observed through the 169 h period, while in samples of LB and NB media supplemented with glycerol, a sudden decreased of the contact angle was noticed at 24 h and no significant oscillations happened until 169 h.



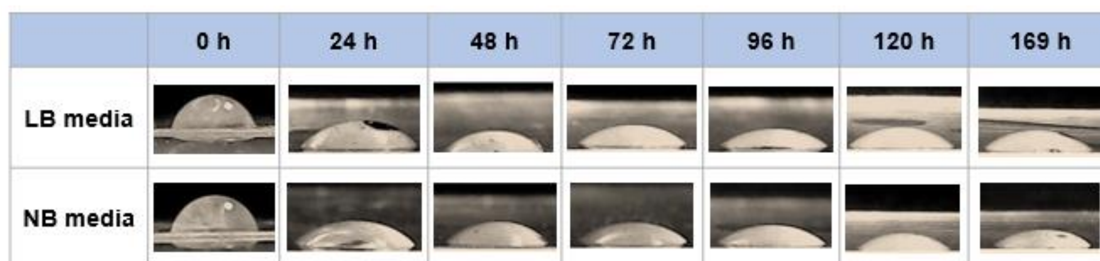
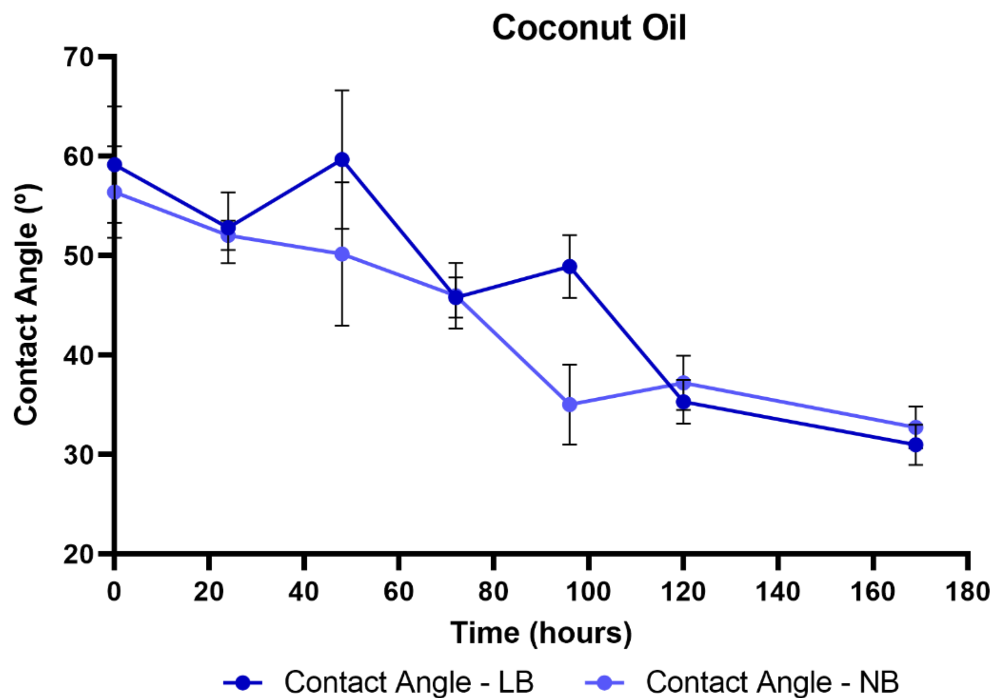


Figure 5. Contact angle of measurements in LB and NB media supplemented with glycerol. Images obtained from AMCap software to determine the contact angle by the sessile drop technique.

The above mentioned results can also be seen in the tables present in Fig. 5 and Fig. 6 where the images were captured show the drops of different samples in LB and NB media supplemented with glycerol and coconut oil, respectively.



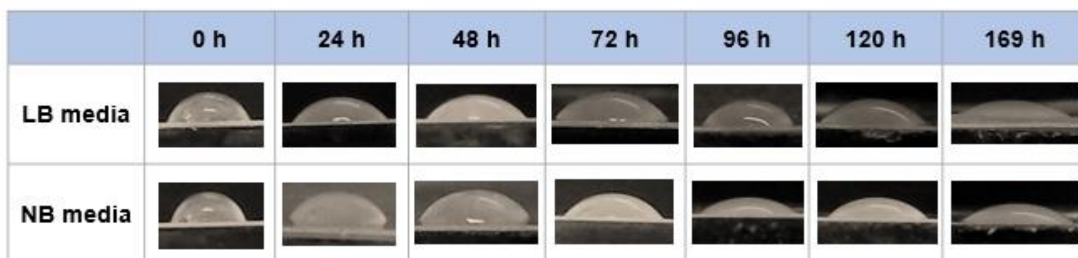


Figure 6. Contact angle of measurements in LB and NB media supplemented with coconut oil. Images obtained from AMCap software to determine the contact angle by the sessile drop technique.

