Universidade de Lisboa

Faculdade de Farmácia

PRODUCTION OF TREHALOSE LIPIDS TOWARDS ANTICANCER APPLICATION

Sara Godinho Lopes

Dissertation supervised by Professor Doutora Maria Henriques Lourenço Ribeiro and co-supervised by Professor Doutor Nuno Guerreiro Oliveira

Master in Biopharmaceutical Sciences

2021

Universidade de Lisboa

Faculdade de Farmácia

PRODUCTION OF TREHALOSE LIPIDS TOWARDS ANTICANCER APPLICATION

Sara Godinho Lopes

Dissertation supervised by Professor Doutora Maria Henriques Lourenço Ribeiro and co-supervised by Professor Doutor Nuno Guerreiro Oliveira

Master in Biopharmaceutical Sciences

2021

Abstract

Having in mind the current environmental concerns in the planet, it is known that it is necessary to investigate and invest in new biologic and eco-friendly compounds that are able to substitute pollutant chemicals.

In this perspective, this work focused on the optimization of the production of new threhaloselipids (TLs) regarding the microorganism's source, medium composition and strategies of growth conditions, as well as the purification conditions and evaluation of the biological activity, as anticancer agents.

The TLs were produced by *Rhodococcus erythropolis* and *Rhodotorula*. This production was carried out with different media, glucose based media and salt media supplemented with glucose, glycerol, *n*-hexadecane or *n*-dodecane in different concentrations (2 and 4%). The production was optimized at 48h for both microorganisms, in order to increase the productivity of the TLs. The glucose consumption during the growth of *R. erythropolis* rounded the 70% but in *Rhodotorula* depended on the initial glucose concentration. For the first time *Rhodotorula* sp. was identified as a producer of trehaloselipids

The extraction was performed through liquid-liquid extraction using a solvent based solution of ethyl acetate and methanol. After drying the solvent, the compound was submitted to a series of tests for the identification and characterization of the produced compounds. TLC, HPLC and UHPLC were the analytical methods used to ensure the presence of the developed TLs. Additionally, conductance, surface tension and critical micellar concentration were determined.

To test their biological activity, as anticancer agents the TLs were solubilized in NaCl (0.9%) submitted to the MTS assay in H1975 non small cell lung cancer cells (NSCLC). It was not possible to observe any cytotoxic effects at the different concentrations of both compounds tested. Nevertheless, these results do not preclude the possibility of continuing the exploration of TLs as a cancer therapeutic in different cancer cells in the future.

Keywords**:** Biosurfactant, trehaloselipid, cancer, *Rhodococcus erythropolis, Rhodotorula* sp

Resumo

Nos dias de hoje, uma das principais preocupações no planeta é a transformação e preservação da Terra como um planeta sustentável. Tendo em conta esta preocupação com o meio ambiente, muitas indústrias estão gradualmente a mudar a visão global sobre o futuro do processo de fabrico. No caso dos surfactantes, que são compostos com várias propriedades e funções, como a capacidade de diminuir a tensão superficial entre substâncias, são produzidos principalmente por síntese química e estão atualmente a ser utilizados em grande escala em diversas indústrias. No entanto, estes compostos não são biodegradáveis e são bastante tóxicos para o meio ambiente. Assim sendo, a indústria atual começa, não só a perceber, como também, a utilizar o potencial das células vivas para um fabrico mais sustentável. Hoje em dia as células vivas são já utilizadas em alguns processos como é o caso do pré-tratamento de matérias-primas, das operações de processamento, das modificações de produtos, da gestão coletiva seletivo de resíduos, da reciclagem e da conservação de energia. Assim, as indústrias começam a substituir os surfactantes pelos biossurfactantes. Estes têm na sua composição uma parte hidrofílica que consiste em aminoácidos ou peptídeos, aniões ou catiões; mono-, di- ou polissacarídeos; e uma parte hidrofóbica que consiste em ácidos insaturados, saturados ou gordos. Os biossurfactantes, tal como os surfactantes têm a capacidade de diminuir a tensão superficial entre dois líquidos, entre um gás e um líquido, ou entre um líquido e um sólido. No entanto, podem ser produzidos por microrganismos a partir de recursos renováveis como solo, água ou óleos. Não se mantendo apenas a propriedades semelhantes aos surfactantes os biossurfactantes apresentam uma concentração micelar crítica entre dez e quarenta vezes mais baixa que a dos surfactantes sintéticos, o que quer dizer que uma menor quantidade de produto é precisa para que a tensão superficial diminuía. Acrescenta-se ainda que os biossurfactantes com um baixo peso molecular são mais suscetíveis a ter boas propriedades de superfície ativa, enquanto os de alto peso molecular são mais eficazes na estabilização de emulsões. De um modo geral, os biossurfactantes apresentam uma eficácia constante numa ampla faixa de temperatura e pH. Isto faz com que estas substâncias possam ser submetidas a condições extremas. No entanto, a produção de biossurfactantes em larga escala apresenta ainda algumas limitações, nomeadamente o alto custo de matéria-prima, o baixo rendimento e o alto custo de purificação. De um modo geral, os biossurfactantes podem ser divididos em cinco categorias principais, os

glicolípidos, os ácidos gordos / fosfolipídios, os lipopeptídos / lipoproteínas, as partículas poliméricas e os surfactantes.

Os glicolípidos são a categoria mais comum e por consequência a mais estudada entre os biossurfactantes, devido ao seu alto potencial de aplicação e alto rendimento de produção. Eles são glúcidos combinados com ácidos alifáticos de cadeia longa ou ácidos hidroxialifáticos. Os glicolípidos biossurfactantes apresentam excelentes propriedades tensoativas, incluindo a emulsificação, a dispersão, a solubilização, a formação de espuma, a penetração e a ação humectante. Os glicolípidos com um ou dois resíduos de açúcar ligados a diferentes estruturas lipídicas podem ser encontrados nas membranas celulares de bactérias, fungos, plantas e animais na forma de esterilglicosídeos, glicosilceramidas e diacilglicerolglicosídeos. Os glicolípidos podem ser divididos em várias categorias sendo as mais conhecidas os ramnolípidos, os soforolípidos, os trehaloselípidos e os manosileritritol lípidos.

Os trehaloselipidos (TLs) são conhecidos por ter um dissacarídeo não redutor no qual as duas unidades de glucose estão ligadas por uma ligação α, α1, 1-glicosídica. Os TLs são normalmente produzidos por bactérias Gram-positivas com alto teor de guanina e citosina. Ao longo dos anos, estes compostos têm mostrado resultados em ambos os casos de inibição e aumento das taxas de biodegradação. Os TLs são capazes de reduzir a tensão superficial de soluções aquosas e a tensão interfacial entre as fases aquosa e oleosa para níveis semelhantes aos observados utilizando os surfactantes sintéticos. Com estas características, eles são capazes de atuar em diferentes indústrias. No que toca ao campo biomédico os TLs têm certas propriedades biológicas já conhecidas como as propriedades antimicrobianas, antivirais e antitumorais.

Tendo tudo isto em conta, este trabalho tem como principal objetivo a otimização da produção de novos TLs quanto à origem do microrganismo, à composição do meio e às estratégias de condições de crescimento, bem como as condições de purificação e a avaliação da atividade biológica, como potenciais anticancerígenos.

Dois microorganismos distintos foram selecionados para serem utilizados neste trabalho para a produção de TLs, *Rhodococcus erythropolis* e *Rhodotorula* sp*. Rhodococcus erythropolis* é uma bactéria gram-positiva já conhecida para a produção de TLs, ao inverso da *Rhodotorula* que se trata de uma levedura nunca antes usada com este propósito. As produções foram realizadas com diferentes meios, uma vez que a produção microbiana

pode ser afetada por fatores como temperatura, fonte de carbono, fontes de nitrogénio, composição do sal e uso de extrato no caldo de cultura. Os meios usados foram à base de glucose e meio com diferentes sais, acrescentado que o meio tinha na sua composição, entre outros sais, carbonato de cálcio, que foi previamente provado eficaz na produção destes compostos. O meio salino foi testado com diferentes fontes de carbono. Essas fontes de carbono testadas foram a glucose, o glicerol, *n*-hexadecano e *n*-dodecano em concentrações de 2 e 4 % cada. A produção decorreu a uma temperatura constante de 28 ºC e uma rotação de 200 rpm durante 72 horas, para que fosse possível otimizar a produção de TLs. O crescimento foi otimizado às 48 horas para ambos os microrganismos com a grande maioria dos diversos meios a cultura. O consumo de glucose durante o crescimento em *Rhodococcus erythropolis* foi em cerca de 70 %, no entanto o da *Rhodotorula* dependeu bastante da concentração inicial de glucose. A extração foi efetuada por uma extração líquido-líquido utilizando uma solução à base de solvente de acetato de etilo e metanol, numa porção de 8:1, sendo depois de separada, levada a secar a parte biológica. Após a secagem, o composto foi pesado e submetido a uma série de testes para identificação e caracterização dos compostos produzidos. TLC e HPLC foram feitos para garantir a presença dos TLs nos compostos extraídos. Posteriormente, a condutância, a tensão superficial e a concentração micelar crítica foram medidas.

Para testar sua atividade biológica, como agentes anticancerígeno, os TLs foram solubilizados em NaCl a 0,9 % (v/v) em diferentes concentrações e adicionados as células para o ensaio do MTS realizado em cancro do pulmão de células não pequenas, H1975 (NSCLC). No final do ensaio de MTS não foi possível observar um efeito citotóxico nas diferentes concentrações do composto dos dois microorganismos nesta linha celular.

Pode-se assim concluir que os resultados obtidos no âmbito deste trabalho revelaram que a levedura de *Rhodotorula* sp é capaz de produzir TLs com propriedades semelhantes aos TLs produzidos pela *Rhodococcus erythropolis*. Contudo, não foi possível ver um efeito citotóxico claro nas diferentes concentrações dos compostos testados nesta linha celular de cancro do pulmão de células não pequenas. No entanto mais ensaios serão necessários de modo a elucidar sobre os mecanismos subjacentes e sobre a possibilidade de continuar a exploração de TLs como uma terapia anticancerígena em outras linhas celulares cancerosas no futuro, utilizando estes compostos produzidos naturalmente.

Palavras-chave: Biosurfactantes, trehaloselípidos, cancro, *Rhodococcus erythropolis*, *Rhodotorula*

Acknowledgments

Em primeiro lugar gostaria de agradecer à minha família e amigos próximos por todo o apoio, carinho e pela disponibilidade a ajudar quando nem tudo corre bem.

Deixo também um muito obrigada às minhas colegas de laboratório pois sem elas não tinha sido a mesma coisa.

Quero também agradecer à Professora Maria Henriques Ribeiro por toda a ajuda e paciência e simpatia para tornarmos este trabalho possível.
Agradeço também ao Prof. Nuno Oliveira e à Rita Manguinhas por toda a ajuda referente aos testes de citotoxicidade.

Um agradecimento também à Professora Célia Faustino pela ajuda na realização dos ensaios de tensão superficial e um agradecimento também à Professora Rosário Bronze e Dra. Andreia Silva permitiram a realização das análises das amostras por HPLC-LC/MS.

Índex

Figure index

Table index

List of Abbreviations

BS – Biosurfactants

C/N - Carbon/Nitrogen

CMC - Critical micellar concentration

DO – Optical density

FTIR - Fourier-transform infrared spectroscopy

GC - Guanine and Cytosine

GS - *Gym Streptomyces*

HPLC- MS - Mass spectrometry High-performance liquid chromatography

HPLC-RC - Reversed-phase High-performance liquid chromatography

MTBE - Methyl tert-butyl ether

MTS - 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium)

Myc-PL – 6-O-mycolyl-b-D-mannopyranosyl-1-phosphoheptaprenol

NMR - Nuclear magnetic resonance

NSCLS – Non small cell lung cancer

SCTL - Succinoyl trehalose lipid

TDCM - Trehalose dicorynomycolate

TDE - Trehalose diesters

TDM – Trehalose dimycolate

TL - Trehaloselipide

TLC - Thin layer chromatography

TMM - Trehalose mono mycolate

TPS - Trehalose-6-phosphate synthetase

1 - Introduction

Nowadays one of the main concerns in the world is the transformation and preservation of Earth as a sustainable planet. In the line with this concern about the environment, many industries are changing global viewpoint on the future of manufacturing. They have recognized the potential of living cells in the pre-treatment of raw materials, processing operations, product modifications selective waste management, energy recycling and conservation.

Surfactants are compounds with interesting properties such as the ability to decrease the surface tension between two liquids, between a gas and a liquid, or between a liquid and a solid. They are mainly produced by chemical synthesis. Biosurfactants (BS) have attracted the attention of research and industry since they are surfactants that can be produced by micro-organisms from renewable resources such as soil or water. It is important to note that on the BSs produced by soil or freshwater microorganisms are less toxic and are mostly biodegradable [3].

BS can be defined as surface-active biomolecules produced by several microorganisms with unique properties such as surface activity, tolerance to pH, temperature and ionic strength, biodegradability, low toxicity, emulsifying and demulsifying activity and a large antimicrobial activity when compared with synthetic surfactants [4].

BSs have a wide range of applications, they can be used in different areas, such as pharmaceutics, cosmetics, agronomy, food, beverages, metallurgy, agrochemicals, organic chemicals, petroleum, petrochemicals, fertilizers and many others [5]. The main applications in the pharmaceutic industry are as antimicrobial, anti-cancer, antiviral, anti-adhesive agents, immunological adjuvants, drug and gene delivery, among others [5].

Since BS are quite adaptable, they have a versatile performance, and therefore, they can be used in a lot of fields. In previous studies, it was reported that BSs have strong antibacterial [6,7], antifungal [8], and antiviral activity [9].

BS are well known for their membrane permeabilization properties as they can induce pore and ion channel formation in lipid bilayer membranes. Moreover, they are able to destabilize membranes disturbing their integrity and permeability. Also, pore formation in membranes

may cause transmembrane ion influxes, including Na^+ and K^+, which result in membrane disruption and cell death [10].

Since BS are a wide group of biocompounds, there are different ways of classification. However, the most usual is to characterize them according to their nature, chemical composition and microbial origin.

BS are normally structured by a hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated or saturated fatty acids [1]. They can be divided into five major categories: glycolipids, fatty acid/phospholipid, lipopeptide/lipoprotein, polymeric and surfactant particles [11].

Glycolipids are indeed the most common and studied category of the BS due to their high application potential and high production yield. They are carbohydrates combined with long-chain aliphatic acids or hydroxyaliphatic acids. Glycolipid BS show excellent surface-active properties, including emulsifying, dispersing, solubilizing, foaming, penetrating, and wetting actions [12].

Glycolipids with one or two sugar residues attached to different lipid backbones can be found in cell membranes of bacteria, fungi, plants and animals in the form of sterylglycosides, glycosylceramides, and diacylglycerolglycosides [13]. The most well-known glycolipids are rhamnolipids, sophorolipids, trehaloselipids and mannosylerythritol lipids.

Trehaloselipides (TLs) are known to have a non-reducing disaccharide in which the two glucose units are linked in an α,α1,1- glycosidic linkage.

TLs are the basic component of the cell wall glycolipids in Mycobacteria and Corynebacteria and are known to be produced by Gram-positive, high GC (Guanine and Cytosine) content bacteria, A*ctinomycetales* such as *Mycobacterium, Nocardia* and *Corynebacterium* and they differ in their structure, size and degree of saturation [1]. In most studies are used TLs produced by *Rhodococcus* and related genera are bound to the cell envelope and are produced mainly when the microorganisms are grown on hydrocarbons. The ability of different microorganism's access hydrocarbons depends on their cell surface hydrophobicity. Cells with high hydrophobicity allow microorganisms to directly contact oil drops and solid hydrocarbons while low cell hydrophobicity allows the adhesion of microbial

cells to the micelles or emulsified oils, formed due to the presence of extracellular biosurfactants or bioemulsifiers.

TLs produced by *Rhodococcus* sp. have been shown to have a great tendency to partition into phospholipid membranes, causing the hemolysis of human erythrocytes by a colloid-osmotic mechanism, most likely by formation of enhanced permeability domains, or pores enriched in the biosurfactant, within the erythrocyte membrane.

In comparison to other microbial glycolipids, TLs have shown contrasting results and achievements with both cases of inhibition and enhancement of biodegradation rates [14]. They can reduce the surface tension of aqueous solutions and the interfacial tension between aqueous and oil phases to levels observed with synthetic surfactants, and have low critical micelle concentrations. With such characteristics they are able to act in different industries, including:

- the food industry, where TLs are especially used as emulsifiers, foaming, wetting, solubilizers antiadhesive, and antimicrobial agents,

- the environmental industry with applications such as microbial-enhanced oil recovery, biodegradation of polycyclic aromatic hydrocarbons or oil-spill treatment,

- the cosmetics industry,

- the biomedical field with properties like antimicrobial [15,16], antiviral [17] and antitumor activities [1,18].

2 - Background and Significance of Trehaloselipids

Trehalose is the general name used for the D-glucosyl D-glucosides [19]. It is a non-reducing disaccharide, wherein two glucose units are linked in an α,α 1,1- glycosidic linkage. It is the carbohydrate group of the cell wall glycolipids in Mycobacteria and Corynebacteria[1]. Trehalose has thermostability, is very resistant to acid hydrolysis and is nonreactive to Maillard reaction.

TLs are glycolipids containing trehalose hydrophilic moiety. First, they were discovered in 1933 [20] and purified in 1956. TLs are among the best known BS distinguished from rhamnolipids and sophorolipids in both activity and composition [21]. In comparison to other

microbial glycolipids, TLs have shown contrasting results and achievements with both cases of inhibition and enhancement of biodegradation rates [14]. They can reduce the surface tension of aqueous solutions and the interfacial tension between aqueous and oil phases to levels observed with synthetic surfactants, and have low critical micelle concentrations.

TLs can be separated into two general sub-classes, as follow:

(i) The first group is the 6,6'-trehalose diesters such as fatty acid trehalose diesters (TDEs, 1), trehalose dicorynomycolates (TDCMs, 2) and trehalose dimycolates (TDMs, 3). The most stated TL is trehalose 6,6'-dimycolate witch is a α-branched- chain mycolic acid esterified to the C6 position of each glucose[1]. Although TDEs are the simplest glycolipids found in the trehalose 6,6'-diester series, can be differentiated by lipid length and the branching of the fatty acids, these can be divided into anionic or non-anionic. It is known that anionic surface-active TDEs have a higher surface activity than non-ionic surface-active [14].

(ii) The second group is the 2,3-trehalose diesters such as diacyl trehalose sulfates (STL) and sulfolipid-1 (SLL) [22,23].

One of the studies showed that TLs, succinoyl trehalose lipids (SCTLs), are promising BS since they are efficiently produced from n-alkanes by *Rhodococcus* bacteria and recovered by precipitation. Among SCTL producers, Hitherto reported, *Rhodococcus* sp. SD-74 providing the best yield (40 g/L) from *n*-hexadecane under the high osmotic conditions [24]. These SCTLs also demonstrated to show growth inhibition against influenza virus [25] and cell-differentiation induction towards human leukemia cells [26–28].