Therefore, to access which media was more suitable to RLs production in both LB and NB media supplemented with coconut oil and glycerol a TLC analysis of extracted samples obtained in the MTPs approach was performed. Samples exhibited more bands than the standard solution of RLs produced by *P. aeruginosa*, which indicated that production of RLs had occurred using the microtiter plates approach (Fig.7)

According to Fig. 7, the TLC of samples obtained from LB and NB media supplemented with glycerol have shown a various number of bands when compared with samples of LB and

NB media supplemented with coconut oil. Addedly, in the samples supplemented with coconut oil, it was observed that the characteristic bands of RLs only appeared more distinguishable in the 169 h samples. In samples of 48 h and 96 h only a big dark band corresponding to coconut oil was seen.

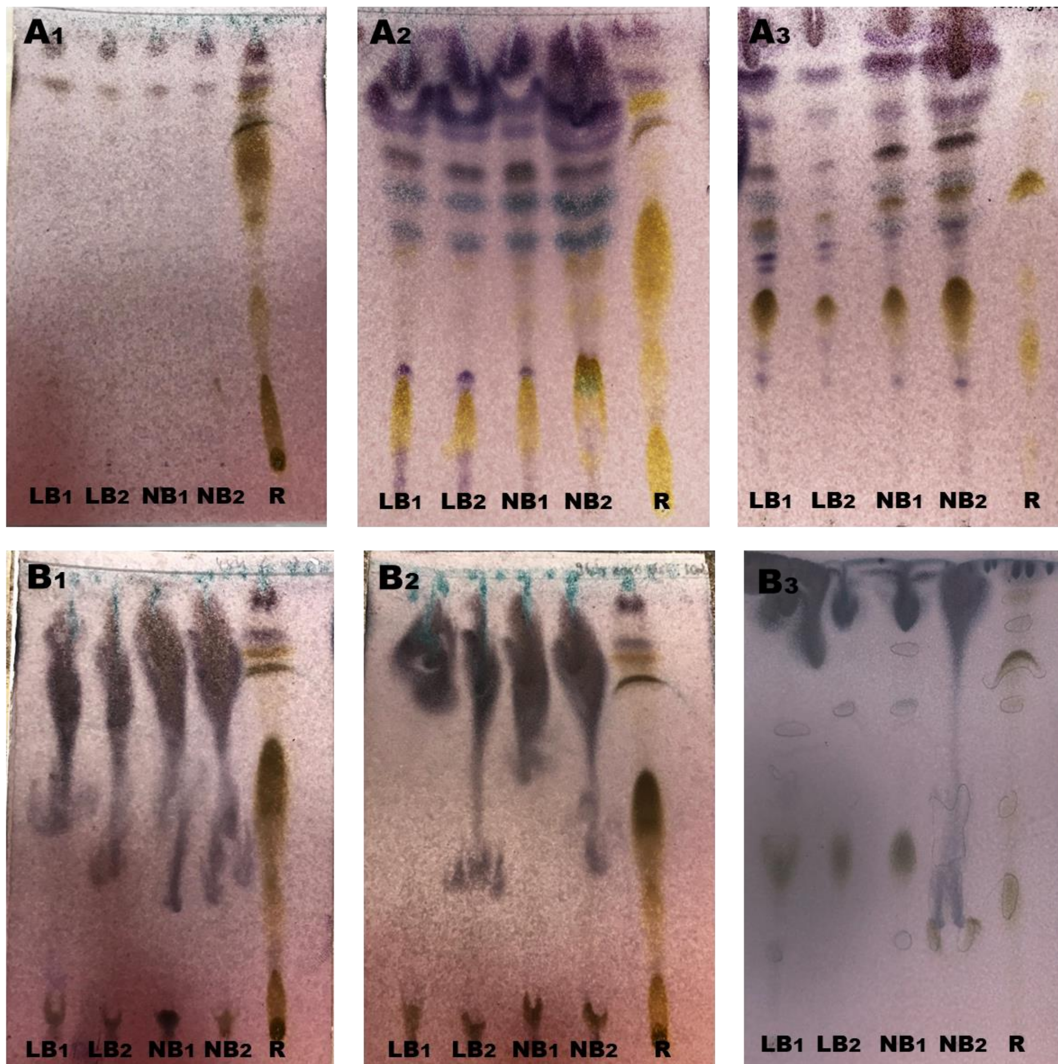


Figure 7. TLC plates from Microtiter Plate Approach at 48 h, 96 h and 169 h of samples in LB and NB media supplemented with glycerol (plates A_1 - A_3) and coconut oil (plates B_1 – B_3) after staining with *p*-anisaldeyde reagent.
(A_1) – Samples of LB and NB media supplemented with glycerol at 48 h; (A_2) – Samples of LB and NB media supplemented with glycerol at 96 h; (A_3) - Samples of LB and NB media supplemented with glycerol at 169 h; (B_1) - Samples of LB and NB media supplemented with coconut oil at 48 h; (B_2) - Samples of LB and NB media supplemented with coconut oil at 96 h; (B_3) - Samples of LB and NB media supplemented with coconut oil at 169 h;

On the other hand, in samples supplemented with glycerol, a high number of bands was seen in samples at 96 h of production. Complementarily, when comparing the samples of NB and LB media supplemented with the different hcarbon sources, the number of bands showed in the TLC assay was similar between the media.