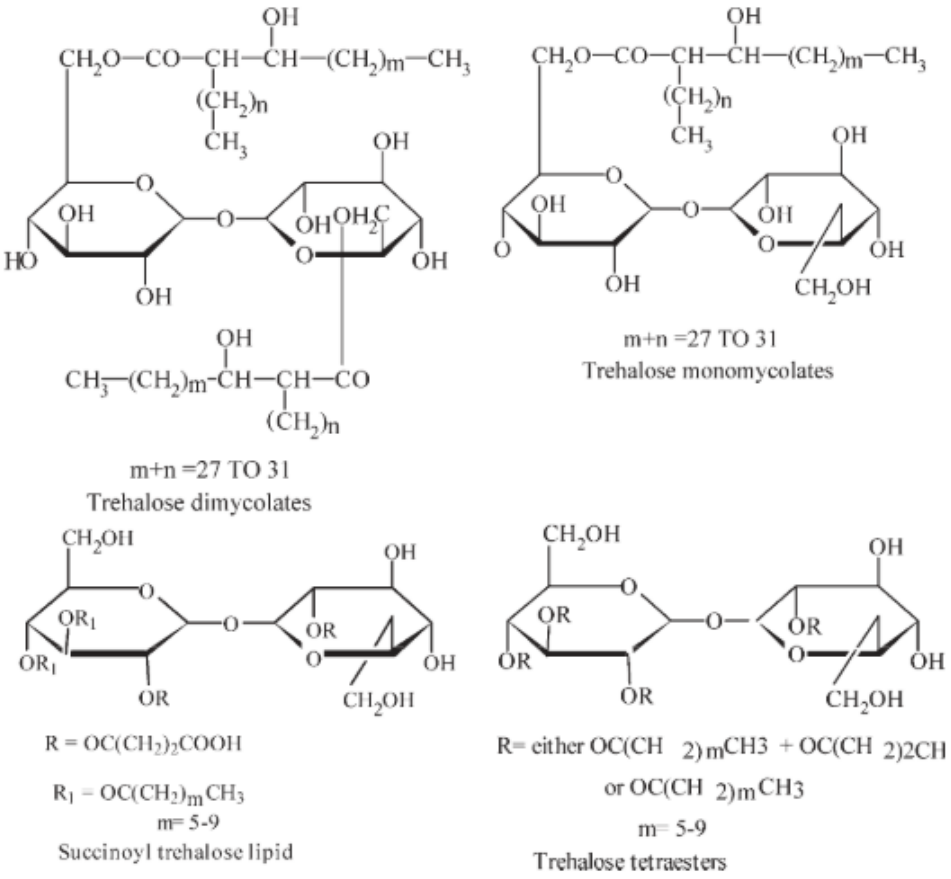


Figure 1. The chemical structures of the main trehaloselipids along with the most commonly reported side chains (adapted from Franzetti, 2010 [1])

2.1 – Biosynthesis

The key reaction for synthesis of the final resulting sugar residue, trehalose-6-phosphate, is catalyzed by a trehalose-6-phosphate synthetase (TPS) which links the two D-glycopyranosyl units at C1 and C1'. UDP-glucose and glucose-6-phosphate act as the immediate precursors. In fact, the trehalose moiety and the fatty (mycolic) acid moiety of trehaloselipid molecules are synthesized independently and are subsequently etherified. Trehalose monomycolate was claimed to be an intermediate of TDM biosynthesis. The formation of the mycolate is considered to be a Claisen-type condensation of two fatty acids, a carboxylated acyl-coenzyme A and an activated acyl chain to yield a 3-oxo intermediate, which would then be reduced to form mycolic acid [2].

The additional reactions involved in the synthesis of TLs have been elucidated for TDMs in which the production happens in the final stages of the synthesis of the cell wall. In this phase, the newly synthesized mycolic acids are transported and attached to the peptidoglycan-arabinogalactan complex of the cell wall, followed by the formation of TDM which occurs by four different reactions.

The biosynthesis proceeds when the first reaction occurs through the transmission of the mycolyl group to D-mannopyranosyl-1-phosphoheptaprenol by a proposed cytoplasmic mycolyltransferase I to form 6-O-mycolyl-b-D-mannopyranosyl-1-phosphoheptaprenol (Myc-PL) (Fig. 2). For the second reaction the mycolyl group is transferred to trehalose 6-phosphate by a membrane-associated mycolyltransferase II to form trehalose mono mycolate (TMM)-phosphate and, after dephosphorylation, results in the formation of TMM. The third reaction happens TMM is transported outside the cell by an ABC transporter. A rapid and efficient transfer of TMM from the inside to the outside of the cell is necessary for the synthesis of cell wall arabinogalactan-mycolate and TDM. The fourth and last reaction occurs by the action of the extracellular mycolyltransferase called Ag85/Fbp/PS1, the final products of the cell wall arabinogalactan-mycolate and TDM are formed from TMM [1].

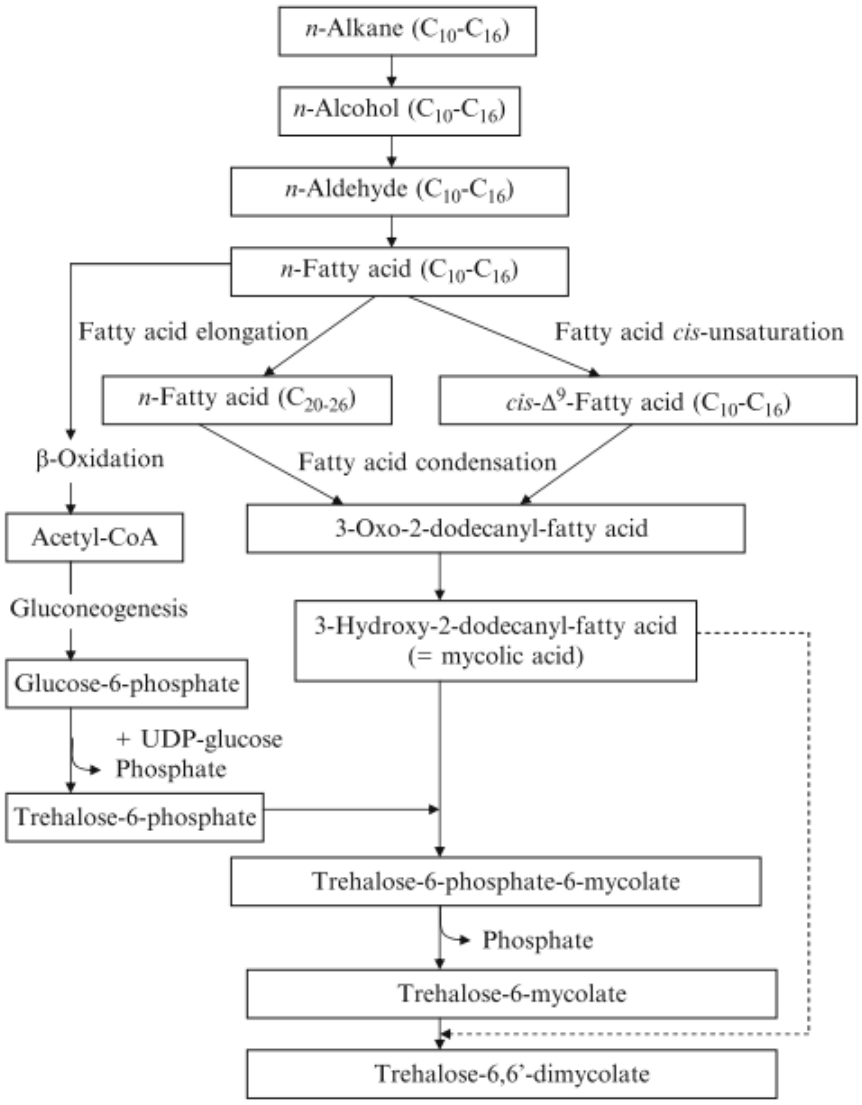


Figure 2. Scheme of trehalose-mono- and -dimycolate synthesis from *n*-alkanes (adapted from Kuyukina & Ivshina, 2010 [2])

2.2 - Production

In the bioproduction of TLs there are a lot of factors to be considered. Firstly, the microorganism must be carefully chosen to produce the TLs aimed, with special attention to the purpose of the application of the biocompound. This microbial production can be affected by factors such as temperature, carbon source, nitrogen sources, salt composition and use of extract in culture broth [5].

Based on previous studies, the yields of TLs production are very low when compared to other glycolipids. Amongst different TLs production systems, only a study described a high trehalose lipid yield, where *Rhodococcus* sp. SD-74 strain was used and 40 g/L of the glycolipid was archived using n-hexadecane under osmotic conditions [24].

2.2.1. Microorganisms

Just like any other BS, TLs can be produced by many microorganisms, they are usually produced by Gram-positive, high GC content bacteria. In many studies, different microorganisms were used, like *Arthrobacter, Nocardia, Rhodococcus*, *Gordonia* [29], *Actinomycetales* such as *Mycobacterium, Nocardia* and *Corynebacterium*, which allowed TLs biocompounds differing in their structure, size and degree of saturation [1], also in mycolate group. Although all of these groups are used for TLs production *Nocardia* or *Rhodococcus* are widely explored [30].

However, some of the producing bacteria are pathogenic mycobacteria (including pathogens in the *Mycobacterium avium*, *M. intracellular* group, *nocardia* (*Nocardia asteroids*), and corynebacteria (*Corynebacterium diphtheria*, *C. matruchotii*, and *C. xerosis*) and the observed or potential pathogenicity of these producer strains, as well as the high toxicity of the synthesized glycolipids, significantly restricted their use, specifically in the biomedical field, in which they have to be GRAS (Generally Regarded As Safe).

For this reason, other non-pathogenic TLs producing bacteria have been studied, in particular, representatives of *Rhodococcus*, *Gordonia*, *Dietzia*, *Tsukamurella*, *Skermania*, *Williamsia*, among others [31].

In the case of TLs synthesized by *Rhodococcus* and related genera, they are usually bound to the cell envelope and are produced mainly when the microorganisms are grown on hydrocarbons sources. This ability of different microorganisms to access hydrocarbons rests on their cell surface hydrophobicity. Cells with high hydrophobicity let microorganisms directly contact oil drops and solid hydrocarbons while low cell hydrophobicity allows the adhesion of microbial cells to the micelles or emulsified oils, formed due to the presence of extracellular BSs [1]. Furthermore, chemical characteristics defining *Rhodococcus* were described by Goodfellow in 1989 and updated by Finnerty in 1992, where cell walls were

found to be chemotype IV, in other words, the only diamino acid in the peptidoglycan is meso-diaminopimelic acid and that the major sugars are arabinose and galactose [32].

There are also the case of *Rhodotorula* species. *Rhodotorula* is a common environmental yeast. It can be found in soil, ocean water, lake water, fruit juice and milk, it is also commonly found in household items like shower curtains and toothbrushes. It is a genus of unicellular pigmented yeasts, part of the division *Basidiomycota*. Nowadays the genus contains 46 species of which three have been described as rare human pathogens: *R. mucilaginosa* (previously known as *R. rubra*), *R. glutinis* and *R. minuta* [33]

2.2.2. Factors affecting TLs production

The production of TLs can be influenced by different factors such as the microorganisms as previously mentioned (c.f. 2.2.1.), the media composition (e.g., carbon and nitrogen sources), bioreactor and environmental conditions

2.2.2.1. Media composition

Carbon Source

To proceed with the growth of the microorganisms for TLs production, there is a need for a carbon source. This carbon source will influence not only the quantity but also the quality of the formed TLs. When carbon sources and insoluble substrates are combined the intracellular diffusion and production of different substances are simplified, this happens because BSs are involved in the emulsification of insoluble substracts [34,35]. Moreover, Microorganism cell adsorption to insoluble substrates and the excretion of surfactant compounds allow growth on carbon sources [35].

In most of the previous studies, n-hexadecane is the one chosen to use as the carbon source [15,36–39]. Furthermore, it has been proved that they produce free fatty acids and glycolipids, glucolipids and TLs [40], in other studies it has been shown that *Rhodococcus* using *n*-hexadecane as a carbon source is able to produce mono- and di-succinyl TLs when using *R. erythropolis* [41,42].

Nonetheless, other sources have been used such as *n*-alkane [43,44], which has been used for the production of TL-1and -2, and *n*-decane to produce trehalose-tetraesters [41], glucose or vegetable oil [35], glyceryltrioleate [23] and a mixture of the mentioned above.

For instance, in 1996 a study was performed to test the effect on different carbon sources where two different mixtures were used, they were called P-120 and P-147. P-120 was composed of *n*-decane, *n*-undecane, *n*-dodecane, *n*-tridecane, *n*-tetradecane. The second mixture had in its composition *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, *n*-heptadecane. At the end of this study, the higher yield was accomplished by using *n*-tridecane alone [45].

Furthermore, using sugars as a carbon source directly may affect the carbohydrate moiety, which might have a good effect on the TLs production and must be less expensive.

Nitrogen Sources

Generally speaking, in fermentative processes, the carbon/nitrogen (C/N) ratio usually affects the buildup of metabolites. High C/N ratios limit bacterial growth. On the other hand, low C/N ratios lead to the synthesis of cellular material and limit the buildup of products [35].

In the specific case of TLs, limiting the nitrogen source may be appropriate. With a lower nitrogen content and an increased level of glycogen, Elbein (1974) was able to maintain the levels of trehalose fairly constant [19]. In 1988, Ramsay (1988) used sodium nitrate ($NaNO_3$) instead of ammonium sulfate ($(NH_4)_2SO_4$) as a nitrogen source [44].

In Uchida paper the effect of the different nitrogen sources was studied, the cell growth was not affected considerately by ammonium sulfate, ammonium dihydrogen phosphate, ammonium nitrate, or urea. However, potassium nitrate allowed a better yield [24]. Also with limited nitrogen conditions, trehalose mycolates formed by *Rhodococcus erythropolis* lead to the production of anionic trehalose tetraesters [29].

2.2.2.2. Environmental factors

The parameters for the fermentative process are of great importance. They vary between temperature, pH, agitation and aeration. For TLs production in bioreactor with control systems (e.g. digital), these parameters must be stable during bacterial growth.

Some authors referred that although TLs produced by *Rhodococcus* sp. formed emulsions that were stable in a pH 2 to 10 and temperatures of 20 to 100 [46,47], the temperature must be stabilized between 28 ºC to 30 ºC during growth and the pH must be neutral and stable as well [15,23,38].

2.3 - Downstream Process

The downstream process is probably the biggest obstacle to overcome, as normally the purification of the target biological compound can account for over half of the production cost in many biotechnology applications [48].

In order to recover TLs from the bacterial medium, solvent extraction is one of the approaches. There are some different mixture of solvents systems that can be used, being the three most used chloroform-methanol, methyl tert-butyl ether (MTBE), or a mixture of ethyl acetate-methanol[1]. The difference between MTBE and chloroform: methanol mixtures don't produce a significant difference in the amount of glycolipid extraction [49]. Some of these systems might represent a problem, due to their toxicity, when TLs is to be applied into the biomedical field.

Prior to the extraction, the compounds must be identified. For this purpose, various methods can be tested, including thin layer chromatography (TLC) [23,37], high-performance liquid chromatography-mass spectrometry (HPLC-MS) [50] and nuclear magnetic resonance (NMR) [23].

TLC has been extensively used to detect TLs, this technique not only purify the compound but also provides information about the structural composition. A few solvent systems have been used to purify TLs although the most used is chloroform:methanol: water (65:15:2) for the mobile phase [37] and to reveal the result usually a chemical revealer is made of acetic acid: anisaldehyde: sulfuric acid, under 150 °C airstream for 2–4 min, TLs will result in the form of spots [23]. With this method, the monomycolates appear near the point of application of the compound and the dimycolates will show its mark a little further.

2.4 - Identification and Characterization

For the identification and characterization of TLs, a lot of techniques can be used.

Resembling other glycolipids, TLs content can be estimated using the anthrone method [51]. Anthrone method is a colorimetric technique based on the reaction of anthrone with the sugar forming a coloured complex that can be quantified in visible region, using a spectrophotometer.

The characterization can be done by breaking down the structure separating the fatty acids from the carbohydrate or just by analyzing the entire molecule. Some of the most commonly used techniques are the mass spectrometry that can be done over Gas Chromatography (GC) [37] and nuclear magnetic resonance spectroscopy [37,52].

Fourier-transform infrared spectroscopy (FTIR) can also be applied for TLs characterization, once these molecules form polymeric aggregates in aqueous media above the critical micellar concentration (CMC). The type of aggregate structure can play a biological role and is determined by the shape of the contributing molecules which is determined by their primary chemical structure and is influenced by ambient conditions such as pH, and concentration of mono- and divalent cations [53]. High-performance liquid chromatography (HPLC) has been used recently for the characterization. With HPLC coupled with mass spectrometry it is possible to reveal the glycoside linkage between two of the sugar moieties [30]. Moreover, reversed-phase HPLC (HPLC-RC) was also used for these compounds identification [15].

2.5 - Surface properties analysis

As is well known, BSs are surface-active compounds capable of reducing surface and interfacial tension at the interfaces between liquids, solids, and gases, allowing them to mix or disperse readily as emulsions in water or other liquids. The surface tension and emulsifier index of TL are one of the main characteristics of the applications in the industry. Additionally, TLs are responsible for lowering the interfacial tension and increasing the miscibility of the liquids. The presence of BS tends to decrease the interfacial tension to values around 1 mN/m and, for this reason, they are very effective in promoting hydrocarbon emulsification and enhancing oil recovery [50].

Moreover, surface tension tends to decrease with the increase of glycolipid concentration. The TLs are able to reduce the surface tension of the water, for instance from 72 to 34 mN/m

[15] which is in agreement with most TLs produced from *Rhodococcus*, showed strong surface activity by lowering water surface below 30 mN/m, lowering the interfacial tension against hexadecane up to 1 mN/m. Depending on the *Rhodococcus* sp., the different trehalose lipids produced vary in interfacial tensions, critical micelle concentrations and emulsifier index therefore all of them show great potential in different fields [49,54].

2.6 - Biologic activity

TL can reduce the surface tension of aqueous solutions and the interfacial tension between aqueous and oil phases to levels observed with synthetic surfactants, and have low critical micelle concentrations [55]

A lot has been studied regarding the biologic activity of TL and their application in the pharmaceutical fields. It is known that they possess antimicrobial [15,16], antiviral [17] and antitumor properties [1,56]. For instance, SCTL has been found to have hemolytic activity [57] and to induce cell differentiation into monocytes instead of cell proliferation in the human promyelocytic leukemia cell line HL60 [26]. Moreover, TDM conferred to mice higher resistance to intranasal infection by influenza virus due to the ability of TL to induce proliferation of the T-lymphocytes bearing gamma/delta T-cell receptors, associated with the maintenance of acquired resistance to the infection [17].

2.7 – Applications

As it was said before, glycolipids have a wide range of applications. Since TLs are able to reduce the surface tension of aqueous solutions and the interfacial tension between aqueous and oil phases and have low CMCs, they are especially used as emulsifiers, foaming, wetting, solubilizers antiadhesive, and antimicrobial agents in the food industry.

Regarding the environmental industry TLs are used as microbial-enhanced oil recovery, biodegradation of polycyclic aromatic hydrocarbons or oil-spill treatment, in the cosmetics industry and most importantly in the biomedical field these compounds are used due to their functions in cell membrane interactions [58]. In the biomedical field, they act as antimicrobial [15,16], antiviral [17] and antitumor activities [1,18,56].

The antitumor activities have been evaluated in some reports in the literature. For instance, in 1980 a tumor regression was seen in guinea pigs bearing transplantable, line-10

hepatocellular carcinomas when synthetic muramyl dipeptides combined with trehalose dimycolate in oil-in-water emulsions were injected directly into the tumors [59]. In 1996 succinoyl trehalose lipid extracted from n-hexadecane culture of *Rhodococcus erythropolis* SD-74 noticeably inhibited the growth of a human monocytoid leukemic cell line [26,60]. In a recent study, TLs were shown to be ineffective on the contractility of rat mesenteric arteries in vitro, as well as reducing breast cancer cell viability [56].

3 - Objectives

The aim of this study was to optimize the production of new threalose lipids (TLs) regarding the microorganisms source, medium composition and strategies of growth conditions, as well as the purification conditions and evaluation of the biological activity, as anticancer agents.

Therefore, the hypothesis of the present study was that different microorganisms, production and purification conditions using a green approach will affect the TLs formed and their biological activity.

4 - Materials and reagents

In order to make all the laboratory work many common materials were used, such as micropipettes (Gilson P1000, P200, P100 and P20), Erlenmeyer's, Eppendorf (1 and 2 mL), graduated pipettes, test tubes, bunsen burners, thermometer, Petri dishes, volumetric flasks, plastic cuvettes (Eppendorf), glass cuvettes, microplates (Fisher Scientific), forceps, Pasteur pipette, inoculation loop and separation funnels.

4.1 – Microorganisms

In order to produce the TLSs two different microorganisms were tested:

- *Rhodococcus erythropolis*, DSM 43066-0917-001, isolated from soil, acquired from DSMZ - German Collection of Microorganisms and Cell Cultures.
  Rhodococcus has been refereed to be a TL producer and be used in many different studies [28,30,43,61]. It is a genus prevenient from *Actinomycete*. It is described as aerobic, Gram-positive, non-motile, mycolate-containing, nocardioform *actinomycete*s.

- *Rhodotorula sp* from the collection of Faculty of Pharmacy of the University of Lisbon.

  Rhodotorula sp, has never been used before with the purpose of producing TLs, however, *Rhodotorula bugoriensis* has been shown to produce extracellular glycolipids [62,63] such as sophorolipids [16].

4.2 - Substrates

Three different media were used in this work for the production of TLs, therefore different reagents and salts were used (Table 1.)

Table 1. Reagents used in the media for bacterial growth and TLs production and their respective brand

Reagent	**Brand**
Trypto-casein soy broth	Biokar
Bacteriological Agar type E	Biokar
Distilled water	Produced internally - MilliQ
Glucose monohydrate	Sigma-aldrich
Malt extract	Sigma-aldrich
Yeast extract	Oxoid
Calcium carbonate	Merck
Dipotassium Hydrogen phosphate trihydrate	Merck
Magnesium sulfate heptahydrate	Merck
Nickel (II) sulfate hexahydrate	Merck
Ferric chloride hexahydrate	Merck
Potassium dihydrogen phosphate	Emsure
Ammonium sulfate	Sigma
Zinc acetate dihydrate	Sigma
Calcium chloride dihydrate	Panreac
Manganese (II) sulfate monohydrate	Scharlau
Disodium hydrogen phosphate dihydrate	Merck
n-Hexane	Sigma
n-Dodecane	Sigma

4.3 - Other Reagents

For the experimental work, some previously made solutions were used, such as DNS reagent, sodiumacetate, acetic acid solution (Table 2.).

Table 2. Chemicals used and their brands.

Chemicals	Brand
Albumin Bovine fraction V (BSA)	Nzytech
Protein Assay dye reagent concentration	Bio-rad
Sodium sulfate granular	Aldrich
Chloroform	Ensure
Sulfuric acid 95-97%	Ensure
Acetic acid (glacial)	Ensure
Methanol	Honywell
Acetonitrile	J.T.Baker
Ethyl acetate	Sigma-Aldrich
4-methoxybenzaldehyde	Sigma
Ethanol	Ensure
Acetone	Ensure
RPMI-1640 with L-glutamine	Biowest
Cisplatin	Sigma-Aldrich
Fetal bovine serum (FBS)	Sigma-Aldrich
Penicillin-streptomycin solution	Sigma-Aldrich
Trypsin	Sigma-Aldrich
Sodium bicarbonate	Sigma-Aldrich
Sodium pyruvate	Lonza
HEPES	AppliChem
D-Glucose	AppliChem

CellTiter 96® Aqueous MTS	Promega

4.4 – Cancer Cell Line

To test the cytotoxicity of the produced compounds (TLs) a lung cancer cell line was used.

The used cell line was from human H1975 non small cell lung cancer (NSCLC). The cell was provided by Dr. Rosell Cancer Institute (Barcelona, Spain). The specific cell line was established in July 1988 and the tissue donor was a non-smoker female. It was originated from lung adenocarcinoma (NSCLC) and it has a duplication time of 30h [64].

4.5 - Equipment

Other equipment were used for the bacterial growth and other assays (Table 3).

Table 3. Equipment used and respective brand.

Equipment	**Brand**
Scale	Precisa 180
Water bath	Memmert
BioPhotometer	Eppendorf
Shaker incubator	Aralab agitorb 160E
Vortex	Retsch
Autoclave	AJC Amaro - 2000
Processor Tenciometer k 12	Krüss
Multiparameter tester, PC5 series	Labbox
Microplate reader	SPECTROstar OMEGA
Laminar flow chamber	Pbinternational (pbiminiflo)
Incubator	Memmert (concessus)
Centrifuge	Heraeus (Biofuge Pico)

5 – Methods

Different methodologies were used to perform the experimental work. For some of the methods, sodium acetate buffer was used. To prepare the buffer 1.36 g of sodiumacetate di-hydratate was solubilized in 400 mL distilled water. This solution was mixed under magnetic stirring when the previously prepared acetic acid solution of 2 M was added until it reached a pH of 4.5. Lastly, it was completed with water at 500 mL.

5.1. Dinitrosalicylic Method (DNS)

DNS is a colorimetric method used to measure the free carbonyl group (C=O), also known as reduced sugars [65]. This method has its bases on the reaction were 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino-5-nitrosalicylic acid under alkaline conditions. With this reaction the yellow colour in the samples will change depending on sugar content, the darker the colour the more sugar it has.

DNS reagent was already prepared by dissolving 5 g 3,5-dinitrosalicylic acid at room temperature and on a magnetic stirred plate in 100 mL NaOH 2 N and 250 mL distilled water. 150 g of La Rochelle salt was added and completed with 500 mL demineralized water. After was stored in the volumetric flask wrapped in aluminum foil to be protected from the light.

To calibrate the method, a stock glucose solution of 1 g/L was prepared by weighing 0.0250 g of glucose and dissolved in 25 mL of distilled water. After, eight different concentrations of glucose were prepared, namely, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L, 500 mg/L, 600 mg/L, 700 mg/L and 800 mg/L.

In the microplate 75 µL of DNS reagent was added to 75 µL of the sample, using water as blank. The plate was then submitted to a water bath at 95-98 ºC for 5 min. After the samples were cooled down and the samples read on a spectrophotometer at 562 nm.

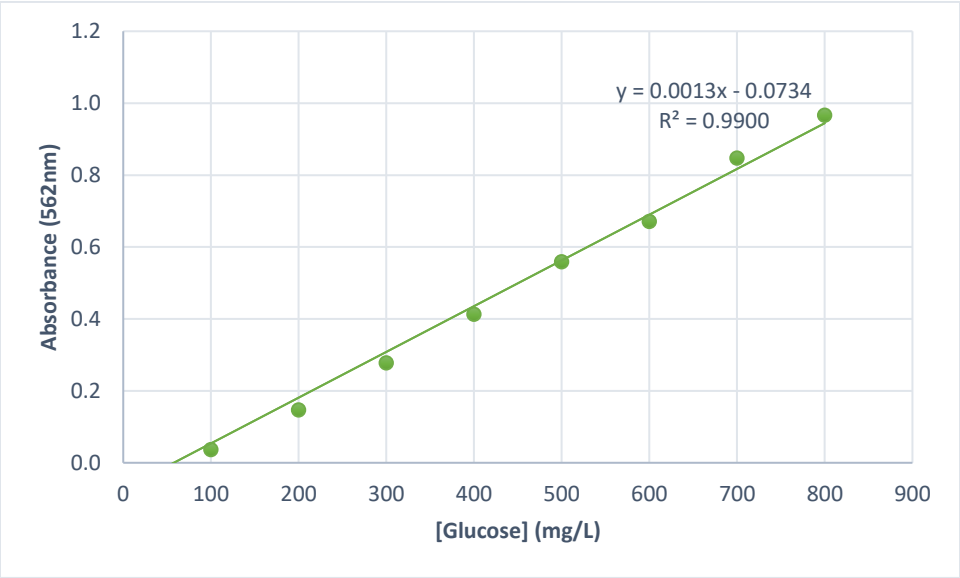


Figure 3. Glucose calibration curve for DNS method

5.2. Bradford Method

The Bradford method is a colorimeter method used to quantify proteins that is based on the formation of a complex between the bright blue cromassie dye present in an acid solution and the proteins in the solution [66,67]. This protein-dye complex causes a maximum change in the dye absorption from 465 to 595 nm and this absorption is proportional to the amount of protein in the solution.

Bradford reagent was made in a dilution of 1:4 with distilled water. For the stock solution (2 mg/mL) 50 mg of albumin was used and diluted in 25 mL of acetate sodium buffer at a pH of 4.5. This stock solution was used to make ten different sample concentration, namely, 0.03 mg/mL; 0.06 mg/mL; 0.09 mg/mL; 0.12 mg/mL; 0.15 mg/mL; 0.18 mg/mL; 0.21 mg/mL; 0.24 mg/mL; 0.27 mg/mL and 0.30 mg/mL.

In different Eppendorf's 50 µL of sample was placed and 250 µL of Bradford reagent was added and the Eppendorf's shaken on the vortex, using buffer as the blank. 5 minutes later the absorbance was read at 595 nm.

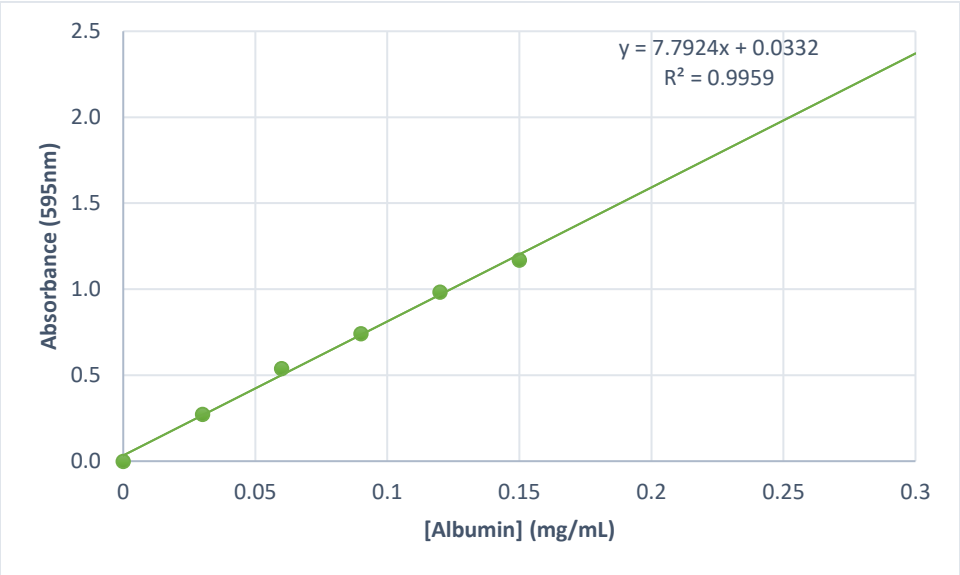


Figure 4. Calibration curve for the Bradford method.

5.3. Growth and TL Production Media

In order to optimize the cell growth as well as the TLs production, 3 different media were prepared, namely:

Medium 535, Tryptic Soy Broth (TSB)

For the liquid medium 15 g of *TSB* was dissolved in 500 mL of distilled water and autoclaved. For the solid medium, the procedure was the same as for the liquid but the solution was warmed up in order to dissolve 7.5 g of agar. It was then autoclaved and used to make plaques of 20 mL each.

Gym Streptomyces (GS):

For this medium 2 g of yeast extract and 5 g of malt extract were diluted in 400 mL of distilled water, 2 g of glucose was solubilized in 50 mL of water and 0.5 g of $CaCO_3$ was also solubilized in 50 mL of water. This last one was submitted to an ultrasonic treatment for better solubilization. The three solutions were autoclaved at 121 ºC for 15 min and

stored. These solutions were made in double to make a liquid and a solid medium. Later, agar was dissolved on one of the flasks of the extracts and autoclaved again in order to make the solid medium.

Mineral Medium (MSM)

To prepare this medium the salts were solubilized separately and join together at the end in a 900 mL solution that was autoclaved at 121 ºC for 15 min. This medium was only prepared as a liquid medium, with the following composition

- Dipotassium hydrogen phosphate trihydrate, 4.8 g/L
- Potassium dihydrogen phosphate, 1.5 g/L
- Ammonium sulfate, 1.0 g/L
- Magnesium sulfate heptahydrate, 0.5 g/L
- Calcium chloride dihydrate, 2.5 mg/L
- Manganese (II) sulfate monohydrate, 1.0 mg/L
- Nickel (II) sulfate hexahydrate, 1 mg/L
- Zinc acetate dihydrate, 2 mg/L
- Ferric chloride hexahydrate, 0.5 mg/L
- Disodium hydrogen phosphate dihydrate, 2.5 mg/L
- Distilled water

.

For this specific medium carbon sources had to be added. These carbon sources were

- *n*-Hexadecane
- *n*-Dodecane
- Glucose monohydrate
- Liquid glycerol

5.3.1. *Rhodococcus erythropolis* DSM 43066 culture

Rhodococcus erythropolis DMS 43066 was purchased dehydrated, in order to open it, a sterile environment was created. Then, the tip of the ampoule was warmed on a bunsen burner to break when the drops of water touched the glass. With a tweezer, the remaining of the glass tip was broken and the insulation material taken out of the vial. The cotton plug was removed and kept under sterile conditions. 0.5 mL of TSB medium was added, the cotton plug was replaced and the pellet set for 30 min to rehydrate. After that time the content was mixed and half of the whole amount (250 µL) was transferred to a test tube with 5 mL of medium and the other half into an agar plate as the manufacturer instructed. Before incubation more plaques and tubes were made. From the fist plaque, we created another two by spreading the inoculate back and forth with an inoculating loop over the solid agar plate. From the liquid culture, the tube was washed with 500 µL of *TSB*, from that wash we washed again with 500 µL of *TSB* and repeated the process with another 500 µL of *TSB*. all of the plaques were incubated at 28 ºC.

In the next day were made more cultures from the previous ones. On four volumetric flasks, two different cultures were made. One culture was made on two flasks with 10 mL of liquid *TSB* and the other two flasks with *GS*. From these liquid cultures, solid cultures were also made in the respective media.

On the third day, the first plaque was taken out of the incubator after 40 hours and put in a 4 ºC camera.

Figure 5. *Rhodococcus erythropolis* DSM 43066

5.3.2. *Rhodococcus erythropolis* DSM 43066 growth

To grow the bacteria an inoculum at 10 % was prepared in the previous day, depending on the medium that was going to be used on the bacterial culture, the medium for the inoculum would vary between TSB or Gym *Sptreptomyces*. For the inoculum 5 mL of the medium was pipetted into a test tube and the bacteria from the plaque was inoculated. The inoculum was then incubated at 28 ºC with a rotation of 200 rpm for about 18 hours.

In 500 mL flasks, 45 mL of medium was added as well as the inoculum. The first samples were collected at that time (t0). The cultures were incubated at 28 ºC and 200 rpm. Depending on the medium samples were collected in different time ranges. Those samples were analyzed for their optical density (DO600), glucose consumption, formation of proteins and TLs formation.

The first three cultures were made on *GS* medium. In the first cultures, the samples were collected every hour, for the following culture's samples were collected every two hours so that bacteria maintained environmental conditions constant in the process. The absorbance of all samples was measured immediately after recovery.

For each growth, DNS and Bradford were performed in order to determine the glucose consumption and their protein content, respectively.

The other cultures were made with MSM varying the carbon source, *n*-hexadecane, glucose, glycerol (Fig 6) and *n*-dodecane at 2 or 4 %. The inoculum was prepared with 5 mL of *TSB*. when inoculating the carbon source was added to the medium. The conditions remained the same (28 ºC, 200 rpm) for all the processes but the samples were collected three times a day, 3 hours apart.

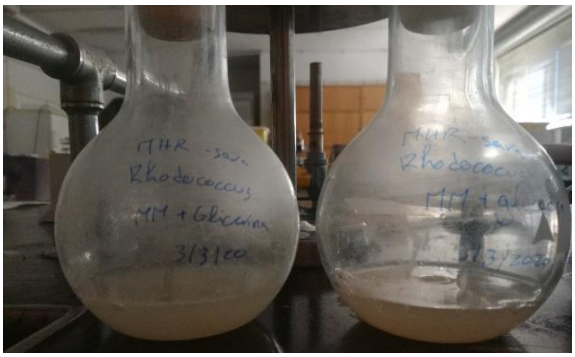


Figure 6. *Rhodococcus erythropolis* cultured in MSM supplemented with 4 % glucose and glycerol at 48h.

5.3.3. *Rhodotorula* sp Growth

To try to produce TL with *Rhodotorula* sp, the same conditions described above were recreated. Firstly, an inoculum of 10 % was made with 5 mL of *Gym Streptomyces* was pipetted into a test tube and the bacteria from the plaque was diluted. The inoculum was then incubated at 28 ºC and 200 rpm, this time and unlike *Rhodococcus*, it was left for 21 hours. In 500 mL flasks, 45 mL of *Gym Streptomyces* was added as well as the inoculum. The first samples were collected immediately (t0). The cultures were incubated at 28 ºC and 200 rpm. The samples were collected at 2, 4, 6, 24, 26, 28, 48, 50 and 72 hours, the optical density (DO600) was measured immediately after recovery.

The following cultures were made with MSM varying the carbon source, *n*-hexadecane, glucose, glycerol (Fig 7) and *n*-dodecane at 2 or 4 %.

The inoculum was prepared at 10 % with *TSB*. After 21 h the MSM and the carbon source was added to the inoculum. The conditions remained the same just like the time of the sample collection.

For each growth, DNS and Bradford were preformed to determine the glucose consumption and their protein content, respectively.

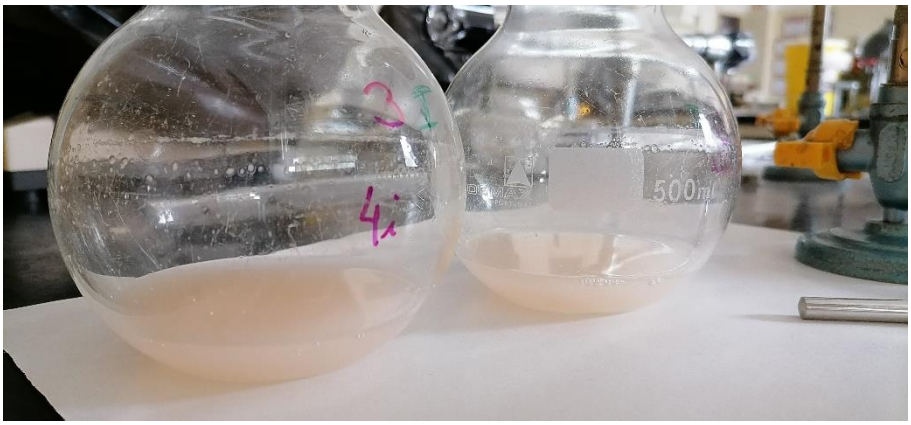


Figure 7. *Rhodotorula* sp cultured in MSM supplemented with 4 % glycerol at 24 h.

5.4. Extraction – Separation and purification

For the separation of the TLs a liquid-liquid extraction was used [30,44]**.** The extraction was made via decantation. A solution of 1:8 of ethyl acetate and methanol was made. That solution was mixed with the whole broth medium in a portion of 1:1 and it was agitated vigorously for 5 min in a separation funnel. It sets for a few minutes in order to settle the cells and be possible to distinguish the different phases and separate them. After the separation, sodium sulphate anhydrous was added in a small portion to the organic phase to evaporate the remaining water, it was then stored in the hotte and left-over night. The next day the dry mixture was scraped off the bottom of the cup and weight. The top part was stored in the refrigerating chamber at 4 ºC.