Afterwards, to evaluate the yield of RLs production in LB and NB media supplemented with coconut oil, an extraction was performed twice with ethyl acetate (1:1) in samples obtained by the shake flask approach. Accordingly to table 1, the yield of RLs production in LB media was 0.3 g L^{-1}, while the yield of RLs production in NB media was 5 g L^{-1}.

Table 1. Yield of RLs production by the shaken flask approach of LB and NB media supplemented with coconut oil.

CULTURE MEDIUM	YIELD OF RLS PRODUCTION
LB	0.3 g L^{-1}
NB	5 g L^{-1}

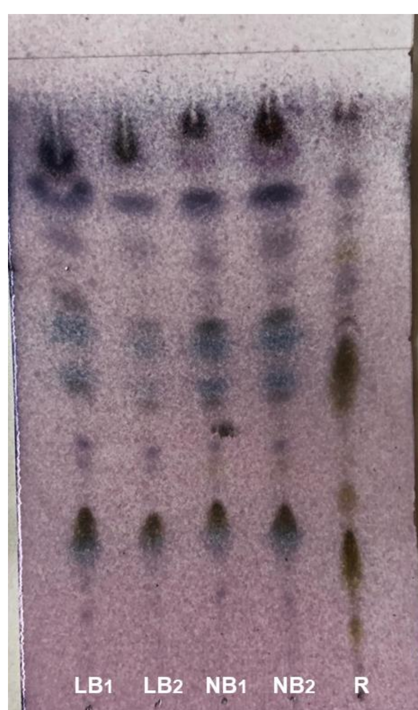


Figure 8. TLC plate from Shake Flask Approach of samples with LB and NB media supplemented with coconut at 169 h after staining with *p*-anisaldeyde reagent.

In last of all, a TLC analysis was performed (Fig. 8) to evaluate the RLs production in samples of LB and NB media supplemented with coconut oil by the shake flask approach. This TLC analysis was also performed with the objective to compare the production between the microtiter plate (microscale) and shake flask (laboratory scale) approach and to validate the microtiter plate suitability on the study of *B. thailandesis* growth and RLs production.

As shown in Fig. 8, samples exhibited diverse bands when compared with the standard solution of RLs produced by *P. aeruginosa*, which indicated that production of RLs had occurred.

3.3. Isolation, analysis, and characterization of RLs

The crude extracts produced by *B. thailandesis* in culture media supplemented with coconut oil were analyzed by liquid chromatography with electrospray ionization mass spectrometry. The mass spectrometric detection was performed in electrospray ionization (ESI) negative mode. The scan chromatogram of the crude showed a wide variety of peaks (Fig. 9). The significant peaks were observed at the retention time of 6.27, 6.93, 7.77, 9.19, 9.94 and 10.86 min.

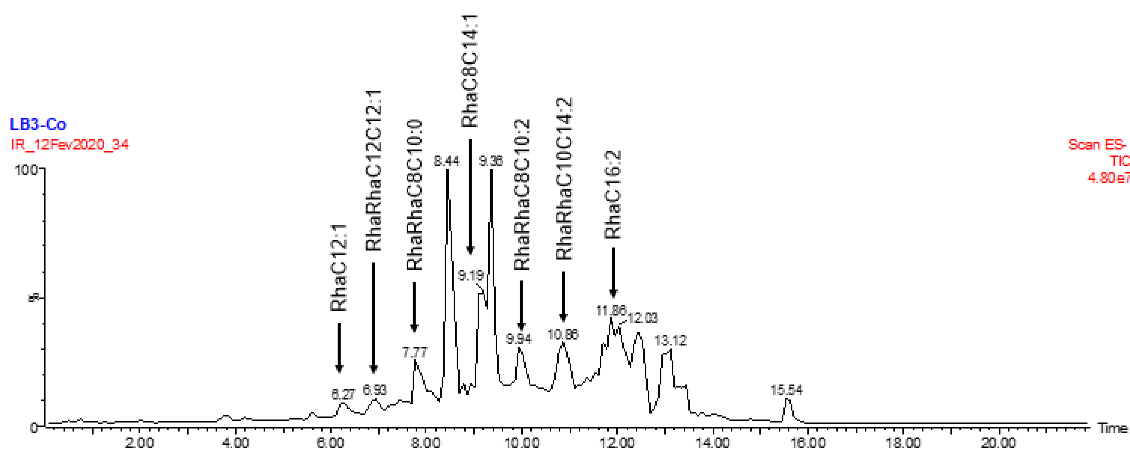


Figure 9. Total Ion Current (TIC) Chromatogram of RLs produced by *B. thailandesis* by HPLC-MS/MS analysis. A reverse phase Waters Atlantis dC18 column was used at 35ºC in a Waters Alliance 2695 HPLC coupled to a Waters Micromass Quatromicro API Tandem Mass Quadrupole Mass Spectrometer equipped with a Waters ESI probe.

The main congener present in the crude extract, with a 50 % relative abundance, has the molecular ion of 530 *m/z* (retention time of 9.19 min) corresponding to the RLs with the empirical formula of Rha-C8-C14:1 (Fig, 10). Succeeding, the second main congener with approximately 40 % of relative abundance, presents the molecular ion at 473 *m/z* (retention time of 11.86 min) corresponding to RhaC16:2.

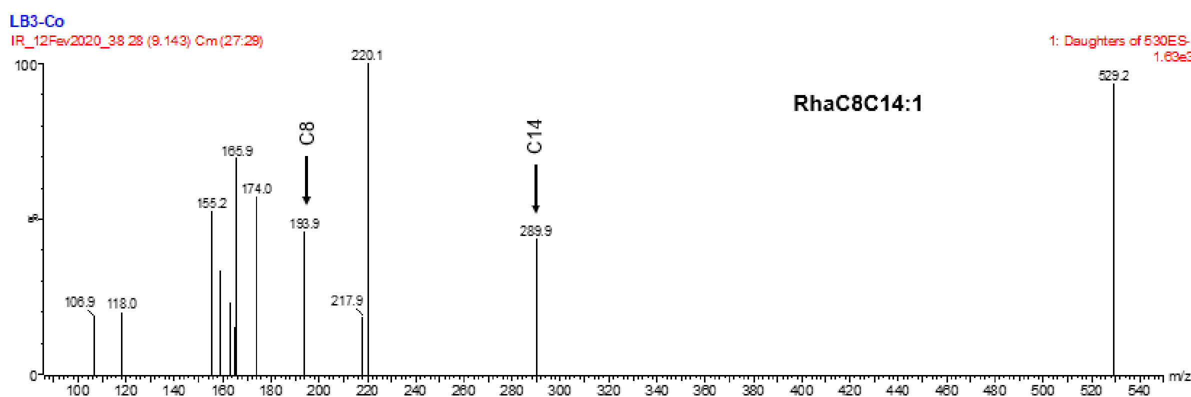


Figure 10. Ion fragments produced upon fragmentation of the mono-rhamnolipid Rha-C8-C14:1 using tandem MS/MS.

Lastly, various mono-rhamnolipids and di-rhamnolipids were identified in the crude produced by *B. thailandesis* in culture media supplemented with coconut oil, such as RhaC12:1; RhaRhaC12C12:1, RhaRhaC8C10:0, RhaC8C14:1 and RhaRhaC14:C14. They differ in the number of rhamnose moieties connected to the lipid part of the molecule. The main congeners are represented in table 2, with the respective RLs identified.

Table 2. Main RLs produced by *B. thailandesis* and identified by HPLC-MS/MS present in RLs production in culture media supplemented with coconut oil.

RETENTION TIME (MIN)	$[MX^-]$	X IN $[MX^-]$	RHAMNOLIPID
6.27	405	$CHOO^-$	Rha-C12:1
6.93	704	H^-	Rha-Rha-C12-C12:1
7.77	621	H^-	Rha-Rha-C8-C10:1
9.19	530	H^-	Rha-C8-C14:1
9.94	677	CH_3COO^-	Rha-Rha-C8-C10:2
10.86	761	CH_3COO^-	Rha-Rha-C14-C14
11.86	473	CH_3COO^-	Rha-C16:2

3.4. Quantification of adsorbed surfactants onto silicone

The RLs incorporation into the silicone segments was measured by the colorimetric anthrone method, which would measure the concentration of RLs present in the supernatant. Indirectly, it is possible to know the concentration that was adsorbed in the silicone specimens.