5.4.1. TLC

TLC is usually used to separate components on non-volatile mixtures. Like any other chromatography, TLC has a stationary and a mobile phase. The stationary phase is an adsorbent layer, while the mobile phase is a solvent or a mixture of solvents that is drawn up the stationary phase via capillary action. The separation is accomplished due to the rate that the different components rise the TLC plate.

To perform the TLC, silica gel was cut in uniform rectangles (7.5 x 2.5 cm) and two lines were drawn on both ends of the silica gel (1 and 0.5 cm). The samples were prepared in Eppendorf's with 0.001 g of the extracted and dried sample 100 µL of chloroform. In the silica gel plaque, 20 µL of the previously prepared samples were pipetted. The TLC was carried out by using a mixture of chloroform, methanol and water, made in a proportion of 65:15:2, used as the mobile phase of the chromatography. Leaving it for about one hour running or until the mobile phase reached the top line, the silica plaque was taken out of the camera, let to dry for a few seconds and has to be sprayed with a chemical solution of acetic acid, sulfuric acid and 4-methoxybenzaldehyde in a proportion of 100:2:1 and heated up to 105 ºC until the stains started to appear.

5.4.2. High Performance Liquid Chromatography-Mass Spectrometry

High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) is an extremely versatile technique that may be applied in the analysis of a wide range of samples. unlike other chromatography's instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres, making it considerably faster and allowing a better separation of the components in the mixture. Some compounds that can be analysed by this method are thermally labile, exhibit high polarity or have a high molecular mass and even proteins. Samples are injected into an HPLC column that comprises a narrow stainless steel packed with silica particles. Compounds are separated based on their relative interaction with the chemical coating of these particles (stationary phase) and the solvent eluting through the column (mobile phase). Components eluting from the chromatographic column are then introduced to the mass spectrometer via a specialized interface. The two most common interfaces used for HPLC/MS are the electrospray ionization and the atmospheric pressure chemical ionization interfaces.

The retention time is the time that it takes for the compound to travel through the column. The time rest on four factors, pressure, nature of the stationary phase, composition of the solvent and temperature of the column during the process. It is through the retention time that it is possible to identify some glycolipids since a lot of them are already published in the literature. Mass spectrometry was used to access the range of the glycolipids, as it gave a pattern that could be compared with a database of known patterns.

The compositional analysis for the extracted TLs was performed by LC–ESI-MS/MS. Twenty samples were analyzed in the total, 10 samples from *Rhodococcus erythropolis* DSM 43066 and 10 from *Rhodotorula* sp to see if it produces the product. Both samples (from *Rhodococcus* and *Rhodotorula*) were prepared at 1 mg/mL with 50 % water and 50 % acetonitrile [68]. A reverse phase Waters™ Atlantis C18 column (100 mm × 2.1 mm ,5 µm particle size) was used at 35 °C in a Waters™ Alliance 2695 HPLC Separation Module connected to a Waters™ Micromass Quatromicro API Tandem Quadrupole Mass Spectrometer equipped with a Waters™ ESI probe. The mobile phase consisted of water (eluent A) and acetonitrile (eluent B). The elution program used was 80 % water and 20 % acetonitrile. The flow rate was constant at 0.3 mL/min and the injection volume was 10 µL. The ionization of the compounds was carried out in a positive mode electrospray source

(ESI +), at different cone voltages (20, 30 and 40 V). Data acquisition and mass spectrometric evaluation were performed with MassLynx Software 4.1.

5.4.3. Ultra-High Performance Liquid Chromatography

Ultra-High Performance Liquid Chromatography **(**UPLC), just like HPLC is a separation technique that enhances principally three areas, just like speed, resolution and sensitivity. The principles of both techniques are the same, the main difference remains in the column material particle size which less than 2 µm. This single variance makes a large difference in the performance and to maximize the advantages of these columns, creating a powerful, robust and reliable solution.

For this method six samples were analyzed in total, 3 samples from *Rhodococcus erythropolis* DSM 43066 extracted TLs and the other 3 samples with the medium of the respective culture. The first three were prepared at 10 mg/mL with 20 % water and 80 % acetonitrile. The other three with 100 µL medium, 800 µL acetonitrile and 20 µL water, the medium was 2 % *n*-hexadecane, 4 % *n*-dodecane and 4 % glucose respectively. All the samples were filtered and samples of TLs from *Rhodococcus erythropolis* with MSM supplemented with 2 % *n*-dodecane and 4 % glucose were diluted in 50 % acetonitrile and filtered once more.

To perform this methodology a Waters AcquityTM Ultra Performance LC (Waters®, Ireland) equipment of UHPLC consisting of a system of binary pumps, degassing, automatic sampler and oven for column was used. The mass spectrometer used was the triple quadrupole type, model Waters AcquityTM (Waters®, Ireland).

A Purospher® STAR RP-18 2 µm (2.1 x 50 mm) column was used at 35 °C. The mobile phase consisted of HCOOH 0.1 % in water (eluent A) and acetonitrile (eluent B). The elution program used was performed during 15 min, with different concentrations of solvents A and B during that time (Table 4). A constant flow rate of 0.3 mL/min and the injection volume was 10 µL. The ionization of the compounds was carried out by a positive (ESI +) and negative electrospray source (ESI-), at different cone voltages (20 to 40 V). Data acquisition and mass spectrometric evaluation were performed with MassLynx Software 4.1.

Table 4. UHPLC conditions.

Time (min)	Eluent A (%)	Eluent B (%)
0	90	10
1	90	10
6	5	95
10	5	95
10.10	90	10
15	90	10

5.5 - Characterization

In addition to the chromatography's more tests must be done to ensure that TLs were formed and presented characterists proprieties of biosurfactants, like, conductivity and surface tension. In line with that the following assays were carried out and conductivity and surface tension were measured.

5.5.1. Conductivity

The conductivity of an electrolyte solution is the quantitative numerical expression of its ability to carry electrical current.

To obtain this measurement a stock solution was made in order to proceed with a serial dilution. The solution was prepared by weighing 0.05 g of the solid from the extraction and 10 mL of distilled water. It was proceeded by doing a serial dilution with water ending up with ten different concentration samples (5.0 mg/mL, 4.5 mg/mL, 4.0 mg/mL, 3.5 mg/mL, 3.0 mg/mL, 2.5 mg/mL, 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL and 0.5 mg/mL). Using a multiparameter tester in the conductivity program as instructed it was proceeded to measure the conductivity in the samples at 21 ºC.

5.5.2. Surface tension and CMC

Surface tension is defined as the force per unit length exerted by a liquid in contact with a solid or another liquid[69]. It can also be considered as a measure of the free energy per unit area associated with a surface or an interface. Among the known liquids, water has the highest surface tension value of 72 dyne/cm or mN/m, this value would be reduced after the addition of surfactant [46,69].

To determine the surface tension the plate method was performed at room temperature using a Krüss tensiometer K12 [30,68] (Fig. 8). The instrument was calibrated against distilled water. Surfactant solutions were obtained by successive dilutions of a 5 mg/mL of TLs samples from both microorganisms. The equipment was calibrated after each measurement, the plate was washed with distilled water, after with acetone, and was then heated in a flame until reaching red hot. All measurements were taken in triplicate and the mean value was calculated.

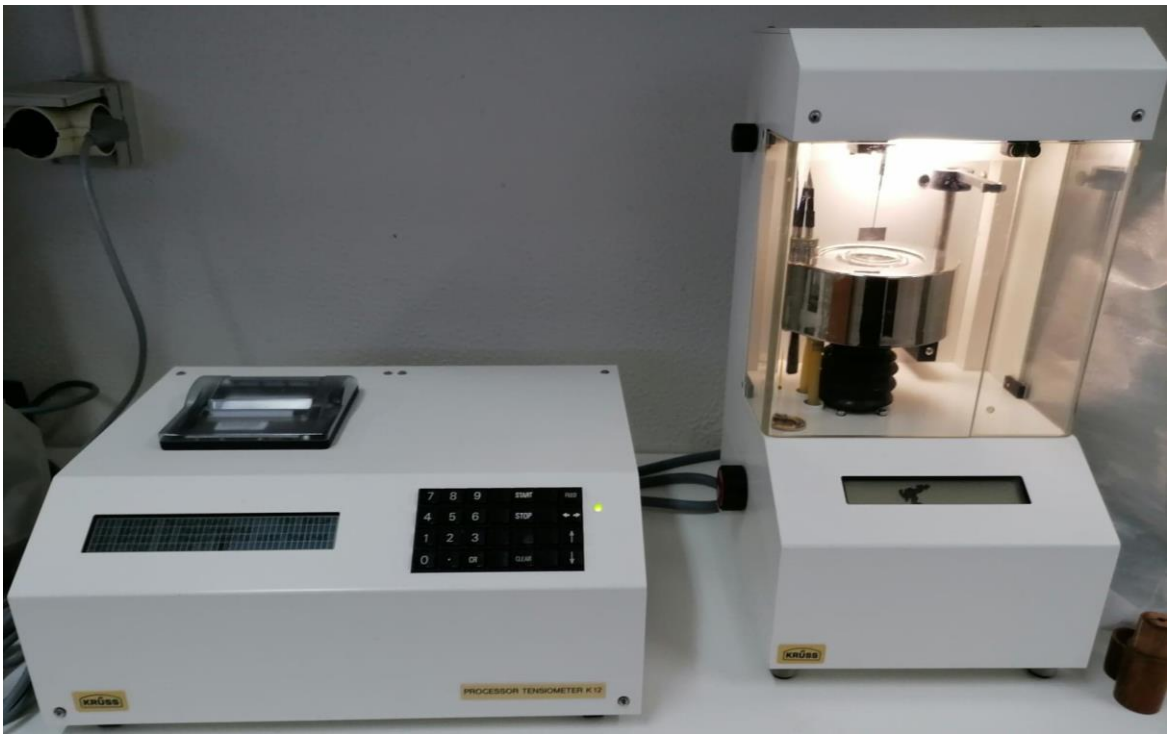


Figure 8. Kruss tensiometer k 12 used in the TLs assays.

The CMC of the TLs was determined by a graphical method [70]. After surface tension was measured for each dilution it was plotted against a logarithm of the dilution. The CMC is the concentration in which the descending line intersects the horizontal one. After estimating the CMC value by the graphical method [36].

5.6. Cytotoxicity Assays

In order to evaluate the cytotoxicity, cell lines H1975 were already cultured in monolayer in RPMI-1640 medium with L-glutamine supplemented with 10 mM HEPES, 2.5 g/L D-glucose, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 10 % FBS and 1 % Pen/Strep (complete cell culture medium), cells were maintained at 37 °C, under a humidified atmosphere containing 5 % CO_2 in air.

The TLs samples were prepared with saline solution at 0.9 %. Two initial samples were prepared from the extraction from *Rhodococcus* and *Rhodotorula*, both with medium supplemented with *n*-dodecane. From the initial samples (10 mg/mL), dilutions were made to end up with concentrations of 8 mg/mL, 4 mg/mL, 3 mg/mL, 2 mg/mL, 1 mg/mL and 0.4 mg/mL.

5.6.1. Trypsinization

Trypsinization is the process of using trypsin, a proteolytic enzyme that breaks down proteins, to dissociate adherent cells from the vessel in which they are being cultured.

This process began with the disposal of the medium, then the cells were washed with PBS. Trypsin was added and it was incubated for 5 min at 37 ºC. After the 5 min, cells were dissociated and the medium was added and centrifuged for 5 min at 1000 rpm. The supernatant was discarded and cells were resuspended in the medium. Cells were counted via Neubauer cell, having a cellular density of 3 x 10^3 cells/well. The cells were resuspended, plated and incubated for 24 hours.

After the 24 hours, TLs were prepared in saline solution 0.9 %. The medium was discharged from the wells and 195 µL of supplemented medium was added, 5 µL of compounds were also added and left for 72 h incubation, noticing that the plaque had a negative control (supplemented medium) as well as a positive control (cisplatin, 50 µM).

5.6.2. MTS

The 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium) (MTS) reduction assay is based on the ability of viable cells to convert the soluble tetrazolium salt into a coloured formazan product by a mitochondrial enzyme (NAD-dependent dehydrogenase).

After treatment with the compounds and extraction of the incubation medium, cells were washed with PBS, followed by the addition of 100 µL of fresh complete growth medium plus 20 µL of MTS substrate prepared from the CellTiter 96® Aqueous MTS (Promega) according to the manufacturer's instructions. Cells were incubated for 2 h with the MTS reagent and the results were measured in terms of absorbance at 490 nm and 690 nm (reference wavelength) using a SPECTROstar OMEGA microplate reader. Three independent assays were carried out and three replicates were used for each condition in each independent experiment.

6 - Results and Discussion

One of the goals of this work consisted in the development of a viable and economic process to produce biosurfactants, namely trehaloselipids (TLs) produced by fermentation of two different microorganisms. The biosynthesis of the glycolipids is dependent on the microorganism and the environmental conditions, for that reason in this work we used *Rhodococcus* that is known to produce TLs and *Rhodotorula* sp to evaluate their capacity to produce them, under different conditions, towards the cytotoxicity evaluation.

6.1. Growth

6.1.1. *Rhodococcus erythropolis*

Cell growth is strongly reliant on the composition of the growth medium including the carbon sources, nitrogen sources and salts. Here, we optimized the carbon sources for the production of TLs. In total five different media were used. Two of the media are strongly supported by glucose (*Gym streptomyces* and MSM supplemented with glucose), and the other based on salts supplemented with different carbon sources (*n*-hexadecane, *n*-dodecane, glycerol and glucose). The growth was carried out in 500 mL shake flasks for 72 h with controlled conditions such as temperature and rotation (28 ºC and 200 rpm). The microbial growth was monitored by biomass measurements by means of optical density observations of the cell suspension at 600 nm.

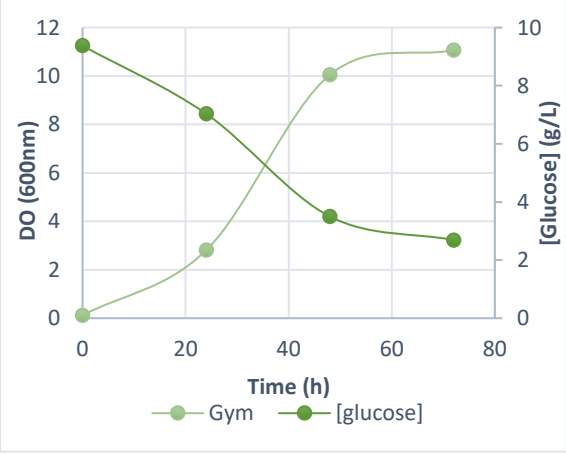


Figure 9. Growth curve of *Rhodococcus* in Gym *Streptomyces* medium and its glucose consumption (g/L)

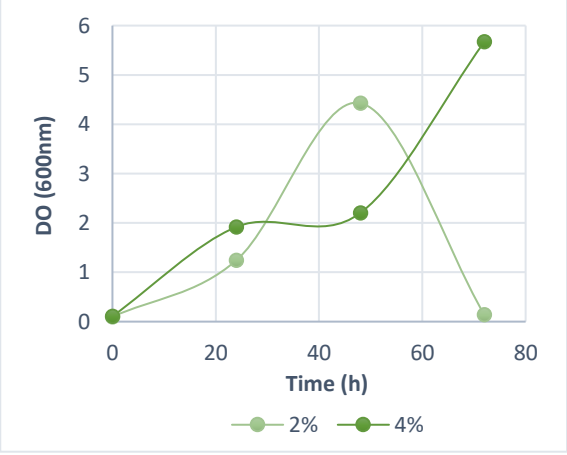


Figure 10. Growth curve of *Rhodococcus* in MSM supplemented with 2 and 4 % of *n*-hexadecane

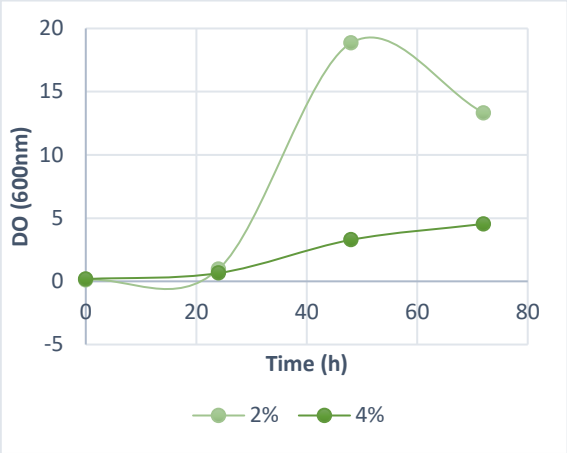


Figure 11. Growth curve of *Rhodococcus* in MSM supplemented with 2 % and 4 % of *n*-dodecane

Figure 12. Growth curve of *Rhodococcus* in MSM supplemented with 4 % of glucose and 4 % glycerol and the glucose consumption (g/L) for the first substrate.

When observing the growth data (Fig 9, 10, 11, 12), we can mention that the media that have a higher value at DO (600 nm) is the MSM supplemented with 2 % *n*-dodecane, reaching that point at 48 h with a microbial optical density of 18.85 (Fig 11). Some microbial growth has a decay after the 48 h which is the case of *Rhodococcus* in MSM supplemented with 2 % *n*-hexadecane (Fig 10) and in MSM supplemented with 4 % *n*-dodecane (Fig 11). In all the other media, Gym Streptomyces, MSM supplemented with 4 % *n*-hexadecane, MSM supplemented with 2 % *n*-dodecane, MSM supplemented with 4 % glucose and MSM supplemented with 4 % glycerol, *Rhodococcus* showed a growth stationary phase (Fig 9, 11, 12) until monotonized at the 72 h.

Overall and in a decreasing order it is possible to say that the better media to attain higher growth density of *Rhodococcus* are MSM supplemented with 2 % *n*-dodecane (Fig 11) followed by Gym *Streptomyce*s (Fig 9), MSM supplemented with 4 % glycerol (Fig 12), MSM supplemented with 4 % *n*-hexadecane (Fig 10), MSM supplemented with 4 % *n*-dodecane (Fig 11) and MSM supplemented with 2 % *n*-hexadecane (Fig 10) and finally, MSM supplemented with 4 % glucose (Fig 12).

Comparing the 24 h in the MSM medium supplemented with glucose, the optical density obtained in this work was very similar to the one obtained by Kurane[71], where the values were around 1.0-1.2

In a general way, and even with different media, *Rhodococcus erythropolis* stopped growing at 48 h, except for the growth with MSM supplemented with 4 % glycerol, which stopped later and MSM supplemented with 2 % *n*-hexadecane that stopped its growth earlier at 24 h. This led us to understand that most likely the log phase would end shortly after the 48-hour growth.

Cell concentration was calculated through the results of the optical density presented in the graphics above (Fig 9,10, 11 and 12) and presented in table 5.

Table 5. *Rhodococcus erythropolis* cells concentration (cell/mL) at different times (h) in the different media.

Media	**Time (h)**		
	24	48	72
	(cell/mL)	(cell/mL)	(cell/mL)
Gym Streptomyces	2.25×10^9	8.04×10^9	8.86×10^9
2 % n-hexadecane	9.94×10^8	3.54×10^9	1.12×10^8
4 % n-hexadecane	1.54×10^8	1.76×10^9	4.54×10^9
2 % n-dodecane	7.65×10^8	1.51×10^{10}	1.07×10^{10}
4 % n-dodecane	5.20×10^8	2.62×10^9	3.63×10^9
4 % Glycerol	1.19×10^9	3.64×10^9	7.86×10^9
4 % Glucose	9.01×10^8	1.88×10^9	2.07×10^9

In agreement with the optical density, *Rhodococcus erythropolis* cultured in MSM supplemented with 2 % *n*-dodecane has a higher cell density of the tested media, it gets that point at 48 h with an optical density (OD 600 nm) of 18.85 and 1.51×10^{10} cells (Fig 11 and Table 5). In the same table, is possible to see with accuracy the highest cell density of the culture in the specific media at a certain time. The higher values were attained at

48 h in the cultures grown in MSM supplemented with 2 % *n*-dodecane and supplemented with 2 % *n*-hexadecane. The highest values of the other media were reached at 72 h cultures, which means that there is a need to culture the bacteria for longer to ensure the time of end of the log phase.