Firstly, it was made a calibration curved, represented in the Fig. 11, with standard solutions between 0.05 mg mL^{-1} and 1 mg mL^{-1}. The equation of $y = 0.6927x + 0.0342$ regression coefficient of 0.9979 were obtained plotting the values of the standard solutions (0.05 mg mL^{-1}, 0.1 mg mL^{-1}, 0.2 mg mL^{-1}, 0.4 mg mL^{-1}, 0.6 mg mL^{-1}, 0.8 mg mL^{-1}, 1 mg mL^{-1}).

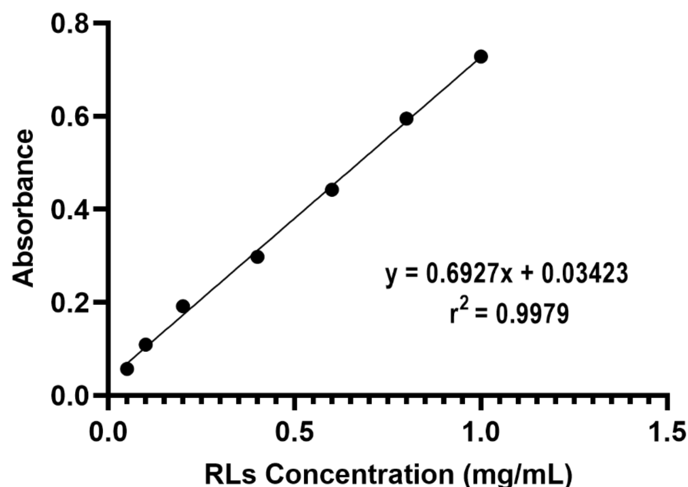


Figure 11. Calibration curve of RLs quantification by the colorimetric anthrone method, using standard solution in ultrapurified water (0.05 mg mL^{-1}, 0.1 mg mL^{-1}, 0.2 mg mL^{-1}, 0.4 mg mL^{-1}, 0.6 mg mL^{-1}, 0.8 mg mL^{-1}, 1 mg mL^{-1})

Subsequently, it was determined the RLs concentration in different samples with the objective of calculating the incorporation capacity (Fig. 12) of the silicone specimens. This assay allowed to determine the maximum quantity of RLs that were incorporated. The adsorption of RLs happened in all silicone rubbers, being that this parameter increased with the concentration of RLs.

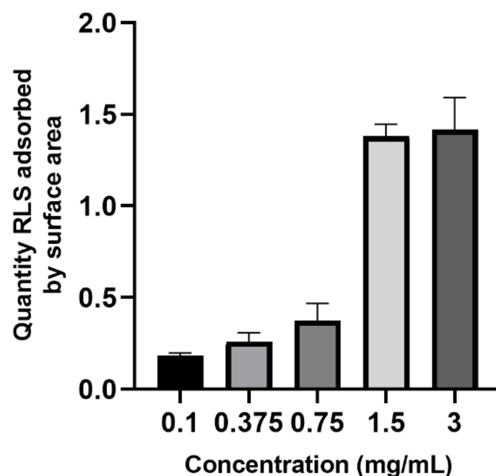


Figure 12. Incorporation Capacity of RLs into the silicone rubbers considering different concentrations of the biosurfactant.

The quantity of RLs absorbed by surface area had the maximum value of 1.4157 ± 2.29E-01 for the concentration 3 mg mL^{-1}. However, the quantity of RLs absorbed stabilize at the 1.5 mg mL^{-1} (1.3780 ± 6.68E-02).

3.5. Characterization of silicones with adsorbed surfactants

Additionally, the wettability properties of the silicone specimens with RLs adsorbed onto their surface were determined through the contact angle measurements with Mili-Q water (Fig. 13).

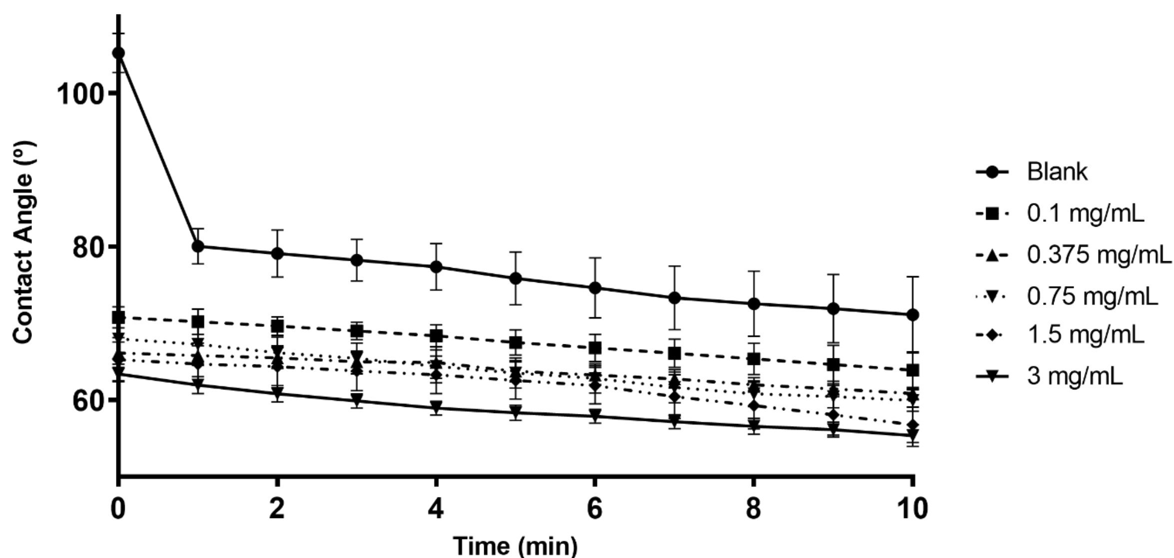


Figure 13. Contact angle measurements of Mili-Q water into silicone specimens with RLs adsorbed

In accordance to Fig. 13, the contact angle in RLs-functionalized surfaces decreased in a concentration-dependent manner. This parameter was used as an indirect measure of the RLs adsorption onto the silicone rubbers.

Additionally, by observing Fig. 13, a gradual decreased of the contact angle was observed through the period of 10 minutes in the selected range of concentrations.

The above mentioned results can also be seen in Fig. 14 where the images captured of the drops of Mili-Q water into the silicone without RLs adsorption and into the silicone with the RLs concentration of 3 mg mL^{-1} after 10 min.

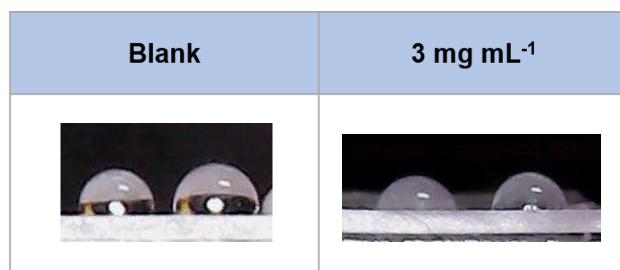


Figure 14. Images obtained from AMCap software to determine the contact angle of Mili-Q Water in silicone specimens with RLs adsorbed.

3.6. Assessment of antimicrobial activity of RLs

After the study of RLs production by *B. thailandesis*, it was possible to study the *S. aureus* susceptibility towards these compounds, by using the assay of the determination of the minimum inhibitory concentration. The choice of this bacteria is related to the fact that *S. aureus* is known to cause infections related to medical devices, particularly venous catheters, which are responsible for several dangerous and systemic infections [42].

Table 3. Minimum inhibitory concentration (MIC) of RLs in LB and NB culture media supplemented with coconut oil.

CULTURE MEDIUM	MIC
LB	0.250 µg L^{-1}
NB	0.125 µg L^{-1}

Accordingly, to this, it was important to determine the minimal inhibitory concentration which stands for the concentration of RLs where there is no microbial growth. After incubation for 24 h at 37ºC, a visual inhibition was noticed at a concentration of 0.250 µg L^{-1} and 0.125 µg L^{-1} for RLs produced by *B. thailandesis* in LB and NB culture media supplemented with coconut oil, respectively.

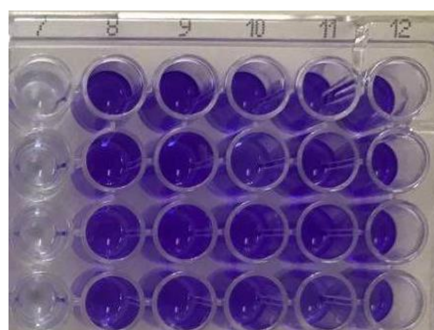


Figure 15. Crystal violet assay to determine the RLs antimicrobial activity (concentrations: 0.1 mg mL^{-1}, 0.375 mg mL^{-1}, 0.75 mg mL^{-1}, 1.5 mg mL^{-1}, 3 mg mL^{-1}) against *S. aureus.*

Moreover, the crystal violet staining method was used to assess the biofilm formation under the different experimental conditions. Firstly, in the pre-treatment of the silicone specimens, it was possible to verify that the *S. aureus* adhesion decreased in a concentration-dependent manner. However, the RLs-functionalized surfaces only inhibited *S. aureus* biofilm formation when concentrations higher than 3 mg mL^{-1} were used, as shown in Fig. 15 (wells D2 – D5). Additionally, the samples with the concentration of 3 mg mL^{-1} inhibited *S. aureus* biofilm formation in an extension of only 18%, when compared to control (Fig. 16).