Regarding glucose consumption, the concentration decreases considerably in the cultures grown in the media supplemented with glucose (Gym *Streptomyces* and MSM with 4 % glucose). From the 9.37 g/L glucose concentration in the initial medium of Gym *Streptomyces* only 2.69 mg/mL remained in the culture. In the culture grown on MSM supplemented with 4 % glucose, the initial glucose concentration was 0.88 g/L and only 0.26 g/L remained in the culture. This led to the conclusion that around 70 % of the glucose was consumed in both cultures.

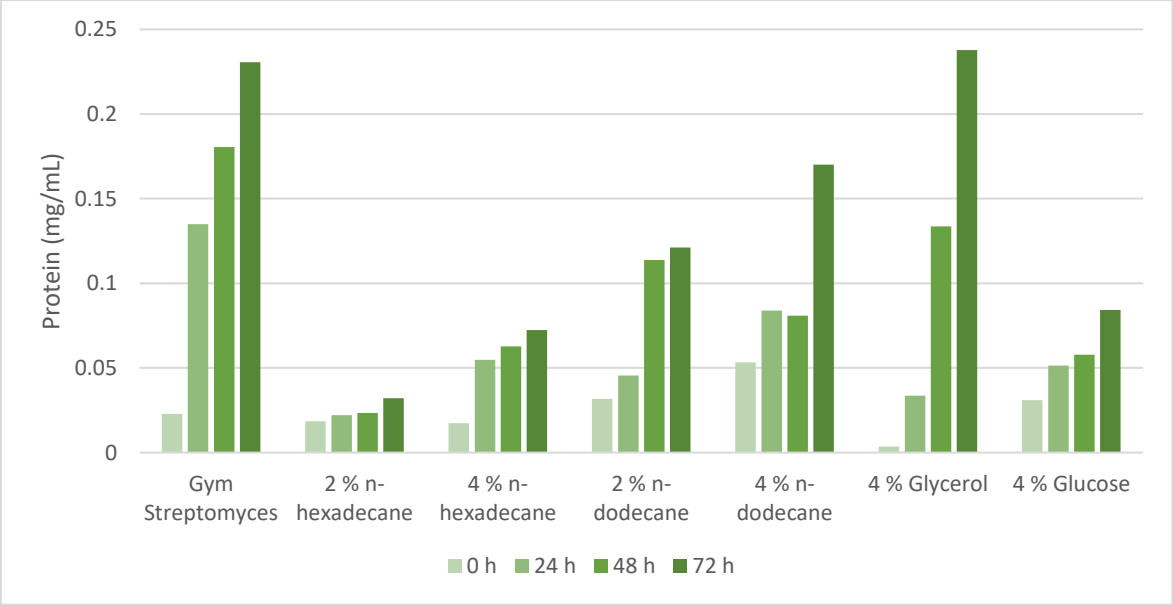


Figure 13. *Rhodococcus erythropolis* protein concentration at different growth times per medium of the whole broth.

Using the data from Fig. 4, a new figure with all protein values was made in order to be easier to compare the *Rhodococcus erythropolis* protein production on every media used in this project.

Unlike the growth, the bacteria produced more protein content when grown on Gym *Streptomyces*, although as expected, the maximum was achieved at 48h, when it also reached a maximum of bacterial growth.

6.1.2. *Rhodotorula* sp

Just like *R. erythropolis*, the growth of *Rhodotorula* sp. is strongly dependent on the composition of the medium therefore, we optimized the carbon sources for the production of TLs and analyse the glucose consumption and the protein production. Herein we use the same media that was used for the previously. The growth was made in the same conditions, in shake flasks of 500 mL for 72 h with controlled temperature and agitation (28 ºC and 200 rpm). The microbial growth was also monitored by biomass measurements through optical density of the cell suspension at 600 nm.

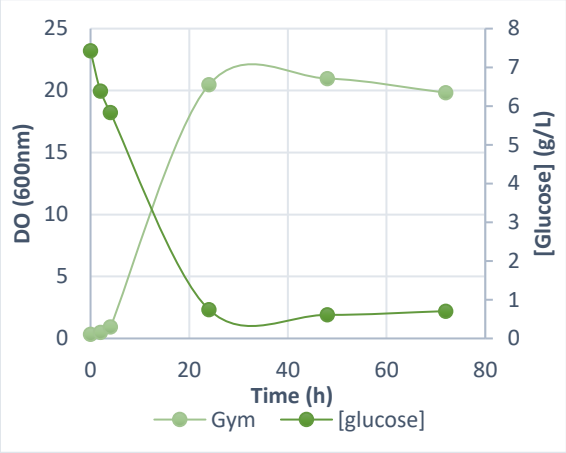


Figure 14. Growth curve of *Rhodotorula* sp in Gym *Streptomyces* medium and glucose consumption (g/L)

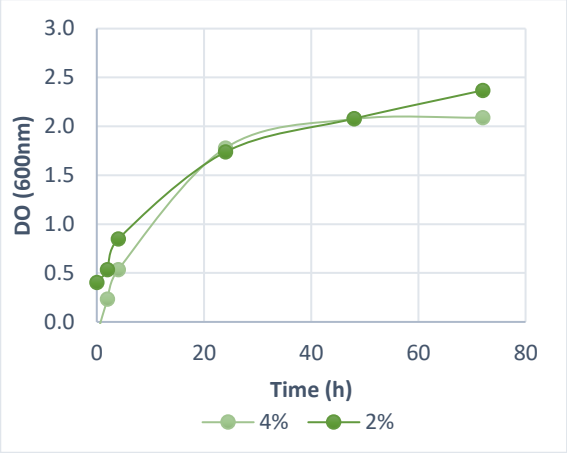


Figure 15. Growth curve of *Rhodotorula* sp in MSM supplemented with 2 and 4 % of *n*-hexadecane

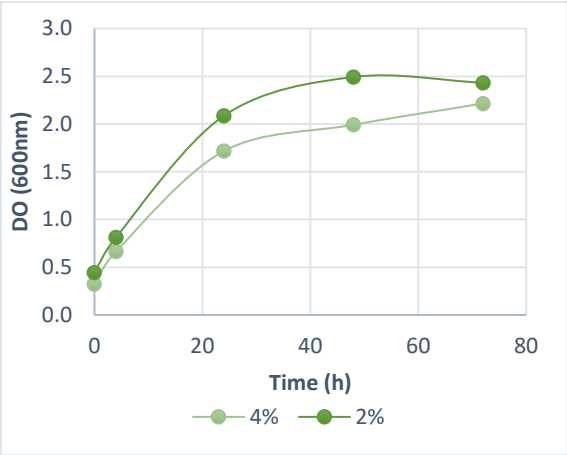


Figure 16. Growth curve of *Rhodotorula* sp in MSM supplemented with 2 and 4% of *n*-dodecane

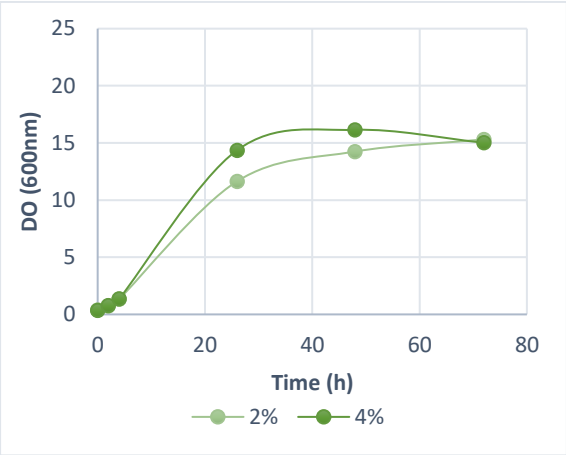


Figure 17. Growth curve of *Rhodotorula* sp in MSM supplemented with 2 and 4 % of glycerol

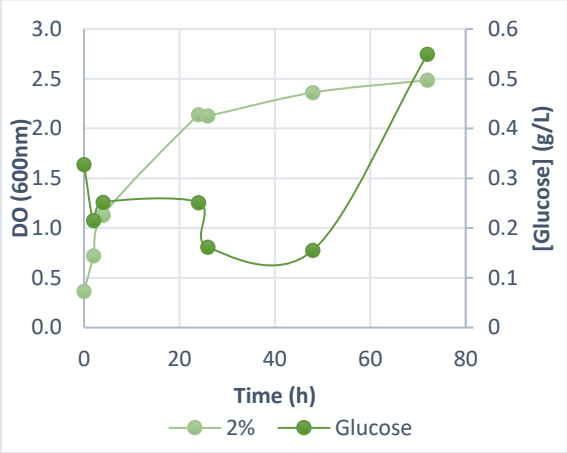


Figure 18. Growth curve of *Rhodotorula* sp in MSM supplemented with 2 % glucose and glucose consumption (g/L)

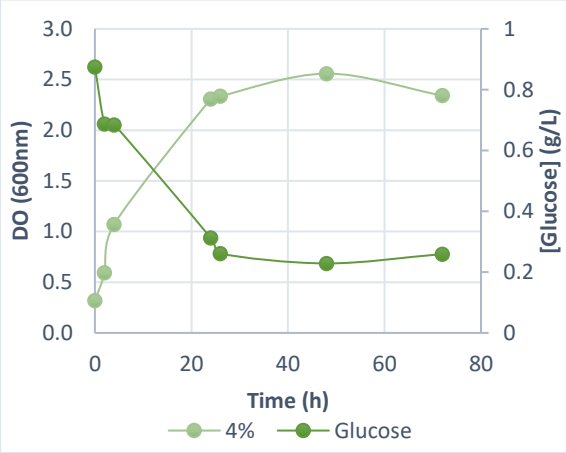


Figure 19. Growth curve of *Rhodotorula* sp in MSM supplemented with 4 % glucose and glucose consumption (g/L)

Looking to the data, two main differences can be noticed in the growth. Growth culture on Gym *Streptomyces* (Fig 14) and MSM supplemented with glycerol (Fig 17) were able to reach values around 20 which are eight times higher compared to the others *Rhodotorula* sp cultures. Most cultures seem to stop growing between 24 and 48 h with some exceptions

such as MSM supplemented with 2 % of *n*-hexadecane (Fig 15), MSM supplemented with 4 % of *n*-dodecane (Fig 16), MSM supplemented with 2 % glycerol (Fig 17) and MSM supplemented with 2 % glucose (Fig 18). The glucose consumption stopped in the three cases (Fig 14, 18 and 19) at 24 h.

Cell concentration of *Rhodotorula* sp cultures was also calculated through the results of the optical density (DO 600 nm) obtainable in the graphics above (Fig 14, 15, 16, 17, 18 and 19) and are presented in table 6.

Table 6. *Rhodotorula* sp cell concentration (cell/mL) at different times (h) in the different media.

Media	**Time (h)**		
	24	48	72
	(cell/mL)	(cell/mL)	(cell/mL)
Gym Streptomyces	1.64×10^{10}	1.68×10^{10}	1.59×10^{10}
2 % n-hexadecane	1.39×10^{9}	1.66×10^{9}	1.89×10^{9}
4 % n-hexadecane	1.42×10^{9}	1.66×10^{9}	1.67×10^{9}
2 % n-dodecane	1.67×10^{9}	1.99×10^{9}	1.95×10^{9}
4 % n-dodecane	1.37×10^{9}	1.59×10^{9}	1.77×10^{9}
2 % Glycerol	9.32×10^{9}	1.14×10^{10}	1.22×10^{10}
4 % Glycerol	1.15×10^{9}	1.29×10^{10}	1.2×10^{10}
2 % Glucose	1.71×10^{9}	1.89×10^{9}	1.99×10^{9}
4 % Glucose	1.85×10^{9}	2.05×10^{9}	1.87×10^{9}

In table 6, it can be observed with accuracy the highest cell density of the culture in the specific media at a certain time. Similar to the optical density, *Rhodotorula* sp cultured Gym *Streptomyces* show a slight variation in cell density between 24 and 48 hours, with values

of 1.68 x 10^{10} and 1.59 x 10^{10} cell/mL respectively. With such similar values, it is possible to settle that the log phase ended at 24 h and entered the stationary phase.

The higher values were mostly reached at 48 h in the cultures grown in MSM supplemented with 4 % *n*-hexadecane, 2 % *n*-dodecane, 4 % glycerol and 4 % glucose. The maximum values of cell density on the other media were reached at 72 h cultures, which are the case of cultures grown in MSM supplemented with 2 % *n*-hexadecane, 4 % *n*-dodecane, 2 % glycerol and 2 % glucose, meaning that there´s a need to culture the bacteria for longer to ensure the time of end of the log phase.

From the 7.42 g/L glucose concentration in the initial medium, only 0.71 mg/mL remained in the culture with Gym *Streptomyces* (Fig 14). In the culture with MSM supplemented with glucose the initial glucose was 0.33 and 0.88 g/L in cultures with 2 and 4 %, respectively, only 0.16 and 0.26 g/L remained on the respective cultures. This led to the conclusion that more than 90 %, 50 % and 70 % of the glucose was consumed in the cultures with Gym *Streptomyces*, MSM supplemented with 2 % glucose and MSM supplemented with 4 % glucose, respectively.

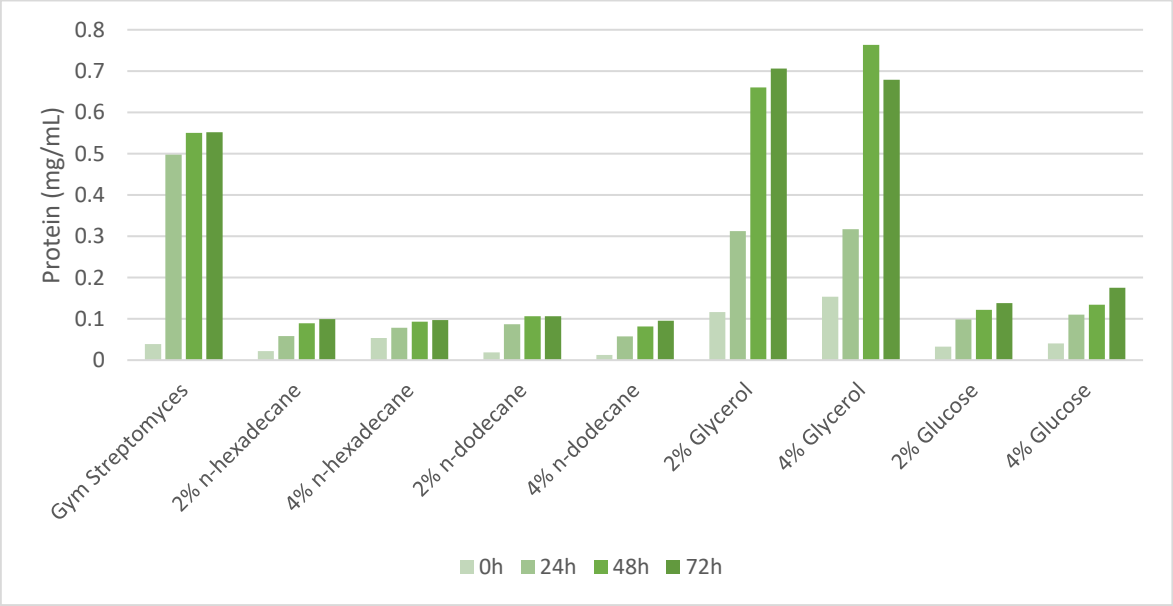


Figure 20. *Rhodotorula* sp protein concentration at different times per medium of the whole broth.

In agreement with *Rhodotorula* sp growth, the ones that produced more protein were the same that produced a higher amount of bacteria (Fig 14, 17 and 20), and comparing with *Rhodococcus erythropolis* the medium that allowed a better protein production was the same (glycerol). Regarding the media supplemented with alkanes, all four produced a similar amount of protein with a maximum of around 0.1 mg/mL. The media supplemented with glucose produced a larger amount with more glucose but not a significant value not reaching 0.2 mg/mL. In a study preformed in 2013, the protein content on the whole broth of TL produced by marine bacterium *Rhodococcus* sp. strain PML026 with a carbon source of 2 % sunflower oil, was around 0.35 mg/mL and 0.60 mg/mL at 24 and 72 h respectively[47]. When comparing it with the results showed in this work, *Rhodotorula* sp. produces a more similar amount of protein in that amount of time than *Rhodococcus erythropolis.*

6.2. Extraction – Separation and purification

The extraction process from culture broth, via liquid-liquid extraction method. The method used ethyl acetate:methanol (8:1) solution and, after the decantation the extract was dried out with anhydrous sodium sulfate, samples were then collected and weighed.

The extraction of TL from *Rhodococcus* medium most of the time got separated into three layers (Fig 21) where the two bottom layers were extracted and dried individually (Fig 23 and 24). While the extraction of TL from *Rhodotorula sp* medium divided into only two phases (Fig 22), being the organic dry (Fig 25).

Figure 21. Three layer extraction from *Rhodococcus erythropolis* in MSM supplemented with 2 % *n*-hexadecane

Figure 22. Extraction of TLs from *Rhodotorula* sp in MSM supplemented with 2 % *n*-hexadecane

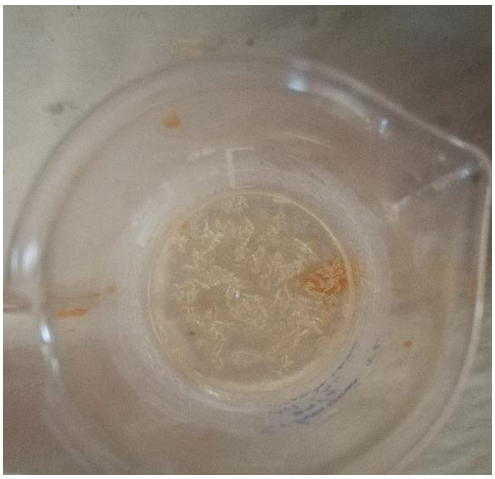


Figure 23. Extraction of TLs from bottom layer of *Rhodococcus erythropolis* in MSM supplemented with 4 % *n*-hexadecane

Figure 24. Extraction of TLs from middle layer of *Rhodococcus erythropolis* in MSM supplemented with 4 % *n*-hexadecane

Figure 25. Extraction of TLs from *Rhodotorula* sp in MSM supplemented with 4 % *n*-hexadecane

After dried, the samples were separated and prepared to further analysis. To identify the different components of the samples for the subsequent tests samples from *Rhodococcus erythropolis* media were enumerated from 1 to 10 and samples from *Rhodotorula* sp were identified by letter and number, being the letter related to the media and the number to the incubation time (Fig 26).

Figure 26. Final samples of TLs extracted from *Rhodococcus erythropolis* and *Rhodotorula* sp

As depicted in fig 26, the samples had different colours, depending on the microorganism and the substrates. All the samples from *Rhodococcus erythropolis* culture have a similar colour except for sample 4, from MSM supplemented with 4% *n*-hexadecane, that ended up with a dark color. The samples from *Rhodotorula* sp culture have a light coloration except the samples "A"s from Gym *streptomyces* medium due to the natural colour (rose) of the microorganism and the elevated cell concentration.