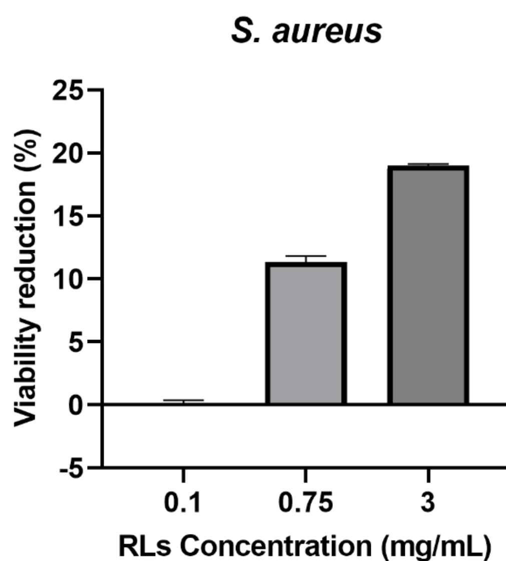


Figure 16. Reduction of biofilm viability by RLs in *S. aureus* (crystal violet assay).

4. Discussion

The main goals of this study were the optimization of *B. thailandesis* growth and RLs production, using different culture media and carbon sources (hydrophobic and hydrophilic), as well as using two different type of approaches, and the determination of the antimicrobial properties of RLs and their viability to reduce the biofilm formation.

For this purpose, firstly it was studied the best strategies for the *B. thailandesis* growth and the RLs production. In the first assay, the study of *B. thailandesis* growth and RLs production was carried out by using the microtiter plate (MTP) approach as microreactors in the selective production of RLs through the utilization of different samples supplemented with two different carbon sources, glycerol and coconut oil. Accordingly to Chong H. *et al.* (2017), the use of economic carbon sources such as glucose, glycerol, mannitol and vegetable oils can be used to replace some conventional substrates [10]. Additionally, in this study, the CFU results represent the number of cells in the samples, which allows the determination of the growth curves. In conclusion, although the best performance of microorganism growth was in LB media supplemented with coconut oil in this experiment as it exhibited with the highest log (CFU) value, the results were very similar between the different culture media and carbon sources used.

Subsequently, the contact angles measurements were used to characterize the RLs production. The drops with low concentrations of RLs remained stable during time. In contrast, the drops containing higher concentrations of RLs collapsed easily. As expected, the contact angles decreased with an increase in surfactant concentration until a limiting value of the surface tension was reached at some critical concentration [53].

Moreover, when comparing the laboratory scale and microscale approach, it is possible to conclude that both methods are suitable for RLs production. This can be proven by comparing TLC plates with samples of 169 h from the shake flask approach in LB and NB media supplemented with coconut oil (Fig. 8) and from microtiter plate approach (Plate B_3 in Fig. 7). It was possible to conclude that RLs productions occurred in the plate B_3 in Fig. 7, which proved that the mini-scale approach (MTPs approach) could be used as a method to study the growth of *B. thailandesis* and RLs production.

However, it was possible to see more distinguished bands in the samples obtained by the shake flask method as well as darker bands (Fig. 8). This indicated that even the microtiter plate approach (microscale) was a suitable method to study the RLs production by *B.*

thailandesis, the production was increased in the shake flask (laboratory scale). By this reason, it is possible to conclude that the thin chromatography method is a very common and useful method used to analysis glycolipids such as RLs. However, in order to identify and characterize these compounds, it is necessary to use more specific methodologies [50].

Subsequently, about the isolation and characterization of RLs, the separation of the RLs derivatives is made by the HPLC system, while MS can reveal the molecular weight as well as some structural information of RLs congeners when compared with the reported MS data [54]. Therefore, in accordance with Funston S. *et al.* (2016) [55], a peak representing a molecular ion of 761 *m/z* (retention time of 10.86 min), which correspond to the di-rhamnolipid C14-C14 was shown in the analysis of the crude extracts produced by *B. thailandesis* [55]. Moreover, Elshikh M. *et al.* (2017), refers that the main congener present in the crude extract is the long chain di-rhamnlipid C14-C14, which is not compatible with the results presented in this study, due to the fact that the main congener identified was the mono-rhamnolipid C8-C14:1 [13].