Table 7. Extracted product from *Rhodococcus erythropolis*, media, concentration and yield

	Rhodococcus erythropolis				
Sample	**Media**	**Incubation time (h)**	**Total Dry Weight (mg/mL)**	TLs production (mg/mL)	**Yield (TLs/Carbon source) (mg/mg)**
1	MSM with 2 % *n*-hexadecane	24	60.1	60.06	1.50
*2 **	MSM with 2 % *n*-hexadecane	24	4.7	4.74	0.24
3	MSM with 4 % *n*-hexadecane	72	48.8	48.76	1.22
4	MSM with 4 % *n*-hexadecane	48	72.8	72.76	1.82
5	MSM with 4 % *n*-dodecane	24	34.8	34.83	0.87
6	MSM with 4 % *n*-dodecane	48	27.9	27.94	0.70
7	MSM with 4 % glucose	24	72.7	72.735	1.82

8	MSM with 4 % glucose	72	26.0	25.98	0.65
9	MSM with 4 % glycerol	72	16.5	16.52	0.41
10	MSM with 4 % glycerol	48	43.6	43.57	1.09

Table 8. Extracted product from *Rhodotorula* sp, media, concentration and yield

	***Rhodotorula* sp**				
Sample	**Media**	**Incubation Time (h)**	**Total Dry Weight (mg/mL)**	**TLs production (mg/mL)**	**Yield (TLs/Carbon source) (mg/mg)**
A3	Gym Streptomyces	24	35.3	39.31	0.65
A5	Gym Streptomyces	72	26.2	35.26	0.48
B1	MSM with 4 % *n*-dodecane	24	19.0	26.18	0.75
B3	MSM with 4 % *n*-dodecane	72	29.8	19.00	0.47
C1	MSM with 4 % *n*-hexadecane	24	18.7	29.80	3.11
C3	MSM with 4 % *n*-hexadecane	72	62.3	18.68	1.02

E1	MSM with 2 % *n*-hexadecane	24	20.4	62.29	1.16
E2	MSM with 2 % *n*-hexadecane	48	46.3	20.42	0.89
G2	MSM with 4 % glucose	48	35.7	46.35	0.65
G3	MSM with 4 % glucose	72	35.3	35.69	0.48

As it can be noticed in Tables 7 and 8, the main difference between the extractions from the two different microorganisms is that *Rhodococcus erythropolis* has a higher weight of extracted product when compared with *Rhodotorula* sp.

6.2.1. Separation and identification

Separation and identification of TLs were carried out by TLC, HPLC and UHPLC as described in 5.4.

6.2.1.1. TLC

For the TLC we ended up using pure chloroform to dilute the samples but also a mixture of chloroform, methanol and water in the same proportion used for the mobile phase (65:15:2).

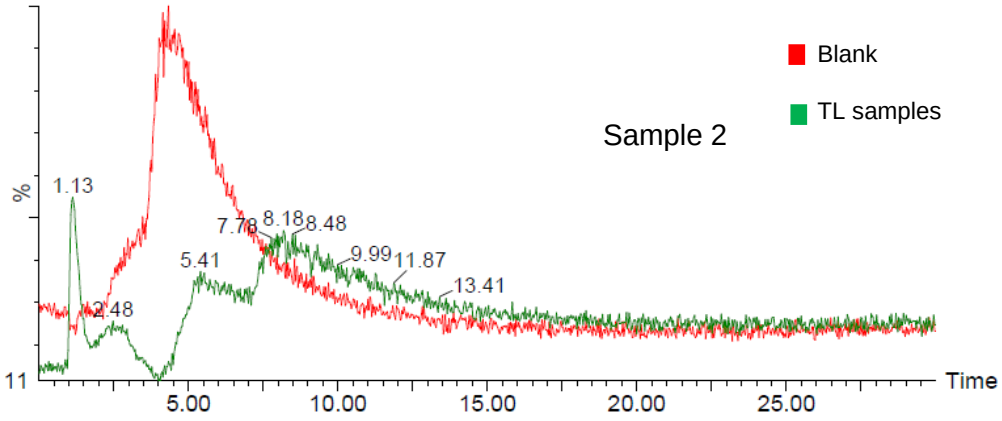
After the elaboration of the TLCs, the results showed a green-coloured product, in both cases, the samples diluted in pure chloroform (Annex 2 and 3) as well as in the chloroform:methanol:water mixture (Annex 1), just like Franzetti [72] mentioned in 2010 which give the impression that TLs are present in the extract in both microorganisms. Our green-product in particular did not appear as a spot but as a line instead. To ensure that the green-coloured line was not prevenient from the solvents, a distinct TLC was made with the same conditions but without the extracted samples, where no green lines or spots appeared (Annex 4). This led us to conclude that TLs are present in the extracts not only from *Rhodococcus* erythropolis but also from *Rhodotorula* sp in different media.

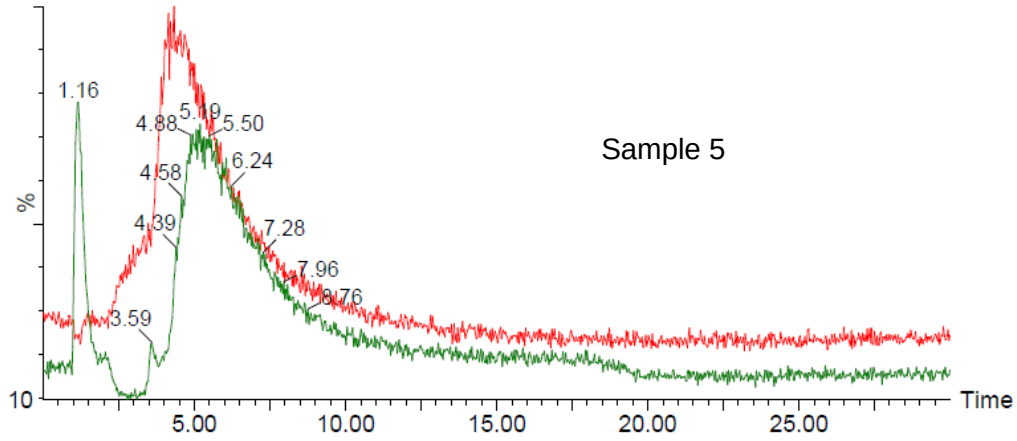
6.2.1.2. **HPLC** and **UPLC**

The HPLC method developed for the identification of TLs allowed a preliminary identification of TL's. The samples of the TL's were solubilized in acetonitrile and water, in a proportion of 1:1, in a concentration of 1 mg/mL.

Trehaloselipids are made up of a disaccharide, trehalose, linked by an ester bond to α-branched β-hydroxy fatty acids. The α-branched β-hydroxy fatty acids are connected at the C6 and C6' of the carbohydrate structure in the case of the trehalose dimycolates and at C6 for the monomycolates, also succinoyl trehalose lipids.

Preliminar identification studies of trehaloselipids were carried out.





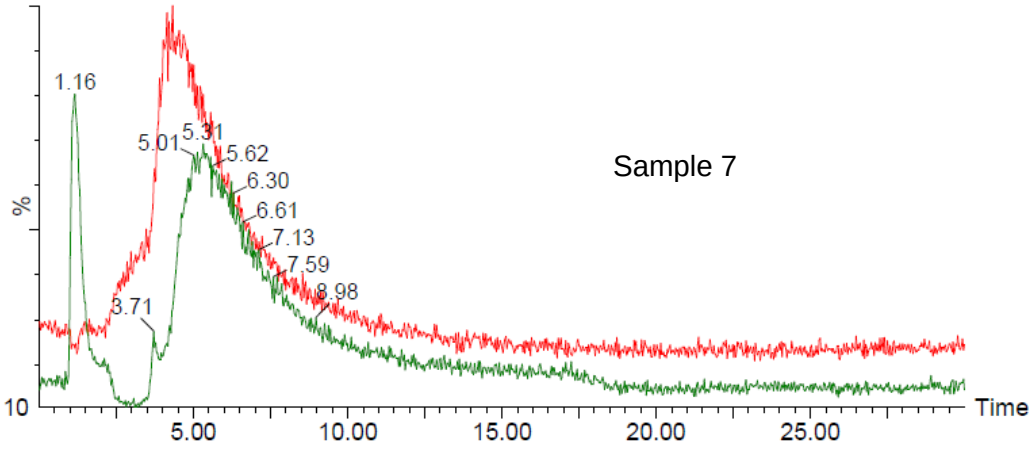
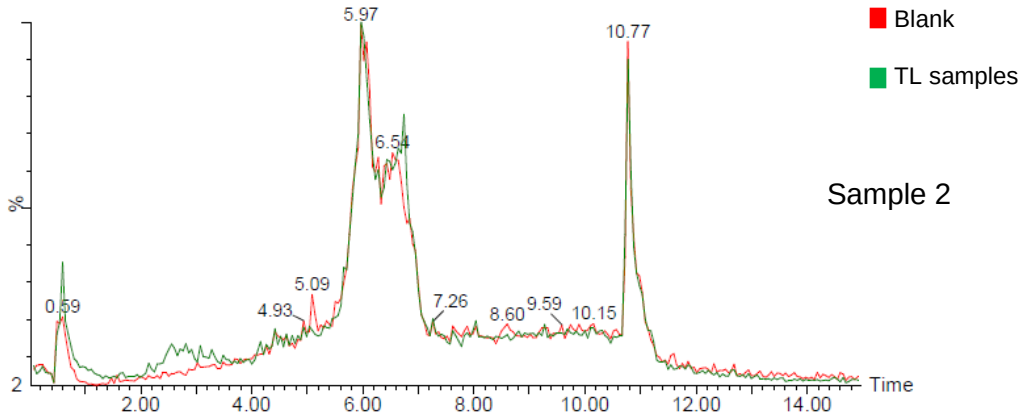
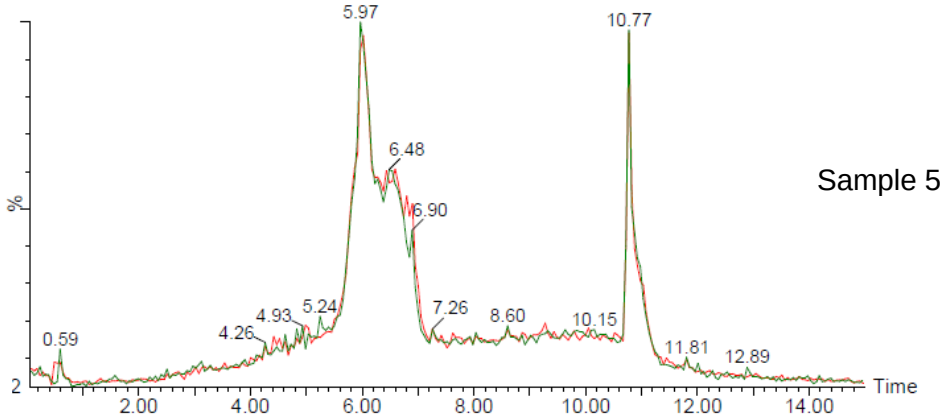


Figure 27. Chromatograms of *HPLC* of different samples from *Rhodococcus erythropolis.*





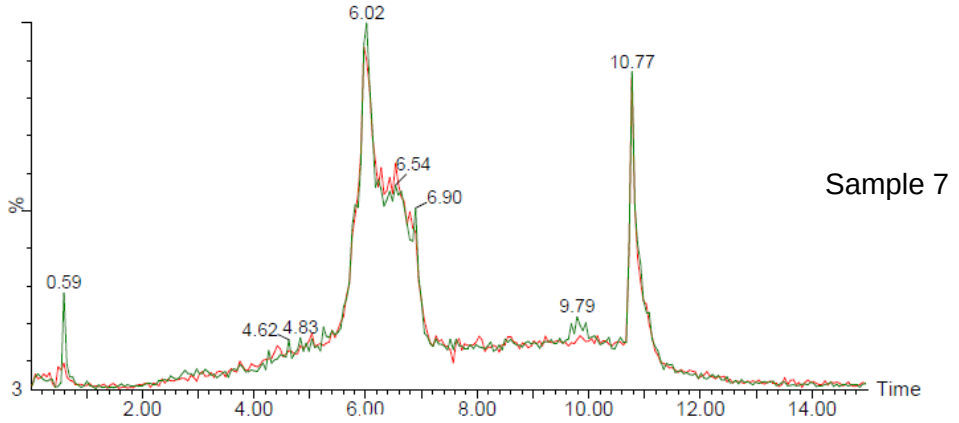
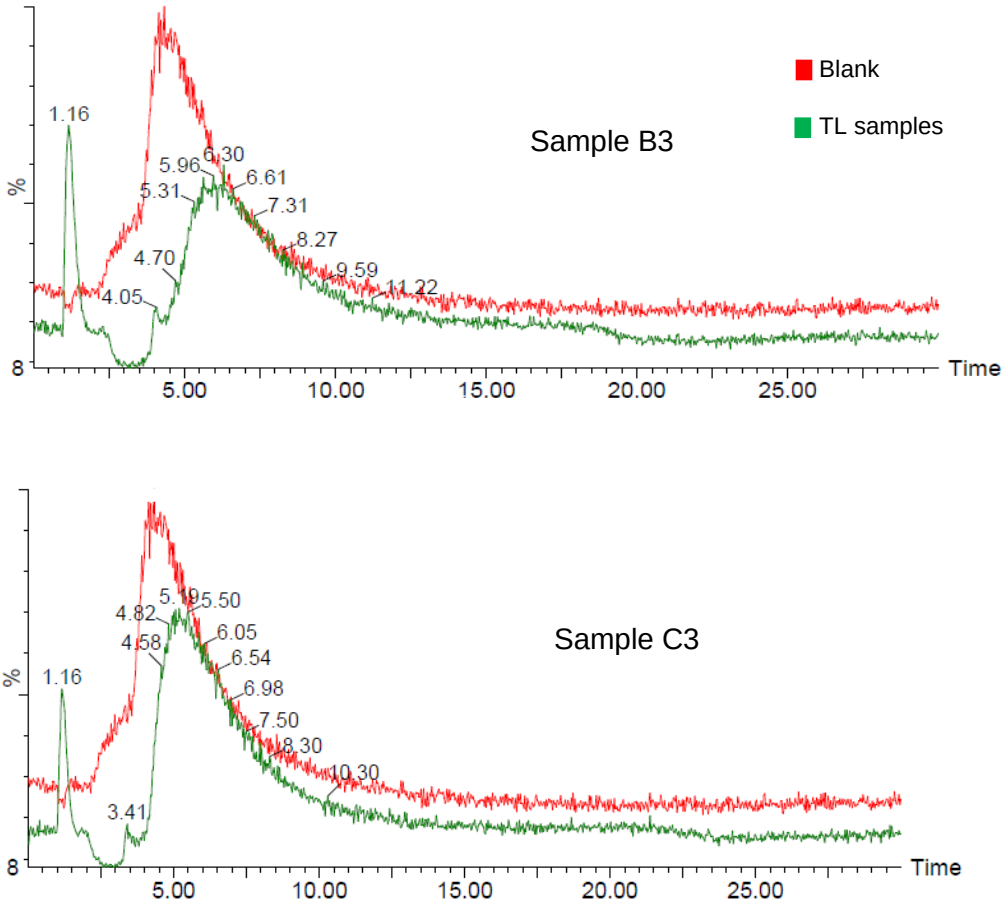


Figure 28. Chromatograms of *UPLC* of different samples from *Rhodococcus erythropolis.*



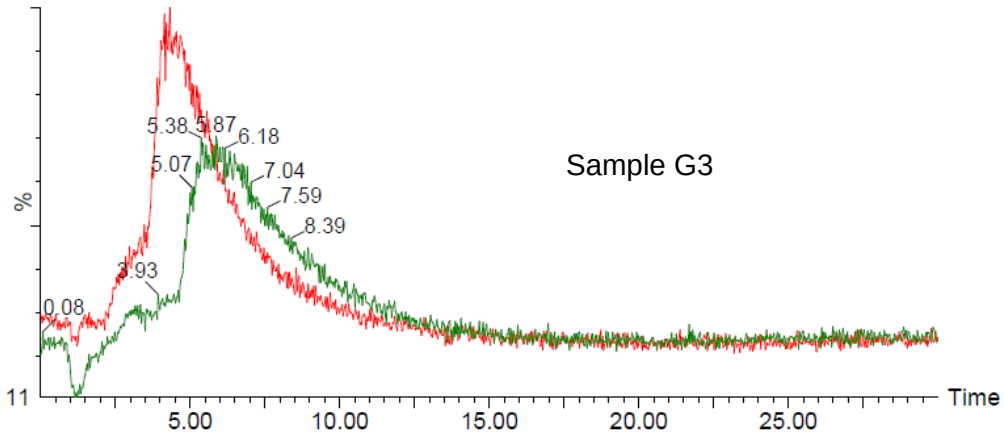


Figure 29. Chromatogram of *HPLC* of different samples from *Rhodotorula* sp.

6.2.1.3. Conductivity

With the diluted TL samples at 21 ºC, the conductivity was measured at different concentrations using a multiparameter tester. In Fig 30 it is possible to see the conductivity measured in function of the concentrations as well as the estimated CMC values.

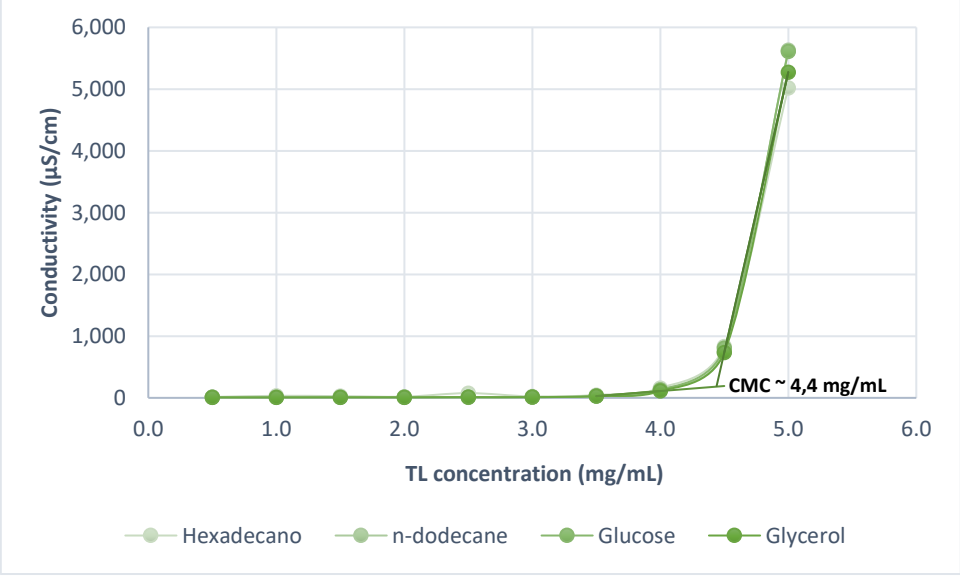


Figure 30. Conductivity of TL extracted from *Rhodococcus erythropolis* and estimated CMC

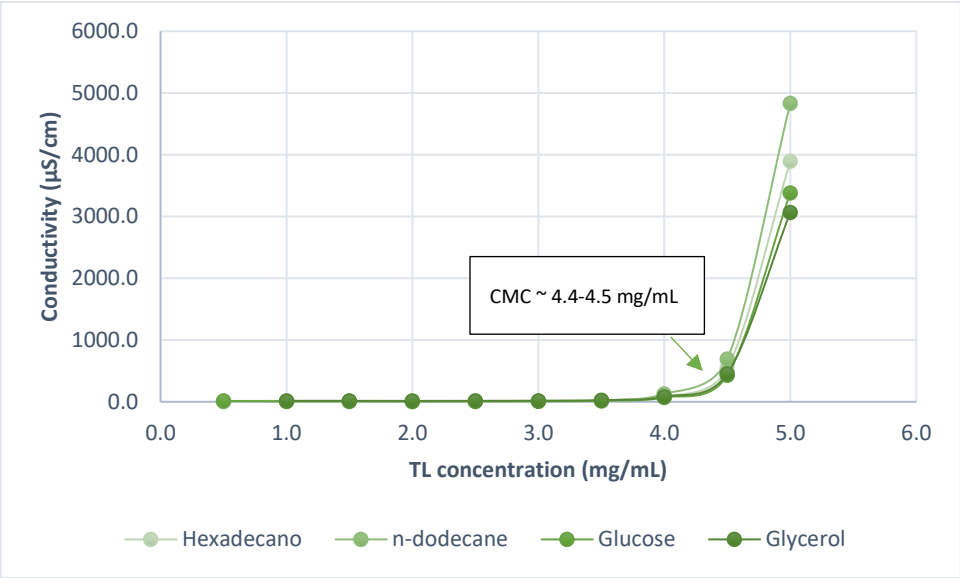


Figure 31. Conductivity of TL extracted from *Rhodotorula* sp. and estimated CMC

Through the analysis of the graphics (Fig 30 and 31), it is possible to estimate the CMC values, which in both cases are around 4.4 mg/mL. However, it is not possible to treat the results mathematically because the values under the CMC are not linear and above there are only two values.

CMC values in buffer solutions are smaller than CMC values of pure surfactant aqueous solutions presumably due to the stronger screening effect of the ions in the buffer which reduced the electrostatic repulsion of the surfactant headgroups thus promoting the micellization process.

The conductivity increased linearly with surfactant concentration till the breakpoint (corresponding to the CMC value) and thereafter also increased but with a larger slope. The increase in slope can be attributed to the cooperative binding of large amount of surfactant after CMC resulting in more released counterions from micelles thereby causing a larger increase in conductivity [73]. Pre-micelle formation in the CMC region is also a possibility since double chain surfactants and surfactants with bulky hydrophobic headgroups, such as bile salts, are known to form highly ionized small aggregates, such as dimers, trimers and small oligomers with reduced counter-ion binding [74]. On the other hand, traditional single-chain surfactants usually show an increase of conductivity after the CMC but with a smaller slope

due to the increase in size as a result of aggregation with increased counterion condensation, which overcomes the increase in charge yielding larger aggregates [74,75].

The values in this study have presented higher CMC compared to the published results. For instance, TL produced by marine bacterium *Rhodococcu*s sp. strain PML026 with a carbon source of 2 % sunflower oil shown a CMC of 0.25 mg/mL [47]. TL produced by *R. erythropolis* DSM 43215, both trehalose-dicorynomycolates and trehalose-monocorynomycolates presented a CMC of 0.004 mg/mL and trehalose-2,2' ,3,4-tetraester a CMC of 0.015 mg/mL [76]. On the other hand, succinoyl trehalose lipids produced by *R.erythropolis* SD-74 presented a CMC of 0.2 mg/mL [77].

6.2.1.4. Surface tension

The surface tension is one of the parameters used for the characterization of the compounds. In Fig 32 and 33 we can see the graphs of the surface tension in function of the logarithmic of TLs concentration.

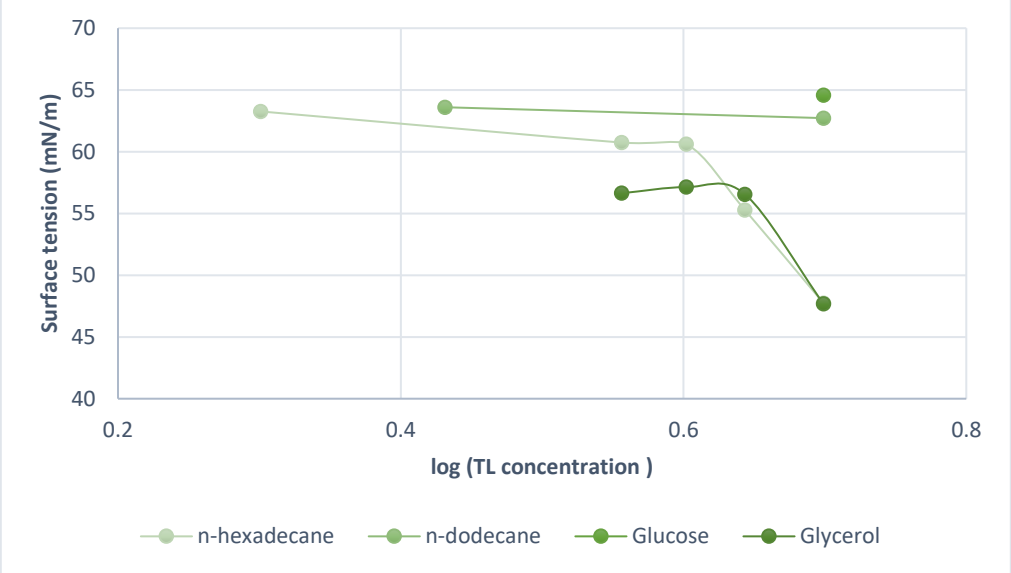


Figure 32. Surface tension of TL extracted from *Rhodococcus erythropolis*

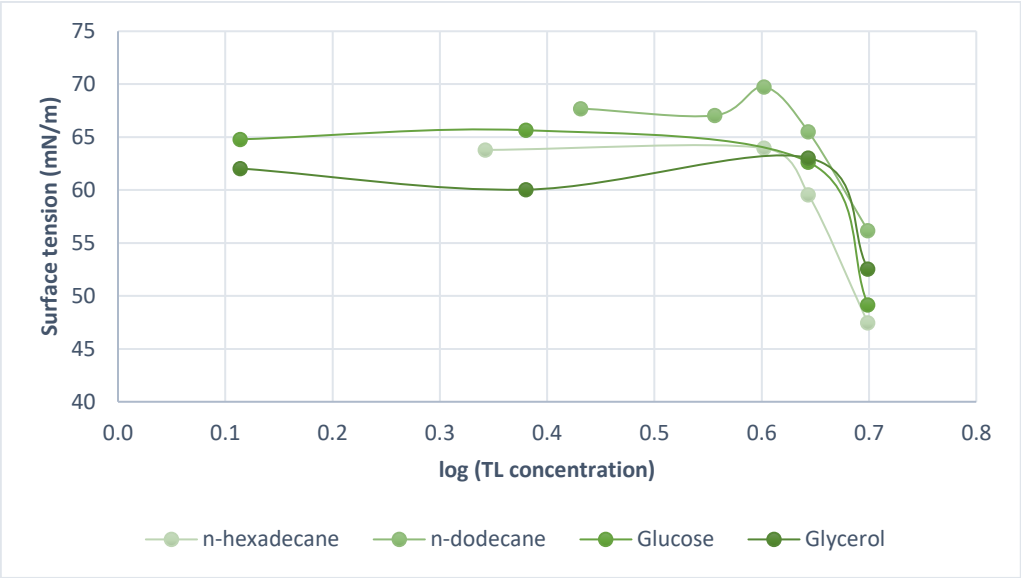


Figure 33. Surface tension of TL extracted from *Rhodotorula sp.*

As can be observed (Fig 32 and 33) the surface tension in all the samples decreased with the increase of TL concentration, although it is not possible to acknowledge the stabilization of the tension. Moreover, according to these graphics it is not possible to observe the reach of the CMC values, which should mean that it is above the concentrations used (higher than 5 mg/mL), even of the conductivity assays the CMC was obtained around 4.5 mg/mL.

The pC_{20} is a parameter that measures adsorption efficiency. However, it is not going to be used for the comparison because the profile is incomplete, nevertheless the comparison will be done through the surface tension at 5 mg/mL.

In both cases (TLs from *Rhodotorula* and *Rhodococcus*) the lower values for the surface tension was reached by media supplementation with *n*-hexadecane. The TLs extracted from *Rhodococcus erythropolis* both *n*-hexadecane and glycerol have similar values 47.74 and 47.69 mN/m respectively. Regarding TLs production in MSM supplemented with *n*-dodecane and glucose do not show significant values.

The surface tension of TL extracted from *Rhodotorula* sp is represented in all the MSM media. The lower values of surface tension with TLs obtained, respectively, in media supplemented with *n*-hexadecane with 47.57 mN/m (similar to *Rhodococcus*) followed by glucose with 49.16 mN/m, by glycerol with 52.55 mN/m and *n*-dodecane with 56.18 mN/m

Both microorganisms were able to produce TL that decreased the surface tension from 74 mN/m to 47 mN/m on water. Although typically, surfactants lower the surface tension between water and air from 72 to 35 mN/m [21]. For instance, TL produced by marine bacterium *Rhodococcu*s sp. Strain PML026 with a carbon source of 2 % sunflower oil shown surperficial tension of 35 mN/m [47], TL produced by *R. erythropolis* DSM 43215, both trehalose-dicorynomycolates, trehalose-monocorynomycolates and trehalose-2,2' ,3,4-tetraester presented a surface tension of 36, 32 and 26 mN/m, respectively [76] and succinoyl trehalose lipids produced by *R. erythropolis* SD-74 presented a value of 26 mN/m [77].

6.3 – Cytotoxicity evaluation

The general effects of glycolipids have been studied over the years. Therefore, it is known that some have antimicrobial [15,16], antiviral [17] and antitumor activities [1,56]. TLs are already known for inducting cell differentiation in the human promyelocytic leukemia cell line HL60 [26], inducting neuronal differentiation in PC12 cells [42] and antiviral activity against HSV and influenza virus [17]. Just like it was performed with leukemia cells, in this work, we also aimed at the evaluation of how cytotoxic can TLs be. The TLs being evaluated are those produced in this work prevenient from *Rhodococcus erythropolis* and *Rhodotorula*, both of them cultured in MSM supplemented with 4 % *n*-dodecane. The cancer cell line used was H1975 from NSCLC.

To test the anticancer activity of the produced TLs, the product was added in different concentrations to the cells and they were left to incubate for 72 hours. These 72 h are due to the cell cycle in order to properly see the effect of the product. The MTS reduction assay was used to evaluate the cell viability after those 72 hours, with saline solution as the negative control and cisplatin (50 µM) as the positive control. The negative control should present an extensive decrease in cell viability towards % values lower than 10-20 % and the positive control should present a very large percentage of cell death.

The absorbance was then read, in all the triplicates, the results were the following

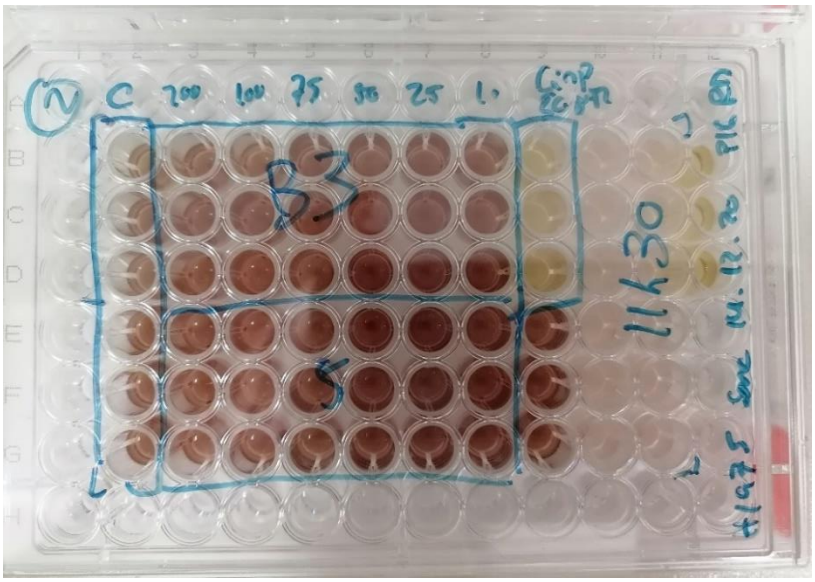


Figure 34. microplate resultant from the MTS assay with the concentrations and controls drawn on the lid.

The absorbance was then read, in all microplates, and the results were presented in figures 35 and 36

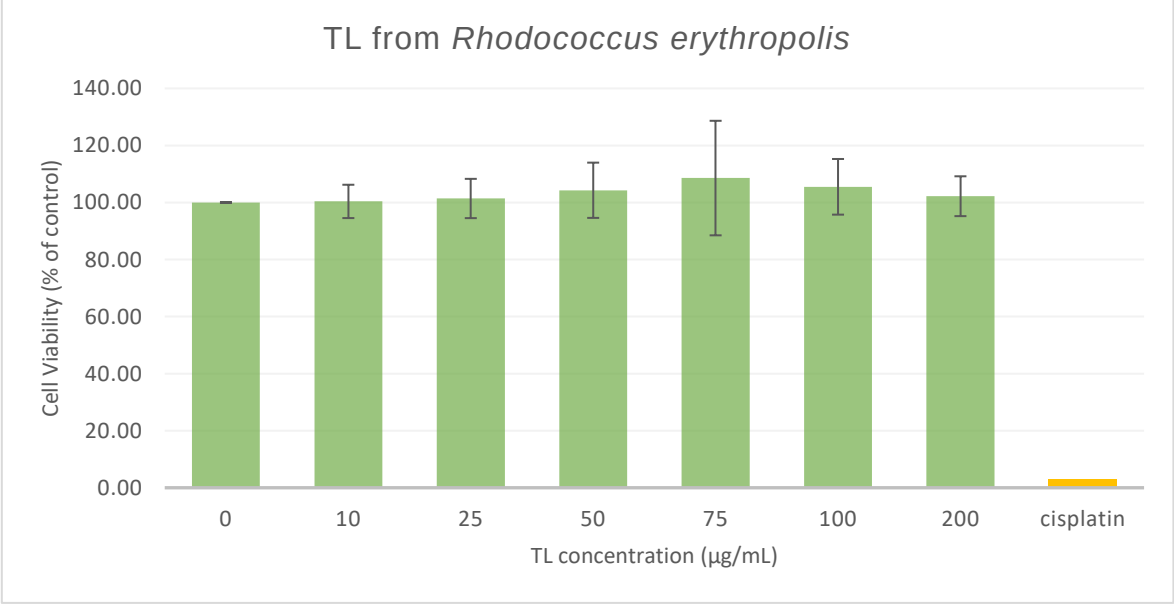


Figure 35. Cell viability of different concentrations of TLs from Sample 5 provenient of *Rhodococcus erythropolis*. Values represent mean +/- SD (n = 3) and are expressed as

percentages of the vehicle-treated control cells. Cisplatin (50 microM) was used as the positive control.

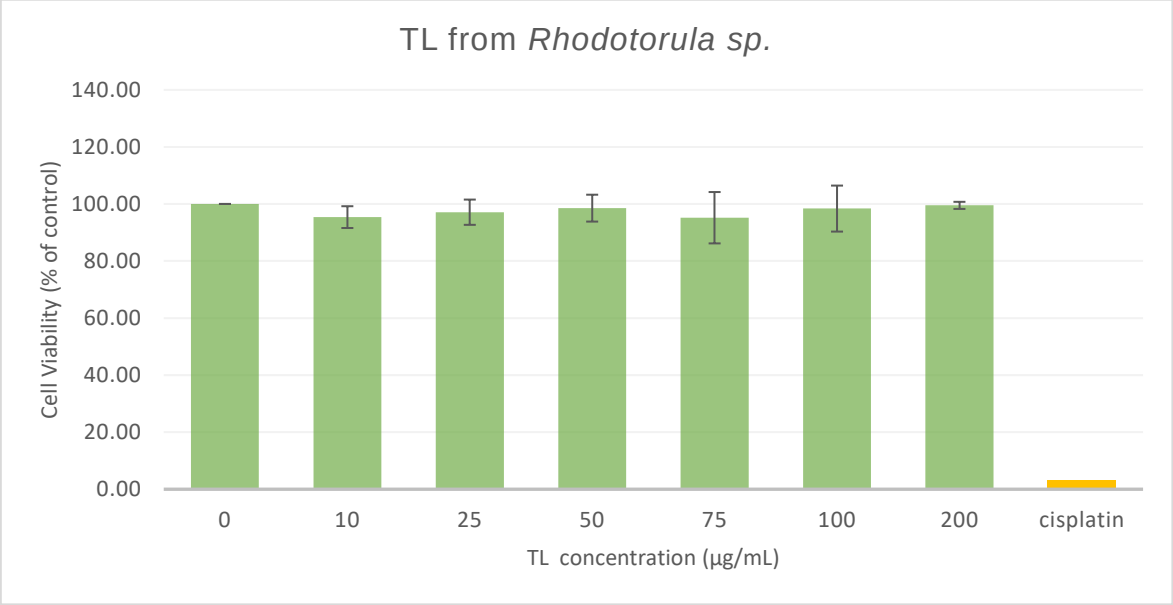


Figure 36. Cell viability of different concentrations of TLs from Sample B3 provenient of *Rhodotorula sp*. Values represent mean +/- SD (n = 3) and are expressed as percentages of the vehicle-treated control cells. Cisplatin (50 microM) was used as the positive control.

As we can clearly observe in Fig. 35, the TL produeced by *Rhodococcus erythropolis* in the H1975 cells was not cytotoxic for H1975 cells. The cell viabilities were in the same range as the negative control (100%). Cisplatin used as a postive control led to a reduction in cell viability to values less than 5%.

The results were similar in the samples of TLs produced by *Rhodotorula* sp, with the cell viabilities also similar to the negative control (100%). Likewise, cisplatin was clealy cytotoxic.

Other studies also tested the anticancer effect of TL. For instance, TLs were used in different concentrations (10–100 µM) for 24 and 48 h against two types of breast cancer cell lines high-metastatic MDA-MB231 and low-metastatic MCF7, as well as against the control non-cancer epithelial cell line MCF10A. After a MTS assay the results showed a decrease in the

percentage of viable cells with increasing TL concentrations and exposure times, thus suggesting the cytostatic/cytotoxic effect of TL on the treated cancer cell lines [78].

7 – Conclusion

This study demonstrates that it is possible to produce TL using two different microorganisms, *Rhodococcus erythropolis* DSM 43066-0917-001 and *Rhodotorula* sp. Different media was used for the production of the TL with several different carbon sources, glucose, glycerol, *n*-hexadecane, or *n*-dodecane. Timewise, the production was optimized at 48 h for *Rhodotorula* sp. and 24 h for *Rhodococcus erythropolis.* Although both microorganisms present a higher DO 600 at 48 h, *Rhodococcus erythropolis* tend to produce more TLs at 24 h time, on the other hand, *Rhodotorul*a sp. seems to have a production of TL equivalent to its growth. For the first time *Rhodotorula* sp was identified as a producer of trehalose lipids.

When comparing the produced compound to the literature, it is possible to see that the TLs produced in this work have a weaker component regarding surface tension and sequentially have a higher CMC. Nevertheless, most samples were able to reduce surface tension of water from 74 mN/m to 47 mN/m, with the exception of TL produced with *Rhodococcus erythropolis* from MSM supplemented with 4 % *n*-dodecane and MSM supplemented with 4 % glucose.

The evaluation of the TLs produced in this work did not reveal any cytotoxic effects in the NSCLC cell line H1975 for both products and in all concentrations tested. It is well known that there is a large variability of the sensitivity displayed by a given antitumor agent among cell lines even from the same type of cancer. Differences can also be the result of the methodology used to assess cell viability. In this sense, and in order to extend our knowledge about the impact of TL as anticancer agent further studies must be done. This work shows the possibility of continuing the exploration of TLs as a cancer therapeutic in different cancer cells in the future. This emphasizes the possibility to have new therapeutics against cancer, by using naturally produced biocompounds.