Therefore, the absorbance of RLs onto medical grade silicone was studied with two main goals: as modifiers of surface properties for preventing bacteria adhesion and as biocide towards colonizable bacteria [51]. To assure differences on biosurfactant coating extension on silicone, distinct RLs concentrations were tested to promote the RLs adsorption onto the material [51]. The coating with RLs was confirmed by the contact angle measurements with Milli-Q water. The results have shown that the Milli-Q water drops on silicone specimens with higher concentrations of RLs adsorbed collapsed easily, when compared to the blank sample (silicone specimens without RLs adsorbed).

Lastly, referring to the antimicrobial properties of RLs, the *in vitro* antibiofilm assay was performed using *S. aureus,* one of the the most frequently microorganisms isolated from HAIs by the point of prevalence survey (PPS) of healthcare-associated infection and antimicrobial use in European acute care hospitals elaborated by ECDC in 2011-2012 [45]

Accordingly to Shen Y. *et al* (2020) [56], gram negative bacteria are less susceptible to RLs, due to the fact of having thicker cell walls. In contrast, they show better antimicrobial activity against gram-positive bacteria as *S. aureus*. It is also mentioned that the biofilm formation involves different intermolecular forces (van der Waals forces, surface hydrophobicity and Lewis acid-base interactions, which allows RLs, as surfactants, position themselves between the bacteria and the surface, inhibiting bacterial adhesion. Additionally, the efficacy of antibiofilm agents depends on bacteria characteristics as surface hydrophobicity

and zeta potential [56]. Furthermore, the mechanism behind RLs antibacterial activity resides in the interaction of these compounds with the cell membrane and disintegration of this structure that leads to penetration of cell wall and plasma membrane by formation of pores on cell membrane and leakage of inner cytoplasmatic content leading to cell death [57].

In the present study, RLs produced by *B. thailandesis* only showed a reduction of biofilm viability of 18 % when used a concentration of 3 mg mL^{-1}, which is in conformity with the results in Shen Y. *et al.* (2020) that demonstrated only a reduction of approximately 40 % when used a concentration of 5 mg mL^{-1} [56]. Moreover, in Silva S. *et al.* (2017), the percentage of S. aureus biofilm removal after 2 h of treatment with RLs at 25 ºC was only 35 % when using 0.1 % concentration [58]. The low reduction of the biofilm viability can be due to the composition of the extracellular polymeric substances of the bacterial biofilm created by *S. aureus* [56].

5. Conclusion

This study investigated a wide variety of parameters, starting at the optimization of the RLs biosynthesis using different culture media (LB or NB) supplemented with different hydrophobic and hydrophilic carbon sources. Additionally, two different pathways for the biosynthesis were used. On the one hand, the laboratorial scale approach (shake flask technique) and on the other hand the microscale approach (microtiter plates). Furthermore, in the shake flask approach, NB culture medium supplemented with coconut oil was shown to be the one with better yield of production, when compared with the LB media supplemented with the same hydrophobic carbon source. However, when the analysis of the thin layer chromatography was made, RLs production was better in samples with culture media supplemented with glycerol in the microtiter plate approach due to the number and shade of bands.

Subsequently, it was made the identification of the main constituents of the RLs in the biosynthesized mixture by HPLC-MS/MS. It was used a crude extract of RLs produced in culture media supplemented with coconut oil. Different congeners were observed, being that the most abundant one is the mono-rhamnolipid C8-C14:1 with a relative abundance approximately of 50 %.

Lastly, it was studied the antimicrobial properties of RLs and their viability to reduce biofilm formation. For this purpose, minimum inhibitory concentration assay and crystal violet assay were performed using *S. aureus* as the pathogen. The first method had demonstrated that RLs were capable of inhibit *S. aureus* growth, which makes them a possible approach to prevent medical devices related infections. However, in the second assay, the reduction of the biofilm formation was lower than expected, with only a percentage of 18 % for the inhibition of *S. aureus* biofilm formation.

Therefore, this work has represented a step towards the exploration of biosurfactants, such as RLs, in the creation of new approaches for bacterial biofilm inhibition. Nevertheless, further investigations will be carried out with the objective of study the RLs production in culture medium supplemented with different hydrophobic and hydrophilic carbon sources. In addition, antimicrobial properties of RLs will be studied against other pathogens, such as *E. coli*, *P. aeruginosa* and *Klebsiella spp.*.

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