8 – References

1. Franzetti A, Gandolfi I, Bestetti G, Smyth TJP, Banat IM. Production and applications of trehalose lipid biosurfactants. *Eur J Lipid Sci Technol*. 2010;112(6):617-627. doi:10.1002/ejlt.200900162

2. Kuyukina MS, Ivshina IB. Rhodococcus Biosurfactants: Biosynthesis, Properties, and Potential Applications. *Microbiol Monogr 16*. 2010:291-313. doi:10.1007/978-3-642-12937-7_11

3. Shekhar S, Sundaramanickam A, Balasubramanian T. Biosurfactant producing microbes and their potential applications: A review. *Crit Rev Environ Sci Technol*. 2015;45(14):1522-1554. doi:10.1080/10643389.2014.955631

4. Geys R, Soetaert W, Bogaert I Van. ScienceDirect Biotechnological opportunities in biosurfactant production. *Curr Opin Biotechnol*. 2014;30:66-72. doi:10.1016/j.copbio.2014.06.002

5. Arrigoni-Martelli E. First European Congress of Pharmacology. *Drugs of Today*. 1995;31(8):635-637. doi:10.4172/2157-7463.1000124

6. Sana S, Datta S, Biswas D, Sengupta D. Assessment of synergistic antibacterial activity of combined biosurfactants revealed by bacterial cell envelop damage. *Biochim Biophys Acta - Biomembr*. 2018;1860(2):579-585. doi:10.1016/j.bbamem.2017.09.027

7. Solaiman DKY, Ashby RD, Birbir M, Caglayan P. Antibacterial Activity of Sophorolipids Produced by Candida bombicola on Gram-positive and Gram-negative Bacteria Isolated from Salted Hides. *J Am Leather Chem Assoc*. 2016;111(November):358-364.

8. Sarwar A, Brader G, Corretto E, et al. Qualitative analysis of biosurfactants from Bacillus species exhibiting antifungal activity. *PLoS One*. 2018;13(6):1-15. doi:10.1371/journal.pone.0198107

9. Borsanyiova M, Patil A, Mukherji R, Prabhune A, Bopegamage S. Biological activity of sophorolipids and their possible use as antiviral agents. *Folia Microbiol (Praha)*. 2016;61(1):85-89. doi:10.1007/s12223-015-0413-z

10. Fracchia L, Cavallo M, Giovanna M, M. I. Biosurfactants and Bioemulsifiers Biomedical and Related Applications – Present Status and Future Potentials. *Biomed Sci Eng Technol*. 2012. doi:10.5772/23821

11. Cortés-Sánchez A de J, Hernández-Sánchez H, Jaramillo-Flores ME. Biological activity of glycolipids produced by microorganisms: New trends and possible therapeutic alternatives. *Microbiol Res*. 2013;168(1):22-32. doi:10.1016/j.micres.2012.07.002

12. Morita T, Fukuoka T, Imura T, Kitamoto D. *Glycolipid Biosurfactants*. Elsevier Inc.; 2016. doi:10.1016/B978-0-12-409547-2.11565-3

13. Warnecke D, Heinz E. European Journal of Cell Biology Glycolipid headgroup replacement : A new approach for the analysis of specific functions of glycolipids in vivo. *Eur J Cell Biol*. 2010;89(1):53-61. doi:10.1016/j.ejcb.2009.10.009

14. Li Z, Lang S, Schulz W, et al. United States Patent (19) O b-7 / OH gh ph SH : (H). 1988;56(19).

15. Janek T, Krasowska A, Czyżnikowska Ż, Łukaszewicz M. Trehalose Lipid Biosurfactant Reduces Adhesion of Microbial Pathogens to Polystyrene and Silicone Surfaces: An Experimental and Computational Approach. *Front Microbiol*. 2018;9(October):1-14. doi:10.3389/fmicb.2018.02441

16. Sen S, Borah SN, Bora A, Deka S. Production, characterization, and antifungal activity of a biosurfactant produced by Rhodotorula babjevae YS3. *Microb Cell Fact*. 2017;16(1):1-14. doi:10.1186/s12934-017-0711-z

17. Masanobu Azuma, Tatsuo Suzutani, Katsuhuko Sazaki, Itsurou Yoshida TS and TY. Role of Interferon in the Augmented Resistance of Trehalose-6 , 6 ' - dimycolate-treated Mice to Influenza Virus Infection. *J Gen Virol*. 1986;(1987):835-843.

18. Gudiña EJ, Rangarajan V, Sen R, Rodrigues LR. Potential therapeutic applications of biosurfactants. *Trends Pharmacol Sci*. 2013;34(12):667-675. doi:10.1016/j.tips.2013.10.002

19. Elbein AD. The Metabolism of α,α-Trehalose. *Adv Carbohydr Chem Biochem*. 1974;30(C):227-256. doi:10.1016/S0065-2318(08)60266-8

20. Lesuk A, Anderson RJ. Lipids of Tubercle Bacilli . LX11. *J Biol Chem*.

1940;(136):603-613.

21. Soberón-Chávez G. *Biosurfactants*.; 2011. doi:10.1016/B978-0-12-809633-8.09184-6

22. Khan AA, Stocker BL, Timmer MSM. Trehalose glycolipids — synthesis and biological activities. *Elsevier*. 2012;356:25-36. doi:10.1016/j.carres.2012.03.010

23. Kügler JH, Muhle-Goll C, Kühl B, et al. Trehalose lipid biosurfactants produced by the actinomycetes Tsukamurella spumae and T. pseudospumae. *Appl Microbiol Biotechnol*. 2014;98(21):8905-8915. doi:10.1007/s00253-014-5972-4

24. Uchida Y, Misawa S, Nakahara T, Tabuchi T. Factors affecting the production of succinoyl trehalose lipids by rhodococcus erythropolis sd-74 grown on n-alkanes. *Agric Biol Chem*. 1989;53(3):765-769. doi:10.1080/00021369.1989.10869386

25. Noel Masihi K, Lange W, Brehmer W, Ribi E. Immunobiological activities of nontoxic lipid A: Enhancement of nonspecific resistance in combination with trehalose dimycolate against viral infection and adjuvant effects. *Int J Immunopharmacol*. 1986;8(3):339-345. doi:10.1016/0192-0561(86)90116-5

26. Isoda H, Shinmoto H, Kitamoto D, Matsumura M. Differentiation of Human Promyelocytic Leukemia Cell Line HL60 by Microbial Extracellular Glycolipids. *AOCS Press*. 1997;32(3).

27. Sudo T, Zhao X, Wakamatsu Y, et al. Induction of the differentiation of human HL-60 promyelocytic leukemia cell line by succinoyl trehalose lipids. *Cytotechnology*. 2000;33(1-3):259-264. doi:10.1023/a:1008137817944

28. Zaragoza A, Teruel JA, Aranda FJ, Ortiz A. Interaction of a trehalose lipid biosurfactant produced by Rhodococcus erythropolis 51T7 with a secretory phospholipase A2. *J Colloid Interface Sci*. 2013;408(1):132-137. doi:10.1016/j.jcis.2013.06.073

29. Lang S, Philp JC. Surface-active lipids in rhodococci. *Antonie van Leeuwenhoek, Int J Gen Mol Microbiol*. 1998;74(1-3):59-70. doi:10.1023/A:1001799711799

30. Patil HI, Pratap AP. Production and Quantitative Analysis of Trehalose Lipid Biosurfactants Using High-Performance Liquid Chromatography. *J Surfact Deterg*. 2018;(June):553-564. doi:10.1002/jsde.12158

31. Kuyukina MS, Kochina OA, Gein S V., Ivshina IB, Chereshnev VA. Mechanisms of Immunomodulatory and Membranotropic Activity of Trehalolipid Biosurfactants (a Review). *Appl Biochem Microbiol*. 2020;56(3):245-255. doi:10.1134/S0003683820030072

32. Bell KS, Philp JC, Aw DWJ, Christofi N. A review: The genus Rhodococcus. *J Appl Microbiol*. 1998;85(2):195-210. doi:10.1046/j.1365-2672.1998.00525.x

33. Ellis D. Mycology Online - Rhodotorula. https://mycology.adelaide.edu.au/descriptions/yeasts/rhodotorula/.

34. Marchant R, Funston S, Uzoigwe C, Rahman P, Banat I. Production of Biosurfactants from Nonpathogenic Bacteria. *Biosurfactants*. 2014:73-82. doi:10.1201/b17599-7

35. Santos DKF, Rufino RD, Luna JM, Santos VA, Sarubbo LA. Biosurfactants: Multifunctional biomolecules of the 21st century. *Int J Mol Sci*. 2016;17(3):1-31. doi:10.3390/ijms17030401

36. Chang JS, Radosevich M, Jin Y, Cha DK. Enhancement of phenanthrene solubilization and biodegradation by trehalose lipid biosurfactants. *Environ Toxicol Chem*. 2004;23(12):2816-2822. doi:10.1897/03-608.1

37. Christova N, Lang S, Wray V, Kaloyanov K, Konstantinov S, Stoineva I. Production, structural elucidation, and in vitro antitumor activity of trehalose lipid biosurfactant from Nocardia farcinica strain. *J Microbiol Biotechnol*. 2015;25(4):439-447. doi:10.4014/jmb.1406.06025

38. Kuyukina MS, Ivshina IB, Korshunova IO, Stukova GI, Krivoruchko A V. Diverse effects of a biosurfactant from Rhodococcus ruber IEGM 231 on the adhesion of resting and growing bacteria to polystyrene. *AMB Express*. 2016. doi:10.1186/s13568-016-0186-z

39. Tokumoto Y, Nomura N, Uchiyama H, et al. Structural Characterization and Surface-Active Properties of a Succinoyl Trehalose Lipid Produced by Rhodococcus sp . SD-74. *J Oleo Sci*. 2009;102(2):97-102.

40. Peng F, Liu Z, Wang L, Shao Z. An oil-degrading bacterium: Rhodococcus erythropolis strain 3C-9 and its biosurfactants. *J Appl Microbiol*. 2007;102(6):1603-

1611. doi:10.1111/j.1365-2672.2006.03267.x

41. Kitamoto D, Isoda H, Nakahara T. Functions and potential applications of glycolipid biosurfactants - From energy-saving materials to gene delivery carriers. *J Biosci Bioeng*. 2002;94(3):187-201. doi:10.1263/jbb.94.187

42. Uchida Y, Tsuchiya R, Chino M, Hirano J, Tabuchi T. Extracellular accumulation of mono- and di-succinoyl trehalose lipids by a strain of rhodococcus erythropolis grown on n-alkanes. *Agric Biol Chem*. 1989;53(3):757-763. doi:10.1080/00021369.1989.10869385

43. Inaba T, Tokumoto Y, Miyazaki Y, et al. Analysis of genes for succinoyl trehalose lipid production and increasing production in Rhodococcus sp. strain SD-74. *Appl Environ Microbiol*. 2013;79(22):7082-7090. doi:10.1128/AEM.01664-13

44. Ramsay B, McCarthy J, Guerra-Santos L, Kappeli O, Fiechter A, Margaritis A. Biosurfactant production and diauxic growth of Rhodococcus aurantiacus when using n -alkanes as the carbon source . *Can J Microbiol*. 1988;34(11):1209-1212. doi:10.1139/m88-212

45. Espuny MJ, Egido S, Mercade ME. M.J. Espuny, S. Egido, I. Rodon A. Manresa*, M.E. Mercade Laboratorio de Microbiologia. Facultad de Farmacia. Universidad de Barcelona. Espafia. *Biotechnol Lett*. 1996;I(5):521-526.

46. Mnif I, Ghribi D. Microbial derived surface active compounds: properties and screening concept. *World J Microbiol Biotechnol*. 2015;31(7):1001-1020. doi:10.1007/s11274-015-1866-6

47. White DA, Hird LC, Ali ST. Production and characterization of a trehalolipid biosurfactant produced by the novel marine bacterium Rhodococcus sp., strain PML026. *J Appl Microbiol*. 2013;115(3):744-755. doi:10.1111/jam.12287

48. Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. *Microbiol Mol Biol Rev*. 1997;61(1):47-64. doi:10.1128/.61.1.47-64.1997

49. Kuyukina MS, Ivshina IB, Philp JC, Christofi N, Dunbar SA, Ritchkova MI. Recovery of Rhodococcus biosurfactants using methyl tertiary-butyl ether extraction. *J Microbiol Methods*. 2001;46(2):149-156. doi:10.1016/S0167-7012(01)00259-7

50. Ortiz A, Teruel JA, Espuny MJ, Marqués A, Manresa Á, Aranda FJ. Interactions of a

bacterial biosurfactant trehalose lipid with phosphatidylserine membranes. *Chem Phys Lipids*. 2009;158(1):46-53. doi:10.1016/j.chemphyslip.2008.11.001

51. Hodge, J. E., Hofreiter BT. Determination of reducing sugars and carbohydrates. Methods in Carbohydrate Chemistry. *Acad Press*. 1962.

52. RAPP P, BOCK H, WRAY V, WAGNER F. Formation, Isolation and Characterization of Trehalose Dimycolates from Rhodococcus erythropolis Grown on n-Alkanes. *J Gen Microbiol*. 1979;115(2):491-503. doi:10.1099/00221287-115-2-491

53. Brandenburg K, Seydel U. Infrared spectroscopy of glycolipids. *Chem Phys Lipids*. 1998;96(1-2):23-40. doi:10.1016/S0009-3084(98)00078-4

54. Mutalik SR, Vaidya BK, Joshi RM, Desai KM, Nene SN. Use of response surface optimization for the production of biosurfactant from Rhodococcus spp. MTCC 2574. *Bioresour Technol*. 2008;99(16):7875-7880. doi:10.1016/j.biortech.2008.02.027

55. Ortiz A, Teruel JA, Espuny MJ, Marqués A, Manresa Á, Aranda FJ. Interactions of a Rhodococcus sp. biosurfactant trehalose lipid with phosphatidylethanolamine membranes. *Biochim Biophys Acta - Biomembr*. 2008;1778(12):2806-2813. doi:10.1016/j.bbamem.2008.07.016

56. Kadinov B, Nikolova B, Tsoneva I, Semkova S, Kabaivanova L, Dimitrova D. Trehalose lipid biosurfactant reduced cancer cell viability but did not affect the isometric contraction of rat mesenteric arteries in vitro. *Int J Bioautomation*. 2020;24(1):79-86. doi:10.7546/ijba.2020.24.1.000708

57. Zaragoza A, Aranda FJ, Espuny MJ, et al. Hemolytic activity of a bacterial trehalose lipid biosurfactant produced by rhodococcus sp.: Evidence for a colloid-osmotic mechanism. *Langmuir*. 2010;26(11):8567-8572. doi:10.1021/la904637k

58. Egea J, Yahyaoui R, González Y, et al. Prevalência ao nascimento dos défices da β -oxidação mitocondrial dos ácidos gordos na Península Ibérica. (tabela 1):26-29.

59. Mclaughlin CA, Schwartzman SM, Horner BL, et al. Regression of tumors in guinea pigs after treatment with synthetic muramyl dipeptides and trehalose dimycolate. *Science (80-)*. 1980;208(4442):415-416. doi:10.1126/science.7189295

60. Publishers KA, Ministry T. Succinoyl trehalose lipid induced differentiation of human

monocytoid leukemic cell line U937 into monocyte-macrophages. *Cytotechnology 19*. 1996:79-88.

61. Rodrigues L, Banat IM, Teixeira J, Oliveira R. Biosurfactants: Potential applications in medicine. *J Antimicrob Chemother*. 2006;57(4):609-618. doi:10.1093/jac/dkl024

62. Cajka T, Garay LA, Sitepu IR, Boundy-Mills KL, Fiehn O. Multiplatform mass spectrometry-based approach identifies extracellular glycolipids of the yeast Rhodotorula babjevae UCDFST 04-877. *J Nat Prod*. 2016;79(10):2580-2589. doi:10.1021/acs.jnatprod.6b00497

63. Spencer JFT. Extracellular glycolipids of rhodotorula species. *Can J Chem*. 1964;42(7801):831-835.

64. Zhao BX, Wang J, Song B, et al. Establishment and biological characteristics of acquired gefitinib resistance in cell line NCI-H1975/gefinitib-resistant with epidermal growth factor receptor T790M mutation. *Mol Med Rep*. 2015;11(4):2767-2774. doi:10.3892/mmr.2014.3058

65. Miller GL. Use of DinitrosaIicyIic Acid Reagent for Determination of Reducing Sugar. *Anal Chem*. 1959;(lll).

66. Kruger NJ. The Bradford Method For Protein Quantitation. *Methods Mol Biol*. 2009:17-24.

67. Bradford MM. A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem*. 1976;254:248-254.

68. Marqués AM, Pinazo A, Farfan M, et al. The physicochemical properties and chemical composition of trehalose lipids produced by Rhodococcus erythropolis 51T7. *Chem Phys Lipids*. 2009;158(2):110-117. doi:10.1016/j.chemphyslip.2009.01.001

69. Satpute SK, Banpurkar AG, Dhakephalkar PK, Banat IM, Chopade BA. Methods for investigating biosurfactants and bioemulsifiers: A review. *Crit Rev Biotechnol*. 2010;30(2):127-144. doi:10.3109/07388550903427280

70. Park AJ, Cha DK, Holsen TM. Enhancing solubilization of sparingly soluble organic compounds by biosurfactants produced by Nocardia erythropolis . *Water Environ*

Res. 1998;70(3):351-355. doi:10.2175/106143098x124984

71. Kurane R, Toeda K, Takeda K, Suzuki T. Culture conditions for production of microbial flocculant by rhodococcus erythropolis. *Agric Biol Chem*. 1986;50(9):2309-2313. doi:10.1080/00021369.1986.10867747

72. Franzetti A, Gandolfi I, Bestetti G, Smyth TJP, Banat IM. Production and applications of trehalose lipid biosurfactants. *Eur J Lipid Sci Technol*. 2010;112(6):617-627. doi:10.1002/ejlt.200900162

73. Yin T, Qin M, Shen W. Physicochemical investigations on the interactions between Gemini/single-chain cationic surfactants and bovine serum albumin. *Colloids Surfaces A Physicochem Eng Asp*. 2014;461(1):22-29. doi:10.1016/j.colsurfa.2014.07.012

74. Manabe M, Kawamura H, Kameyama K. Premicelle formation of double-chain surfactants and bile salts in the neighborhood of the CMC region: Application of a differential conductivity technique to the determination of micellization parameters. *J Oleo Sci*. 2011;60(10):515-525. doi:10.5650/jos.60.515

75. Faustino C, Martins T, Duarte N, Ribeiro MH. Self-Assembly of Lipoaminoacids-DNA Based on Thermodynamic and Aggregation Properties. *J Surfactants Deterg*. 2020;23(3):581-593. doi:10.1002/jsde.12391

76. Kim JS, Powalla M, Lang S, Wagner F, Lünsdorf H, Wray V. Microbial glycolipid production under nitrogen limitation and resting cell conditions. *J Biotechnol*. 1990;13(4):257-266. doi:10.1016/0168-1656(90)90074-L

77. Yutaka Ishigami, Shigeru Suzuki, Tadashi Funada, Masako Chino YU and TT. Surface-Active Properties of Succinoyl Trehalose Lipids as Microbial Biosurfactants. *J Japan Oil Chem Soc*. 1987;36(11):847-851. doi:10.5650/jos1956.36.847

78. Nikolova B, Antov G, Semkova S, et al. Bacterial natural disaccharide (trehalose tetraester): Molecular modeling and in vitro study of anticancer activity on breast cancer cells. *Polymers (Basel)*. 2020;12(2):1-11. doi:10.3390/polym12020499

9 – Annexs

Annex 1. TLC plates with samples from *Rhodotorula* sp and *Rhodococcus* with samples diluted with chloroform:methanol:water (marked with the sample identification) and pure chloroform (marked as C)

Annex 2. TLC plate made with samples 4 and 5 from *Rhodococcus erythropolis* diluted in pure chloroform

Annex 3. TLC plate made with samples A3 and A5 from *Rhodotorula* diluted in pure chloroform

Annex 4. TLC without diluted sample